



**International
Standard**

ISO 8259

**Soil quality — Bioaccessibility of
organic and inorganic pollutants
from contaminated soil and soil-like
materials**

*Qualité du sol — Bioaccessibilité des polluants organiques et
inorganiques provenant d'un sol ou de matériaux de type sol
pollués*

**First edition
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Foreword

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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).

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

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

The concept of bioavailability is used during risk assessment of contaminants and contaminated land. It is used during human health risk assessment by application of in vitro methods designed to assess the bioaccessible fraction of a chemical such as the one described in this document.

ISO 17402 provides a general overview of the definitions and concept of bioavailability and of available methods to assess the bioavailability for several exposure pathways. ISO 15800 provides guidance on the soil and site characterization necessary for the evaluation of human exposure to substances present in soil including bioaccessibility and bioavailability. ISO 17924 describes a method to assess the bioaccessible fraction of metals in a contaminated soil after soil ingestion. This document describes a test procedure for the estimation of human bioaccessibility after oral uptake of both metals and non-volatile organic contaminants from soil.

To evaluate the health effects of the oral ingestion of contaminated soil or soil-like materials by humans, it is necessary to consider the total concentration of a contaminant in soil, the quantity of soil ingested, the dissolution of the contaminants from soil in the gastrointestinal tract as well as their absorption through membranes. Simulation of human absorption and bioavailability of contaminants can be determined in animal tests. For ethical reasons, and to reduce the amount of work and time needed, in vitro methods have been developed determining the quantity of contaminants that can be dissolved from the contaminated soil and soil-like materials by the digestive juices in the digestive tract.

Contaminants bound to soil and soil-like materials are generally only dissolved to a partial extent by the digestive juices in the digestive tract. The degree of dissolution depends on the type of contaminant, the characteristics of the soil and soil-like materials and the components of the digestive juices in the digestive tract. The dissolved contaminants can be absorbed into the organism in the gastrointestinal tract. Contaminants that remain bound are largely excreted in an unchanged state. The quantity actually resorbed is always less than or, at most, equal to the dissolved quantity.

Digestive juices are complex mixtures of electrolytes, enzymes and digestive aids. The composition of the digestive juices varies depending on their type, characteristics and quantity, and depends on exogenous and endogenous factors. The type of food consumed is a significant exogenous factor in this regard.

The presence of food has an impact on the dissolution process of contaminants from soil particles ingested by humans to their gastrointestinal juices. Therefore, it is relevant to add a food surrogate in an in vitro test, to mimic the true situation in vivo. Food surrogates like milk powder are acting as emulsifiers because the absorption of lipophilic substances depends significantly on the presence of lipids, for example from food, which causes the secretion of bile salts, as well as the formation of micelles resulting in higher bioaccessibilities for hydrophobic organic contaminants.^[56,61] In addition, they can also have an impact on inorganic contaminants. The use of food additives in in vitro testing simulates dissolution in children after a standard meal (e.g. baby food) to achieve “realistic worst case” estimates for risk assessment especially for organic contaminants^[34].

This document standardizes a test system to assess the bioaccessible fraction of pollutants in contaminated soils and soil-like materials with the aid of artificial digestive juices. The composition of the used artificial digestive juices corresponds approximately to the average composition of natural digestive juices of humans in the case of stimulated secretion, as occurs during the intake of foodstuffs. The treatment duration of the samples with gastric juice is 2 h and corresponds to the average residence times of foodstuffs in the stomach. In contrast, the treatment duration with intestinal juice is 3 h and based on the residence time of foodstuff components in the upper section of the small intestine, which is the main site of absorption.^[61] To simulate food consumption and, hence, take the influence of foodstuff into account, milk powder is added to the test system as food surrogate. Soil and soil-like sample material that has not been crushed or ground is generally used after sieving at ≤ 2 mm simulating soil ingestion by eating without extensive mastication (e.g. by young children).

This method has been validated in vivo for arsenic, lead, cadmium, chromium, nickel and mercury by animal testing.^[40] Polycyclic aromatic hydrocarbons (PAHs) including Benzo[a]pyrene (BaP) were also used in in vivo animal testing. An interlaboratory trial was carried out using arsenic, lead, cadmium and antimony as well as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and trinitrotoluene.^[61]

Several in vitro methods have been developed for the estimation of contaminant bioaccessibility. There are simplified approaches simulating the human gastrointestinal physiology by separate gastric and/or intestinal tests or by using gastric and intestinal phases subsequently. The test method described in ISO 17924 uses both a separate gastric phase as well as combined gastric and intestinal phases to assess the bioaccessibility of contaminants. The methodology used in this document is based exclusively on a combined gastric and intestinal phase. In contrast to ISO 17924, milk powder is used as food surrogate in the method described in this document. In addition, the method described in this document is not limited to inorganic contaminants but is also applicable to organic contaminants like PAHs or PCBs. The methods of both standards are in vivo validated and comparisons of both methodologies have been published.[\[42,43,46,47\]](#)

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Soil quality — Bioaccessibility of organic and inorganic pollutants from contaminated soil and soil-like materials

1 Scope

This document specifies a method for testing the bioaccessibility of substances from contaminated soil and soil-like materials. The method is not applicable for volatile contaminants. Furthermore, the method is only applicable if suitable analytical methods for extraction and detection of substances and/or elements from complex digestion assays are available.

NOTE During the in vivo validation with minipigs, the PAHs naphthalene, acenaphthylene, acenaphthene and fluorene were not evaluated due to their volatility.^[40] However, the results of the overall recovery indicate if volatilisation has occurred during the test.

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/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

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

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

3.1

contaminated soil and soil-like material

<assessment of human bioaccessibility> soil and soil-like material containing *contaminants* (3.2) that can pose health risks if ingested by humans

3.2

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 harms results from the presence of the contaminant.

3.3

total content

analyte concentration in a sample that is measured using a largely exhaustive extraction method, e.g. after aqua regia digestion (heavy metals) or solvent extraction (organic components)

3.4

duplicate determination

carrying out the method twice to determine the *bioaccessibility* (3.6), each time with a new subsample

3.5**bioavailability**

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

3.6**bioaccessibility**

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

Note 1 to entry: Bioaccessibility is expressed and calculated as percentage transfer of a substance from the solid sample into the liquid phase (the gastrointestinal phase solution) of the in vitro test system specified by this document, where the reference quantity is the conventional *total content* (3.3) of the solid sample as analysed after digestion or extraction.

3.7**solid phase after centrifugation**

solid phase remaining after centrifugation at the end of simulated digestion

Note 1 to entry: The solid phase after centrifugation contains the non-bioaccessible content and/or fraction.

3.8**mass balance**

relationship between input and output of a specified substance in a defined system

Note 1 to entry: In this context, mass balance is the calculation of the contents of a *contaminant* (3.2) in the gastrointestinal phase (supernatant) and in the pellet after centrifugation as well as the calculation of their sum.

