The Standard Operating Procedure for Bioelution Testing of Metals, Inorganic Metal Compounds, and Complex Metal-Containing Materials: Simulated Gastric Fluid

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1. Purpose of the test

The systemic toxicity of most metals and metalloids is associated to a large degree with the release of metal ions and their uptake by the body and/or interaction at their target organ sites (i.e. their bioavailability). The simple presence of a metal or inorganic ion in a substance does not necessarily impart to that substance the biological properties of the released metal/inorganic species; it is the bioavailability of the released metal at the site of action in the organism that is the most important factor determining toxicity for metals and minerals.

Information on bioavailability can be derived from *in vivo* sources such as toxicokinetic or toxicological test data, or predicted using different models. In situations where the **bioavailability** of a metal or mineral substance is not known or where it is not feasible to determine this *in vivo*, the amount of released metal "available for absorption" may be measured using *in vitro* methods (**bioaccessibility**). **Bioelution** refers to these *in vitro* methods, which are used to measure the degree to which a substance is released (e.g. as metal ions) into simulated biological fluids. The purpose of such physico-chemical tests is thus to determine the bioaccessibility of metals from various metal-containing materials in test media mimicking physiological conditions.

The proposed gastric bioelution test method is designed to measure metal releases in artificial gastric fluid and provide an *in vitro* estimate of oral bioaccessibility of metals from metal containing substances, complex metal-containing materials like alloys, UVCBs and pigments. This test can be used to compare the metal releases from reference and target materials as described under section 2. Applicability and section 6.5. Data analysis.

This Standard Operating Procedure (SOP) specifically addresses a bioelution test in simulated gastric fluid, relevant for the oral route. This SOP builds upon the experience gained from a round robin study examining the performance of the gastric bioelution test system when applied to various metal-containing materials (Henderson et al. 2014).

2. Applicability of the test

The gastric fluid bioelution test applies to all metals, and inorganic metal compounds, or complex metal containing materials (e.g. alloys, UVBCs, pigments), in massive form (≥ 1mm) or powder form (≥ 0.1µm - <1mm).

Bioelution test data in gastric fluid can be used in different applications, but always in a comparative way, so as to determine relative metal bioaccessibility. Below are the two main applications of this SOP under consideration by ECVAM.

Application 1: using relative bioaccessibility for grouping and read-across

Bioelution test data are used to group substances of a given metal or complex materials containing the metal, based on similar metal bioaccessibility in gastric fluid. Relative oral bioaccessibility is considered to be predictive of relative oral bioavailability. Test data can be used to contribute (in a weight of evidence approach) to read-across (for substances) and bridging (for mixtures), so as to fulfil human health data requirements and/or classification under chemical management programmes.

Application 2: calculate the bioaccessible concentration of metal ingredients in alloys.

The test can also be used to compare metal releases from alloys to those from their pure metal ingredients and to calculate the bioaccessible concentration of the metal in the alloy for classification purposes.

The way to process the data for these two application is further explained under Annex 1.

Importantly, this SOP **does not apply to** nanomaterials as defined by the EU Commission in 2011 (e.g. a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm).

3. Test materials

3.1. Types of test material

Test materials shall be metals, inorganic metal compounds, or complex metal-containing materials like e.g. alloys, UVCBs, pigments.

Materials can be in powder or massive form, and should be tested in the form that is placed on the market or in which they can reasonably be expected to be used[1]. Depending on the type of test material and physical form, specific sample preparation and test execution applies. In summary:

- **When materials on the market are present in micron-size powder form (defined as particles < 1 mm diameter, but above 100 nm diameter)**, additionally testing the subfraction of particles <100 µm

[1] For example, Article 5 of the EU CLP states: *The information shall relate to the forms or physical states in which the substance is placed on the market and in which it can reasonably be expected to be used*. The ECHA CLP Guidance (2017) indicates: "*Samples offered for testing must in all aspects be representative of the substance or mixture to be classified."* And it goes on to state that *"In some cases, additional parameters like (e.g.) physical condition, particle size and shape, specific surface area, density, crystal structure, may influence the test result. Therefore, these properties should be mentioned in the test report. The tests must be performed on the substance or mixture in the appropriate physical form where changes in that form may influence the outcome of the test (see also Articles 5 and 6 of CLP).*

in diameter is recommended as it is also relevant to the fraction of the inhalation exposure that is swallowed and becomes bioavailable through the oral route.