3.9**overall recovery**

quality assurance measure whereby the sum of the content in the gastrointestinal phase (supernatant) and the *solid phase after centrifugation* (3.7) is compared with the *total content* (3.3), taking into account *sample inhomogeneity* (3.10)

3.10**sample inhomogeneity**

inhomogeneous distribution of the analyte in a sample that results in subsamples with different analyte concentrations, described by the standard deviation of a replicate determination

4 Principle of the test**4.1 Human digestion**

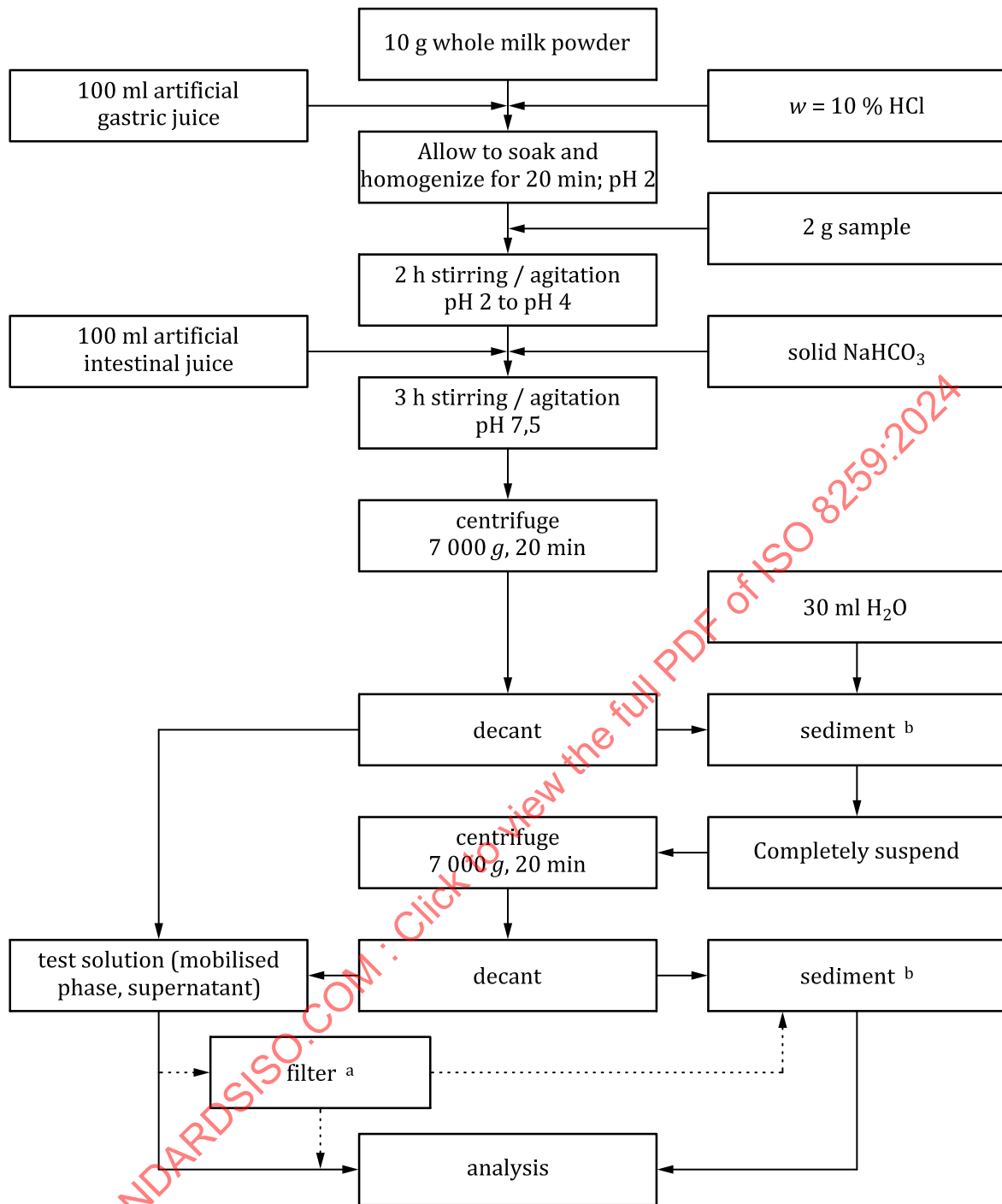
Digestion starts in the mouth, where, in addition to mechanical size reduction, an α -amylase contained in the spittle initiates the hydrolysis of starches. However, experience shows that separate consideration of this partial process is not essential with regard to the release of contaminants.^[40] Food is denatured in the stomach by hydrochloric acid. At the same time, digestion of proteins by peptide hydrolases (pepsins) begins. In addition, lipases that originate from the duodenum initiate the digestion of fats. Maximum secretion of hydrochloric acid is achieved after 1 h. The hydrochloric acid is buffered to a pH value of between 3 and 4 by food components.^[61] The pH value only falls into a range of 1 to 2 again when digestion has progressed. Depending on the characteristics of the food, it is transferred to the duodenum after a residence time of between just a few minutes and several hours. Here, the hydrochloric acid is first buffered to a pH value of 4 by hydrogen carbonate from the duodenum juice, pancreatic juice and bile. The pH value then increases more slowly to between 6 and 7.5. The pancreatic juice contains a range of peptide hydrolases (trypsin, chymotrypsin etc.), α -amylases for lysis of carbohydrates, and lipases for lysis of triglycerides into mono- or diacylglycerides and fatty acids. Lipids can be digested by lipases only in emulsified form. Already in the stomach, fats are mechanically suspended by the movements of the stomach. As a result of the influx of bile, which contains bile acids and lecithins as natural surfactants, strong emulsification of fats and the products of fat decomposition (di- and monoglycerides and fatty acids) occurs. In the small intestine (jejunum and ileum), further digestive enzymes are also present, but these do not differ significantly from the enzymes

in the pancreatic juice in their effect. In total, the body produces an average of 0,5 l to 1,5 l of spittle, 2 l to 3 l of gastric juice, 0,5 l to 1,5 l of pancreatic juice, 0,8 l to 1 l of liver bile, which is concentrated by a factor of approximately 7 in the gall bladder, and 2 l to 3 l of intestinal juice from the small intestine in 24 h. The small intestine is the main organ for the absorption of food and drink components and pollutants. Here, approximately 8 l to 9 l of water is resorbed with approximately 70 g of electrolytes, 300 g of carbohydrates, 100 g of proteins and 70 g of fats every day. In addition, a large majority of the bile acids is recovered as no more than 4 g to 6 g is available to the body while several times this amount is required for digestion.

4.2 General test description

To assess the bioaccessibility of contaminants in soil or soil-like materials after oral ingestion, an in vitro test is described using two artificial digestive juices (artificial gastric juice and artificial intestinal juice) simulating the digestion in the human gastrointestinal tract under close to realistic physiological conditions, i.e. at an elevated temperature (37 °C), with continuous agitation and at a pH value that is typical for gastric or intestinal juices. A schematic workflow of the test is presented in [Figure 1](#).

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^a If necessary when particles floating on the surface of the test solution.

^b Quality assurance measure; if necessary, incl. filtration residue.

Figure 1 — Schematic diagram of test process

4.3 Applicability

The test to assess the bioaccessibility of contaminants in soil or soil-like materials can be used for scientific research as well as for risk assessment of contaminated sites. Concerning the risk assessment, it is presupposed that the national legal context is considered when and how bioavailability estimates can, or should, contribute to risk assessment. Usually, bioaccessibility data are used during a detailed and site-specific risk assessment (see ISO 15800) and in vitro tests are carried out if national threshold values based

on total concentrations are exceeded to a reasonable extent (i.e. which can result in an acceptable risk when considering the bioaccessibility of the contaminant).

4.4 Substances and elements

The test has been validated *in vivo* for arsenic, lead, cadmium, chromium, nickel and mercury as well as for polycyclic aromatic hydrocarbons (PAHs) by animal testing.^[40] However, correlation with bioaccessibility – bioavailability for PAHs could not be clearly shown due to the metabolism of PAHs by the minipigs; and comparison could only be carried out indirectly (by retention)^[41].

An interlaboratory trial was carried out using arsenic, lead, cadmium and antimony as well as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and trinitrotoluene.^[61] Results of the inter-laboratory trial are presented in [Annex B](#).

Up to now, the extractability by artificial digestive juices of organic contaminants from contaminated soil and soil-like materials has been tested on a wide range of soil-like materials and technogenic materials for the following compounds:

polycyclic aromatic hydrocarbons (PAHs or BaP)	soil	[29] , [30] , [57]
p,p-DDT	housedust	[33]
γ-HCH (hexachlorocyclohexane)	soil, housedust	[33] , [51]
petroleum hydrocarbons	soil	[57]
polychlorinated biphenyls (PCBs)	soil, housedust	[29] , [33]
polychlorinated dibenzodioxines (PCDDs) and polychlorinated dibenzofurans (PCDFs)	red slag	[52]
PCP	housedust	[33]
Permethrin	housedust	[33]

The bioaccessibility of inorganic substances, such as the elements arsenic, lead, cadmium, chromium and mercury in their various forms and oxidation states, has also been investigated using a wide range of contaminated soil and soil-like materials:

As	soil	[32] , [40]
Cd	soil, soil-like materials	[32] , [40] , [53]
Cr	soil, soil-like materials	[40] , [53]
Cu	soil, soil-like materials	[53]
Hg	soil	[40]
Mn	soil, soil-like materials	[53]
Ni	soil	[40]
Pb	soil	[30] , [32]
U	soil	[32]
Zn	soil, soil-like materials	[53]

4.5 Contaminated soil and soil-like materials

Both anthropogenically and geogenically contaminated soil and soil-like materials can be investigated in this test system. The test was validated by animal testing^[40] and by an interlaboratory trial^[61] using soils from different contaminated sites.

Other materials, such as fly ash, filter residues, blasting sand, materials from recycling processes, road asphalt, road dust, domestic dust, sewage sludge, sediments from bodies of water, dredged material, metallurgical waste from smelting works, other wastes for recovery and removal, and materials from the mining and processing of ores, can also be investigated.^[36,53]

5 Sampling and pre-treatment of soil and soil-like materials

The sample size shall be sufficient to allow the laboratory investigation as well as the provision of retain samples after preparation of the fraction < 2 mm from the samples. Soil and soil-like sample material that shall not be crushed or ground is used for the test after sieving.

At least 100 g of dry mass (including a duplicate test and retain sample) of inhomogeneous or coarse soil and soil-like materials should be available for the testing of bioaccessibility. Smaller quantities of soil and soil-like sample material are acceptable in exceptional cases, such as an investigation of roof dust.

Total contents and bioaccessible contents should be determined by the same laboratory. If there are more than 6 months between sampling and the measurements of bioaccessible contents, sampling should be carried out again for the determination of bioaccessible contents. The total content should also be determined again using the new sample.

6 Reagents and apparatus

6.1 Reagents

All reagents shall be of analytical quality as a minimum.

6.1.1 Water, deionized.

6.1.2 Sodium chloride, NaCl.

6.1.3 Sodium hydrogen carbonate, NaHCO₃.

6.1.4 Potassium chloride, KCl.

6.1.5 Potassium dihydrogen phosphate, KH₂PO₄.

6.1.6 Calcium chloride dihydrate, CaCl₂ · 2 H₂O.

6.1.7 Magnesium chloride hexahydrate, MgCl₂ · 6 H₂O.

6.1.8 Buffer solutions for calibration of the titration system with pH values of 2,0 and 7,0.

6.1.9 Hydrochloric acid HCl, w_{HCl} = 10 %.

6.1.10 Saturated sodium hydrogen carbonate solution.

6.1.11 Sodium azide for stabilization.

6.2 Enzymes and digestive aids

NOTE See [Annex A](#) for samples of suitable enzymes and suppliers of these enzymes.

6.2.1 Porcine mucin.

6.2.2 Porcine pancreatin.

6.2.3 Pepsin.

6.2.4 Trypsin.

6.2.5 Porcine bile extract.

6.2.6 **Instant whole milk powder**, small portion units, freshly opened, which keeps for a maximum of one week if refrigerated.

6.3 Apparatus

6.3.1 **Autotitration system**, consisting of a control unit, automatic burettes, pH electrodes (single pore, pH value 0 to 14, 30 °C to 60 °C), and a control and evaluation unit (optional).

6.3.2 **Water bath with tangential or circular shaker**, heated with variable holders for incubation vessels or a tempering bath with a magnetic stirrer ($T = 37\text{ °C} \pm 0,5\text{ °C}$).

6.3.3 **Amber glass bottles** (nominal capacity 500 ml, with a screw cap with holes for a pH electrode if necessary and a dosing tip).

6.3.4 **Soxhlet apparatus** (optional).

6.3.5 **0,5 l rotation perforator**, for extraction media with a lower density (optional).

6.3.6 **Centrifuge** (7 000 *g*).

6.3.7 **Analytical sieves for sample preparation**, made of stainless steel (mesh size 2 mm). Mesh size should be adapted if smaller soil fractions are investigated for other national requirements.

6.3.8 **Filter for filtering gastrointestinal phase solutions to remove floating particles**, mesh size 20 µm, for analysis; made of stainless steel for organic compounds and made of plastic for inorganic substances.

6.3.9 **Analytical balance**, for weighing out soil-like materials and reagents for the test system.

6.3.10 **Drying cabinet**.

7 Test system

7.1 Set-up of apparatus

Testing of the dissolution of pollutants from contaminated soil and soil-like materials by artificial digestive juices is carried out under close-to-realistic physiological conditions, i.e. at an elevated temperature (37 °C), with continuous agitation and at a pH value that is typical for gastric or intestinal juices. Testing is carried out in 500 ml amber glass bottles ([6.3.3](#)), in a temperature-controlled water bath with a shaking function

at a shaking frequency of 200 min^{-1} or, alternatively, in a thermostat-controlled water bath ($37 \text{ }^{\circ}\text{C}$) on a magnetic stirrer (6.3.2) with a polytetrafluorethylene-coated stirring rod. The agitation shall be so strong that the soil and soil-like materials to be tested cannot deposit and become compacted at the base of the vessel in the assays. The amber glass bottles (6.3.3) are closed by screw caps.

The pH value can be regulated manually or automatically.

If the pH value of the test assays is monitored using a pH meter and is regulated manually, the pH value should be maintained as close to the specified values as possible. Fluctuations in the pH value around the specified value should not be greater than 0,2 pH units when titrating the assays after the end of the initial adjustment. Deviations in this regard shall be described in the test report.

A number of samples can be continuously agitated in 500 ml amber glass bottles with screw caps in a shaking water bath at $37 \text{ }^{\circ}\text{C}$ at 200 min^{-1} at the same time. The pH value in the test assays is measured and regulated at short intervals (in the range of seconds) using a computer-controlled autotitration system (6.3.1).

For special purposes, anaerobic conditions can be created using an inert gas (e.g. N_2).

7.2 Artificial digestive juices

The composition of the artificial digestive juices in the test system is presented in Table 1 and Table 2. All digestive juices contain electrolytes and biochemical components.

The inorganic components are dissolved in water (6.1.1) in separate stock solutions for gastric and intestinal juices concentrated by a factor of between 10 and 1 000 and are then diluted to produce the relevant inorganic working solution. The biochemical components are added to these solutions with the aid of stirring or agitation immediately before they are used.

Artificial gastric juice contains the concentrations of NaCl , KCl , KH_2PO_4 , pepsin and mucin specified in Table 1 in 100 ml. Artificial intestinal juice contains CaCl_2 , MgCl_2 , KCl , NaHCO_3 , urea, lyophilized bile, pancreatin and trypsin (see Table 2). The concentrations of the enzymes, mucous substances and other components of artificial digestive juices lie within the range of the average concentrations of these components in natural digestive juices.

In order to take into account the influence of foodstuffs such as carbohydrates, proteins and lipids on the dissolution processes of contaminants in the gastrointestinal tract, whole milk powder is added to the test system at the start of the stomach stage.

NOTE The whole milk powder serves as a surrogate for average fat-rich and protein-rich components of human foodstuffs and primarily simulates the influence of foodstuffs on the dissolution process of contaminants in the digestive tracts of babies and toddlers. Whole milk powder largely fulfils the requirements of a standardized material and it is available to buy with a sufficiently constant level of quality. However, opened packages keep for only a limited period of time.

Whole milk powder is added to the artificial gastric juice directly before the start of the test. Artificial gastric juice is adjusted to a pH value of 2 using HCl . 100 g of milk powder is suspended for every 1 000 ml in 20 min with stirring, allowed to soak and then adjusted to a pH value of 2 again. 1 000 ml of artificial gastric juice together with milk powder results in 1 100 ml of gastric juice. The test starts with the addition of 110 ml of this mixture to 2 g of sample.

Table 1 — Composition of artificial gastric juice (see also [Annex A](#))

Stock solution	Addition to the stock solution concentrated by a factor of 50 g/l	Final concentration in gastric juice g/l
NaCl	145	2,9
KCl	35	0,70
KH ₂ PO ₄	13,5	0,27
Enzymes and digestive aids		
pepsin	—	1,0
mucin	—	3,0

Table 2 — Composition of artificial intestinal juice (see also [Annex A](#))

Ingredient	Addition to the stock solution concentrated by a factor of 100 g/l	Final concentration in intestinal juice g/l
Stock solution 1		
KCl	30	0,30
NaHCO ₃	100	1,0
Stock solution 2		
CaCl ₂ · 2 H ₂ O	50	0,50
MgCl ₂ · 6 H ₂ O	20	0,20
Enzymes and digestive aids		
trypsin	—	0,30
urea	—	0,30
pancreatin	—	9,0
bile, lyophilized	—	9,0

8 Preparation and analysis of the sample

8.1 Sample preparation

Sample preparation, including drying of the soil and soil-like materials, is carried out to determine physico-chemical properties and to identify organic and inorganic contaminants. The particle size fraction of ≤ 2 mm is used for chemical investigations of soil-like materials. Soil and soil-like materials that shall not be crushed or ground is generally used for the test.

NOTE The particle size fraction of ≤ 2 mm was selected on the basis of probability considerations for ingestion of soil particles by children^[31] as well as on a practicable solution for handling samples of soil and soil-like materials in routine laboratories. However, it is important that the reference particle size fraction of the bioaccessible content and the total content are the same.

In preparation for the bioaccessibility testing, particles with a particle diameter ≤ 2 mm are sieved out of at least 100 g of the starting soil and soil-like material using an analytical sieve made of stainless steel (6.3.7). If lower quantities of soil or soil-like materials are available, this shall be noted in the test report by the laboratory. Even if coarse soil or soil-like materials is tested in the test system, which can be necessary if the contaminated fractions are larger particles, for example, this shall be stated in the test report by the laboratory.

The retain samples shall be kept for at least 6 months after the end of all tests.

8.2 Test procedure

8.2.1 The in vitro test on bioaccessibility is carried out only on small quantities of contaminated soil-like materials because there are generally only small quantities of contaminated soil-like materials present in the gastrointestinal tract^[31] and because the quantity of soil-like material in the test system affects the dissolution rate of particle-bound contaminants. For this reason, no more than 2 g of contaminated soil-like material should generally be put into a total of 200 ml of digestive juices (with regard to gastric and intestinal stage). At higher contents of contaminated soil and soil-like materials, impairment of the dissolution rate is possible, particularly of organic contaminants as a result of inactivation of digestive juice components, e.g. by sorption of these components onto the soil or soil-like materials.

8.2.2 The test is initially carried out in duplicate with two subsamples.

8.2.3 Each subsample of 2 g of contaminated soil or soil-like materials ≤ 2 mm is suspended in 100 ml of artificial gastric juice and 10 g of whole milk powder (resulting in 110 mL, see 7.2 for preparation). The pH value of the suspension is adjusted to $2,0 \pm 0,05$ using 10 % hydrochloric acid.

8.2.4 The suspension is agitated continuously for 2 h at 37 °C. This can be done in a shaking water bath with a thermostat at a shaking frequency of 200 min^{-1} . Alternatively, the assay can be stirred in a water bath with a thermostat and a magnetic stirrer using a polytetrafluorethylene-coated stirring rod.

If the pH value increases above 4,0, it shall be adjusted back to 2,5 using 10 % hydrochloric acid.

8.2.5 After 2 h, 100 ml of artificial intestinal juice is added to the digestion assay and the pH value of the assay is adjusted to 7,5 during 60 min to 90 min in the course of the test using NaHCO_3 (solid). The intestinal phase lasts 3 h.

8.2.6 After the pH-value plateaus at 7,5, the pH value of the assay is kept at 7,5 using the autotitrator or else manually using hydrochloric acid. The target pH value of 7,5 shall not be exceeded by more than 0,2 pH units at any time. For this purpose, the pH value shall be checked and documented at least every 10 min at the start of the intestinal stage and at larger intervals later on.

When the digestion assays are being agitated, particles from the soil-like material and components of the digestive juices can accumulate at the wall of the vessel above the aqueous phase. These deposited particles shall be shaken back into the suspension manually from time to time.

[Annex C](#) describes an example of an assay with artificial digestive juices, the monitoring of the pH value, and the resulting volumes.

NOTE In the case the pH value exceeds 7,7, it is possible that irreversible precipitation of inorganic contaminants occurs. If precipitation occurs, the measured concentration of the contaminant in the gastrointestinal phase solution would be too low (i.e. not reflecting the conditions at pH 7,5) and, hence, the in vitro test would underestimate the bioaccessible fraction of this contaminant.

8.3 Separation of dissolved contaminants from particle-bound contaminants

8.3.1 Immediately after completion of the intestinal phase, the test mixture is centrifuged for 20 min at $7\,000 \text{ g}$ (see [Figure 1](#)).

8.3.2 The dissolved contaminants remain in the supernatant after centrifugation (gastrointestinal phase solution); the particle-bound contaminants enter the centrifugation solid phase. The supernatant is decanted off.

8.3.3 The solid phase is fully suspended with 30 ml of distilled water and centrifuged again.

8.3.4 The resulting supernatant is decanted off again and is combined with the supernatant of the first centrifugation step. Only if necessary, the combined supernatants are filtered through a stainless-steel sieve

(mesh size 20 µm) for organic contaminants or through a plastic sieve for inorganic compounds (6.3.8). This ensures that any particles possibly present with a lower density (i.e. floating on the surface of the solution) are removed from the supernatant. The particles are added to the centrifugation solid phase.

8.3.5 For measurement of analytes in the centrifugation solid phases, they shall be processed as a whole, i.e. it is not feasible to divide them into aliquot portions. Thus, for each group of analytes, separate absorption assays are necessary.

8.4 Quantification

8.4.1 General

Chemical analysis of the relevant parameters is carried out in accordance with the standards appropriate for the quantification of the contaminant(s) to be measured, taking into account the areas of application specified in these standards (e.g. for inorganic substances ISO 11047, ISO 11885, ISO 17294-2, ISO 22036 or ISO 54321 and for organic substances ISO 18475, ISO 14154, ISO 18287, ISO 22478, EN 15308, EN 16181, EN 16190, and References [21],[24]-[26], or [28]). As a result of the use of whole milk powder, additional pretreatment – e.g. digestion in the case of heavy metals – or clean-up is generally necessary. If analysis cannot be carried out immediately after the experiment is carried out, the centrifugation supernatants and solid phases shall be stabilized by adding 1 ml of 20 % sodium azide solution (6.1.11) to each to prevent microbial decomposition.

8.4.2 Analysis of inorganic substances

To determine the fraction remaining on the particles in the digestion model, the entire quantity of the solid phase after centrifugation is transferred to a digestion vessel, dried and then digested with concentrated nitric acid. Aqua regia digestion is then carried out.

NOTE If predigestion occurs, a higher temperature can occur due to the higher heat capacity of the undiluted nitric acid.

Oxidative digestion is necessary for determination of the element concentration in the gastrointestinal phase solution due to the high fraction of organic components (particularly whole milk powder). Oxidative digestions are suitable, e.g. with nitric acid and hydrogen peroxide under pressure or with microwaves. In this way, 3 ml of nitric acid (65 % mass fraction) and 1 ml of hydrogen peroxide (30 % mass fraction) can be added to 25 ml of gastrointestinal phase solution. Heat to 170 °C for 15 min, hold at 170 °C for a further 15 min and then allow to cool for 15 min. Alternatively, 10 ml of gastrointestinal phase solution is treated with 7 ml of nitric acid (65 % mass fraction) and 2 ml of hydrogen peroxide (30 % mass fraction) for 4 h at 200 °C in a pressure tube.

8.4.3 Analysis of organic substances

For the chemical analysis of organic substances in the centrifugation solid phase and the gastrointestinal phase solution (supernatant), extraction and purification methods suitable for the respective substance should be used.

Due to the components of the artificial juices as well as the digested milk powder, analysis of organic substances can be challenging. The analytical method should include extraction and purification steps specific for the respective substance and should be tested in advance for efficiency and recovery. In the following, extraction methods for polycyclic aromatic hydrocarbons (PAHs) from both centrifugation solid phase and supernatant (i.e. gastrointestinal phase solution) are described as an example.

To carry out analysis of e.g. PAHs, the entire quantity of the centrifugation solid phase is extracted with a suitable extraction medium such as hexane or toluene, with the addition of up to 20 % (volume fraction) acetone if necessary, for at least 12 h at approximately 12 cycles per hour in a Soxhlet apparatus (6.3.4) with a fibre glass sleeve in order to determine the fraction remaining on the particles in the digestion model. Extraction may also be carried out using other suitable methods.

The gastrointestinal phase solution can be extracted using a 0,5 l rotation perforator (6.3.5) (continuous liquid-liquid extraction). If a perforator is used, the gastrointestinal phase solution is first placed in the rotation perforator and this is then made up to 600 ml by adding saturated NaCl solution. A layer of approximately 85 ml of extraction medium is then carefully added on top of this solution. Additionally, 75 ml of extraction medium is added to the collection vessel (100 ml to 250 ml flask). Extraction is carried out using hexane or toluene, with the addition of up to 20 % (volume fraction) ethyl acetate for slightly polar substances if necessary, for at least 8 h, and for polar substances for at least 12 h, at a distillation rate of 0,5 l/h and at a stirring speed of 700 min⁻¹.

Alternatively, the gastrointestinal phase solution can also be extracted in a separatory funnel. In this case, 5 g of NaCl and 10 ml of acetone are added to the solution and these are then shaken vigorously for 3 min with 30 ml of hexane with intermittent aeration. To break the emulsion formed during shaking, 5 ml to 10 ml of ethanol and the same quantity of saturated NaCl solution can be slowly added to the assay while swirling it. If the phase separation achieved in this way is not sufficient, centrifuging or other residue analysis methods can also be used. Extraction shall be repeated at least twice, and for polar substances four times, using 30 ml of hexane.

A further example of the extraction of polycyclic aromatic hydrocarbons (PAHs) from the gastrointestinal phase solution is described in [Annex D](#).

The organic solvent extracts of the centrifugation solid phase and of the supernatant (i.e. gastrointestinal phase solution) are diluted with respect to analytes but contain high concentrations of interfering matrix constituents. They contain components from bile and, as a result of the use of whole milk powder, also partially digested fats and lipids. Purification and concentration of analytes will generally be necessary. Food-chemistry methods or solid-phase extractions are available for this purpose (References [21]-[26], [28], EN 15662, EN 16190).

9 Calculation of bioaccessibility

The dissolved content of a contaminant, expressed as a mass fraction $w_{i,\text{mob}}$ in micrograms per gram of substance i of the solid sample, shall be calculated using [Formula \(1\)](#):

$$w_{i,\text{mob}} = \frac{\rho_i \cdot V}{m_E} \quad (1)$$

where

$w_{i,\text{mob}}$ is the dissolved content of substance i , in micrograms per gram (µg/g);

ρ_i is the measured concentration of substance i in the gastrointestinal phase solution, in micrograms per litre (µg/l);

V is the total volume, in litres (l);

m_E is the mass of solid sample added, in grams (g).

The bioaccessibility R_i , in per cent of substance i , is calculated using [Formula \(2\)](#):

$$R_i = \frac{w_{i,\text{mob}} \cdot 100 \%}{w_{i,\text{solid}}} \quad (2)$$

where

R_i is the bioaccessibility of substance i , in percent (%);

$w_{i,\text{mob}}$ is the dissolved content of substance i , in micrograms per gram (µg/g);

$w_{i,\text{solid}}$ is the total content of substance i in the solid sample, in micrograms per gram (µg/g).

The non-dissolved content (non-bioaccessible content, content in the centrifugation solid phase), expressed as a mass fraction $w_{i, \text{sed}}$ in micrograms per gram of substance i of the solid sample, shall be calculated using [Formula \(3\)](#):

$$w_{i, \text{sed}} = \frac{\rho_{i, \text{sed}} \cdot V_{\text{sed}}}{m_E} \quad (3)$$

where

- $w_{i, \text{sed}}$ is the non-dissolved content of substance i , in micrograms per gram ($\mu\text{g/g}$);
- $\rho_{i, \text{sed}}$ is the measured concentration of substance i , in micrograms per litre of centrifugation solid phase extract ($\mu\text{g/l}$);
- V_{sed} is the total volume of centrifugation solid phase extract, in litres (l);
- m_E is the mass of solid sample added, in grams (g).

10 Quality assurance

10.1 General

The requirements in ISO/IEC 17025 shall be followed. The quantification methods used shall be documented. When the method is (first) established in a laboratory, a known sample that is as homogeneous as possible, ideally a reference soil material or interlaboratory test material, is measured five times using an overall recovery and mass balance for every contaminant to be quantified.

Method performance characteristics for selected bioaccessible inorganic and organic substance fractions are listed in [Annex B](#).

10.2 Quantification of the bioaccessible content

Duplicate determinations are carried out.

The bioaccessible content in the supernatant is measured.

If the deviation between the two measurements is greater than $\pm 10 \%$ of the average value, an additional duplicate determination is carried out. However, if the closer of the two values differs from the national guideline value by more than the difference between the two values, these subsequent measurements are not necessary. After outliers have been eliminated, the bioaccessible content is calculated as the average value of the remaining measured values.

10.3 Mass balance and overall recovery

To check mass balance and overall recovery, the non-bioaccessible content in the centrifugation solid phase is also determined for both subsamples as part of the duplicate determination.

It shall be tested whether the analysed total content of substances and/or elements in the solid sample is equal to the sum of the dissolved content and the content in the centrifugation solid phases from the test system. Based on the duplicate determinations (for bioaccessible contents and contents in the centrifugation solid phase), the average values for the gastrointestinal phase solutions and the solid phases are calculated. If there are significant differences from the theoretical 100 % ($> 20 \%$), additional control measurements (see [10.4](#)) should be carried out.

NOTE Based on experience the user can define a lower limit depending on the soil or soil-like materials used.

10.4 Additional control measurements

If the requirements relating to the quantification of the bioaccessible content (10.2) and/or the mass balance and overall recovery (10.3) are not conformed with, additional control measurements are necessary.

Additional measurements of the total contents of the relevant contaminants shall be carried out based on the freshly prepared original sample in order to determine the sample-specific inhomogeneity. Three subsamples are prepared from the ≤ 2 mm fraction of the original sample and the total content of each of these subsamples is measured twice.

The average value of these 6 measurements and its standard deviation serve as the starting value for quantifying the total content. If additional measurement values are available, these can also be included. The coefficient of variation C_V for the total contents equals the standard deviation divided by the average value, expressed in per cent. The coefficient of variation for the bioaccessible contents is also calculated.

If the coefficient of variation of the measurements of the bioaccessible content is less than 10 % higher than the coefficient of variation of the measurements of the total content, the average value for the bioaccessible content can be accepted.

EXAMPLE $C_{V,\text{total}} = 6,7 \%$ and $C_{V,\text{bioaccessible}} = 15,4 \%$.

In the case of strongly inhomogeneous samples, it shall be decided whether the evaluation of the bioaccessibility can be omitted.

11 Test report

The test report shall contain at least the following information:

- a) a reference to this document, i.e. ISO 8259:2024;
- b) clear identification of the investigated sample;
- c) the type of contaminated soil or soil-like materials (matrix);
- d) the type of contamination;
- e) the available quantity of the investigated soil or soil-like materials, if less than 100 g is available;
- f) the quantity of investigated soil or soil-like materials entered into the test system;
- g) the particle size of the tested soil or soil-like materials (fraction ≤ 2 mm);
- h) the volume of the assays;
- i) deviations from the test procedure;
- j) the analytical methods for quantification of the dissolved and particle-bound chemical compounds and/or elements, and the standard deviation and detection limit of the relevant methods;
- k) the test results:
 - the content of the tested soil or soil-like materials (starting material) as a mass fraction $w_{i,\text{solid}}$, in mg/kg, for total extraction with solvents (organic contaminants) or for aqua regia extraction (inorganic contaminants), if necessary as individual values and as a calculated average value;
 - the dissolved content of the tested soil or soil-like materials as a mass fraction $w_{i,\text{mob}}$, in mg/kg, as individual values and as a calculated average value;
 - the bioaccessibility R_i in accordance with Formula (2) from the calculated average values;
 - the content in the centrifugation solid phase, in mg/kg, as individual values and as a calculated average value;

- the overall recovery and mass balance in accordance with [10.3](#);
- l) the date of testing;
- m) the signature of the person who carried out the test.

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Annex A (informative)

Enzymes and digestive aids

The following products have proven themselves in practice up to now¹⁾:

For 6.2.1	porcine mucin (M 1778)	Sigma-Aldrich®
For 6.2.2	porcine pancreatin (P 1625, P 1750)	Sigma-Aldrich®
For 6.2.3	pepsin (77 160)	Sigma-Aldrich®
For 6.2.4	trypsin (933 615, T 4799)	Sigma-Aldrich®
For 6.2.5	porcine bile extract (B 8631)	Sigma-Aldrich®
For 6.2.6	instant whole milk powder	commercially available product (small portion sizes)

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1) These are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

Annex B

(informative)

Method performance characteristics for selected bioaccessible inorganic and organic substance fractions

Interlaboratory testing was carried out on behalf of the German Federal Environment Agency (FKZ 3714 71 217 0) in 2016 to quantify bioaccessibility in accordance with DIN 19738 and the method performance characteristics were determined^[61].

The following soil materials were investigated for selected bioaccessible inorganic and organic substance fractions and mass balances/overall recoveries:

- soil A: contaminated with arsenic (As), antimony (Sb), lead (Pb) and cadmium (Cd);
- soil B: contaminated with polychlorinated biphenyls (PCBs); quantification was based on the sum of 6 PCBs ("PCB6", sum of PCB 28, PCB 52, PCB 101, PCB 138, PCB 153, PCB 180, nomenclature according to Ballschmitter);
- soil C: contaminated with 2,4,6-trinitrotoluene (TNT);
- soil D: contaminated with polycyclic aromatic hydrocarbons (PAHs); as a trigger value has only been specified for benzo[a]pyrene as an indicator substance for PAHs up to now, the evaluation was confined to this contaminant.

The method performance characteristics for the bioaccessible fractions are summarized in [Table B.1](#) to [Table B.4](#); the method performance characteristics for the overall recoveries are shown in [Table B.5](#) to [Table B.8](#).

Table B.1 — Method performance characteristics for the bioaccessible fractions of soil A

Parameter	l	n	o	$\bar{\bar{x}}$	s_R	$C_{V,R}$	s_r	$C_{V,r}$
As	7	42	12,5	37,2	3,5	9,49	3,2	8,58
Cd	8	48	0,0	38,8	18,4	47,28	7,3	18,85
Pb	7	42	12,5	11,5	4,3	37,44	2,0	17,39
Sb	5	30	37,5	23,7	6,3	26,55	3,2	13,58
Key l number of laboratories after elimination of outliers n number of outlier-free individual analysis values o fraction of outliers, in percent (%) $\bar{\bar{x}}$ total average value, in percent bioaccessible fraction (%) s_R reproducibility standard deviation, in per cent (%) $C_{V,R}$ reproducibility variation coefficient, in percent (%) s_r repeatability standard deviation, in percent (%) $C_{V,r}$ repeatability variation coefficient, in percent (%)								

Table B.2 — Method performance characteristics for the bioaccessible fraction of soil B

Parameter	l	n	o	$\bar{\bar{x}}$	s_R	$C_{V,R}$	s_r	$C_{V,r}$
PCB6	2	12	50,0	95,5	12,3	12,89	10,3	10,78
Key l number of laboratories after elimination of outliers n number of outlier-free individual analysis values o fraction of outliers, in percent (%) $\bar{\bar{x}}$ total average value, in percent bioaccessible fraction (%) s_R reproducibility standard deviation, in per cent (%) $C_{V,R}$ reproducibility variation coefficient, in percent (%) s_r repeatability standard deviation, in percent (%) $C_{V,r}$ repeatability variation coefficient, in percent (%)								

Table B.3 — Method performance characteristics for the bioaccessible fraction of soil C

Parameter	l	n	o	$\bar{\bar{x}}$	s_R	$C_{V,R}$	s_r	$C_{V,r}$
2,4,6-TNT	3	18	25,0	83,0	23,0	28,31	6,0	7,41
Key l number of laboratories after elimination of outliers n number of outlier-free individual analysis values o fraction of outliers, in percent (%) $\bar{\bar{x}}$ total average value, in percent bioaccessible fraction (%) s_R reproducibility standard deviation, in per cent (%) $C_{V,R}$ reproducibility variation coefficient, in percent (%) s_r repeatability standard deviation, in percent (%) $C_{V,r}$ repeatability variation coefficient, in percent (%)								

Table B.4 — Method performance characteristics for the bioaccessible fraction of soil D

Parameter	l	n	o	$\bar{\bar{x}}$	s_R	$C_{V,R}$	s_r	$C_{V,r}$
benzo[a]pyrene	5	30	28,6	57,91	17,02	29,40	4,17	7,19
Key l number of laboratories after elimination of outliers n number of outlier-free individual analysis values o fraction of outliers, in percent (%) $\bar{\bar{x}}$ total average value, in percent bioaccessible fraction (%) s_R reproducibility standard deviation, in per cent (%) $C_{V,R}$ reproducibility variation coefficient, in percent (%) s_r repeatability standard deviation, in percent (%) $C_{V,r}$ repeatability variation coefficient, in percent (%)								

Table B.5 — Method performance characteristics for the overall recoveries of soil A

Parameter	l	n	o	$\bar{\bar{x}}$	s_R	$C_{V,R}$	s_r	$C_{V,r}$
As	8	48	0	96,2	13,8	14,33	6,3	6,57
Cd	7	42	12,5	91,9	12,1	13,20	9,2	9,99
Pb	4	23	52,1	97,7	6,5	6,69	6,2	6,37
Sb	7	42	12,5	82,8	20,8	25,10	6,6	8,03
Key l number of laboratories after elimination of outliers n number of outlier-free individual analysis values o fraction of outliers, in percent (%) $\bar{\bar{x}}$ total average value, overall recovery in percent (%) s_R reproducibility standard deviation, in per cent (%) $C_{V,R}$ reproducibility variation coefficient, in percent (%) s_r repeatability standard deviation, in percent (%) $C_{V,r}$ repeatability variation coefficient, in percent (%)								

Table B.6 — Method performance characteristics for the overall recovery in soil B

Parameter	l	n	o	$\bar{\bar{x}}$	s_R	$C_{V,R}$	s_r	$C_{V,r}$
PCB6	2	12	60,0	101,5	10,2	10,03	10,2	10,03
Key l number of laboratories after elimination of outliers n number of outlier-free individual analysis values o fraction of outliers, in percent (%) $\bar{\bar{x}}$ total average value, overall recovery in percent (%) s_R reproducibility standard deviation, in per cent (%) $C_{V,R}$ reproducibility variation coefficient, in percent (%) s_r repeatability standard deviation, in percent (%) $C_{V,r}$ repeatability variation coefficient, in percent (%)								

Table B.7 — Method performance characteristics for the overall recovery in soil C

Parameter	l	n	o	$\bar{\bar{x}}$	s_R	$C_{V,R}$	s_r	$C_{V,r}$
2,4,6-TNT	3	18	40,0	83,8	22,3	26,64	6,2	7,45
Key l number of laboratories after elimination of outliers n number of outlier-free individual analysis values o fraction of outliers, in percent (%) $\bar{\bar{x}}$ total average value, overall recovery in percent (%) s_R reproducibility standard deviation, in per cent (%) $C_{V,R}$ reproducibility variation coefficient, in percent (%) s_r repeatability standard deviation, in percent (%) $C_{V,r}$ repeatability variation coefficient, in percent (%)								

Table B.8 — Method performance characteristics for the overall recovery in soil D

Parameter	l	n	o	$\bar{\bar{x}}$	s_R	$C_{V,R}$	s_r	$C_{V,r}$
benzo[a]pyrene	7	42	0	87,8	16,5	18,80	5,9	6,74
Key l number of laboratories after elimination of outliers n number of outlier-free individual analysis values o fraction of outliers, in percent (%) $\bar{\bar{x}}$ total average value, in percent bioaccessible fraction (%) s_R reproducibility standard deviation, in per cent (%) $C_{V,R}$ reproducibility variation coefficient, in percent (%) s_r repeatability standard deviation, in percent (%) $C_{V,r}$ repeatability variation coefficient, in percent (%)								

NOTE The number of interlaboratory testing participants is critical in the statistical evaluation of interlaboratory testing results. As a result, the statistical evaluation is regarded as reliable/secure for 8 participants or more, while a relatively high level of uncertainty is associated with the total average values for a lower number of participants and the determined performance characteristics then tend to be regarded as informative in nature. However, to give users of this method the opportunity to test and classify their results with respect to statistical characteristics, the determined performance characteristics of all soils used in interlaboratory testing are given regardless of the number of participants.

Annex C (informative)

Example of an assay with artificial digestive juices, adjusting the pH value and resulting volumes

The samples of 2,0 g each weighed out and placed in the reaction vessel are moistened with a few drops of water so that they are wetted by the gastric juice as quickly as possible.

1 230 ml of artificial gastric juice is prepared for 12 assays (6 samples in duplicate). 120 g of whole milk powder is then suspended in this for approximately 10 min directly before the start of the stomach stage. The pH value is adjusted to 2,0 by adding approximately 12 ml of diluted hydrochloric acid (approximately 18 %). Further addition of acid during the stomach stage is not necessary for most samples. 2 g of each sample is added to 110 ml of this mixture, and this is incubated in the water bath at 37 °C.

At the start of the intestinal stage, 100 ml of artificial intestinal juice and 2,3 g of solid sodium hydrogen carbonate are added to each sample. Experience shows that the pH value then increases exponentially to 7,5 over the course of the three-hour intestinal phase and does not exceed this value. As a result, further addition of acid or base is generally not necessary here either.

2 g of soil sample in 210 ml of absorption assay after centrifugation results in approximately 190 ml of gastrointestinal phase solution (supernatant). The solid phase after centrifugation is suspended in 20 ml of water again, centrifuged again and the supernatant is added to the gastrointestinal phase solution. This results in approximately 210 ml of combined gastrointestinal phase solutions including the supernatant of the second centrifugation step for every 2 g of sample.

Annex D (informative)

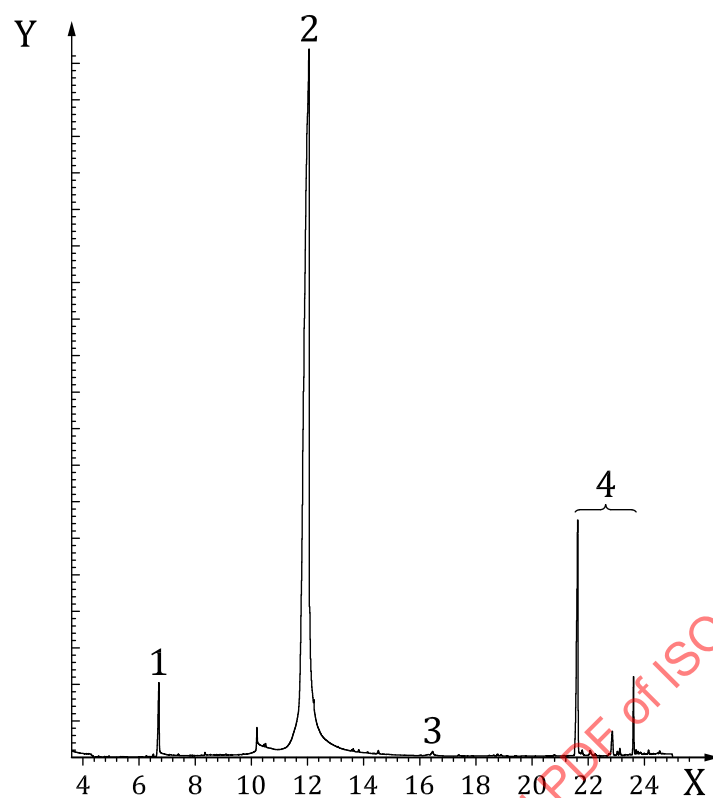
Example of the extraction of polycyclic aromatic hydrocarbons (PAHs) from the gastrointestinal phase solution

210 ml of golden-yellow, turbid suspension is obtained from 2 g of soil sample as gastrointestinal phase solution (including the supernatant of the second centrifugation step). This suspension is homogenized by stirring and 70 ml is removed, corresponding to 0,66 g of soil sample, and processed for PAHs. 5 g of sodium chloride, 5 ml of acetone and an internal standard, such as deuterated PAHs, are added to this. The mixture is extracted three times with 15 ml of hexane each time. The hexane phase can be removed almost completely after centrifugation; the entrainment of a little of the turbid intermediate phase is unproblematic. If a useful phase separation is not obtained, up to 10 ml more acetone can be added.

The centrifugation solid phase cannot be aliquoted. It is completely transferred from the centrifuge beaker with a small volume of water into a suitable glass and shaken with 5 g of sodium chloride, 50 ml of acetone, the internal standard and 25 ml of hexane for 6 h. The mixture is made up with water, the hexane phase is completely removed with a small volume of the aqueous phase, this is extracted again with 50 ml of water and dried over sodium sulfate after centrifugation. The remaining mixture is extracted again with 10 ml of hexane in the same way (based on Reference [25], ISO 18287 or EN 16181).

Subsequently, the hexane extracts are cleaned up individually by means of solid-phase extraction. To do this, the relevant extract is dried over sodium sulfate and fed into a polystyrene column conditioned with hexane (e.g. Chromabond HR-P, Macherey & Nagel), without vacuum if possible (freely dropping). After this, the column is flushed with 5 ml of hexane with injection pressure, dried with argon or nitrogen, and eluted with approximately 8 ml of toluene. The toluene eluate is made up to 10 ml and measured against corresponding standard solutions using GC-MS (based on Macherey & Nagel Application Guide 301290). The signal strengths of the internal standards in the eluates relative to the standard solutions should be recorded in table form.

[Figure D.1](#) shows a chromatogram of a toluene eluate of this type with residual matrix interferences, compared with a chromatogram of a standard solution ([Figure D.2](#)). [Table D.1](#) shows the detected substances from a blank solution (gastrointestinal phase solution obtained without soil or soil-like material), with 10 µg of each component added, in two parallel measurements (target concentration in the toluene eluate: 1 µg/ml).

**Key**

X retention time, in min

Y relative abundance

1 lauric acid

2 palmitic acid, oleic acid and linoleic acid

3 oleic acid monoglyceride

4 steroids

Figure D.1 — Chromatogram of a toluene eluate with residual matrix interferences

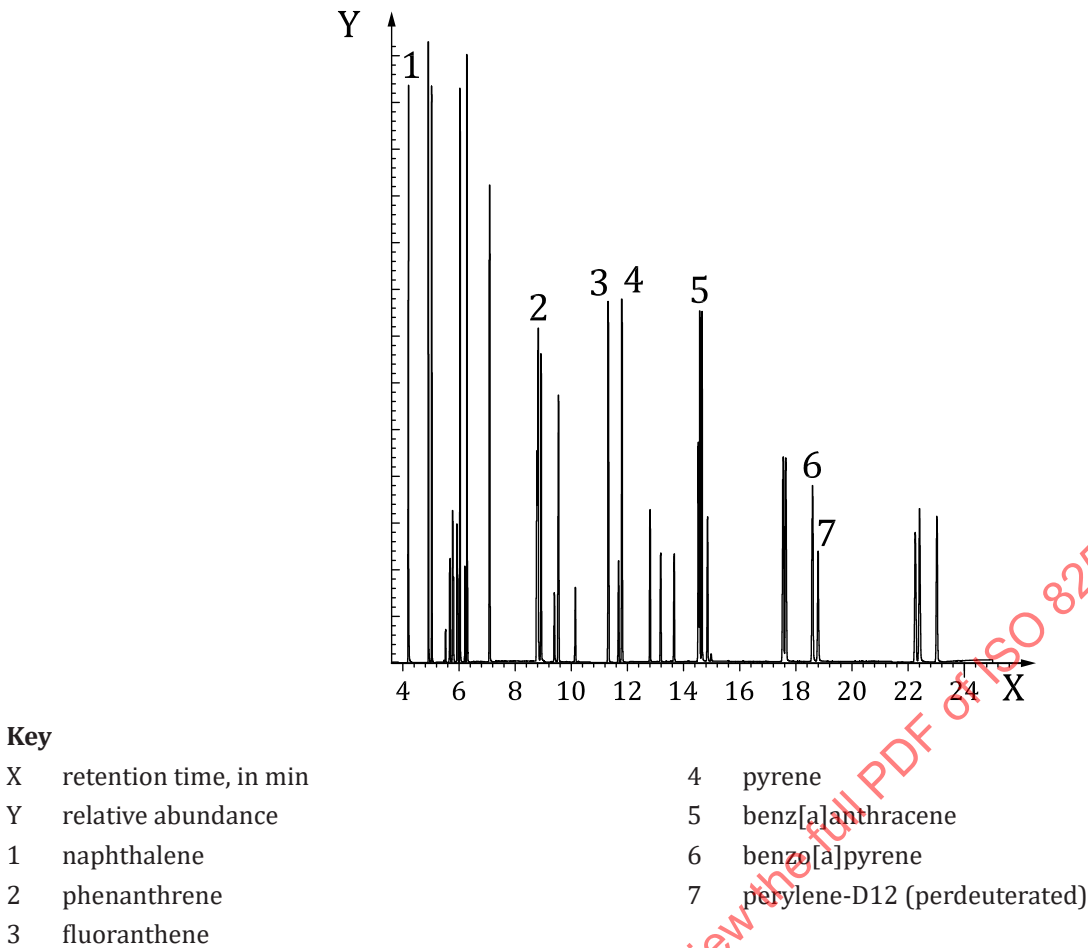


Figure D.2 — Chromatogram of a standard solution