- **If the material on the market is present only as massive surfaces**, this form should be tested. The massive (e.g. metal or alloy) material can be tested as it is or tested as an epoxy embedded sample. Embedding samples in epoxy resin may facilitate sample handling and provide a standardised exposed surface. However, care must be taken since grinding and polishing operations during sample preparation may cause the exposed surface to no longer be representative of the as received material (this may be overcome by allowing the polished surface to be oxidized for a specified period of time), and it may also introduce crevices at the sample/epoxy resin interface that promote enhanced corrosion/metal release (see also 3.4 Test material handling).

- **If particles are expected to be generated from the massive forms during normal handling and use, then representative particles should be tested**. In order to obtain the desired particle size, potential methodologies for sample preparation include crushing, grinding, milling, and/or sieving). Of these methods, sieving is always preferred, as this method does not impart any physical or chemical changes to the sample (e.g. possible surface oxidation/passivation). Crushing, grinding, and milling operations can have a significant effect on metal release from some alloys and should only be considered when sieving is not feasible. Under these circumstances, it will be important to test the metal release from the material both as initially received and after being crushed/ground/milled in order to establish whether the crushing/grinding/milling operation has had an impact on the release properties of the test material. Characterisation of the surface oxides after crushing/grinding/milling operations, can be important to interpret the metal release results before and after crushing/grinding/milling, as this is the interface that governs the release process. Any such change of surface properties (including its reactivity) must be recorded and reported as well as the relative difference in surface area exposed to the test medium.

3.2. Test material identification and characterisation

For reasons of traceability, a test material and accompanying documentation (e.g. certificate of analysis) must be properly identified. The information needed is described under section 6.8. Reporting and Record Keeping.

A Material Safety Data Sheets (MSDS) or equivalent should accompany the test materials for safe handling and storage of the materials.

3.3 Test material storage

For all test materials: It is important to perform a visual inspection and follow any storage instructions provided by the manufacturer or supplier (e.g. powders should be stored under a nitrogen or an inert atmosphere, stored in a sealed container, etc). In the absence of storage instructions from the manufacturer or supplier, materials should be stored at room temperature in a dry place and at dark conditions to protect the test material from moisture and further surface oxidation.

3.4 Test material handing

- **Powder material:** Powder particles shall be weighed in dry, acid- cleaned chemical-resistant glass or polymeric test vessels by using spatulas or cleaned spoons.
  With regard to the dispersion of particles, the following recommendations are provided for guidance. Gentle agitation of the test media may improve dispersion. However, as it may significantly influence the metal release, ultrasonic treatment ***shall not be used*** for the particle dispersion of powder test material in test medium, as it can lead to surface changes[2].

- **Massive material- non-epoxy embedded:** where massive samples (e.g. in the form of discs, sheets, etc.) are received for testing, record the weight, geometry, surface finish and the geometric surface area of the sample. Massive material samples should, generally be tested in their as received condition (i.e. in the form, and with the surface, in which they are placed on the market).
  Where test samples are prepared from the original as received material (**non-embedded)**, cut edges shall be wet ground to 1200 grit (SiC paper) in order to provide flat surfaces to aid area measurements and to prevent lacerations from sharp edges. All wet grinding shall be followed immediately by mild ultrasonic cleaning in ethanol for 10 min and subsequently in isopropyl alcohol for 10 minutes[4]. If, however, the as received surface of the massive material has been damaged by the sampling operation (e.g. scratched), the test surface and cut edges shall be prepared for testing by wet grinding with silicon carbide papers from coarse to fine grit, until a uniform surface finish with 1200 grit is attained. Grinding surface finishing should be followed immediately by mild ultrasonic cleaning in ethanol for 10 minutes and subsequently in isopropyl alcohol for 10 minutes[3]. Afterwards, the samples shall be dried under a stream of cold (not hot) nitrogen gas and stored in a desiccator for a minimum of 168 hours prior to the start of the test.
  During the testing, care shall be taken to avoid the creation of crevices (not normally present in the sample) that may lead to increased corrosion and release of metals. For example, where test samples are drilled and suspended in the solution on cotton or wire ligaments looped through the drilled holes.

- **Massive material - epoxy embedded sample**: massive material can also be tested as an epoxy embedded sample. The preparation of such samples is briefly described below and occurs in three steps: cutting the massive metal, epoxy embedding and surface preparation:

  a. Cutting: typically, coupons are cut from the massive metal by suitable means (e.g. water-cutting, laser cutting, hacksaw, shears, etc.) depending on the size and shape of the sample. It is important that whatever technique is selected, it should not alter the properties of the material. The samples are degreased with ethanol before embedding in epoxy resin.

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[2] This is an important consideration, because for the toxicology testing of nanopowders, sonication is recommended as a way to avoid agglomeration.

[3] Mild ultrasonication is recommended to avoid introducing surface changes in massive materials.

b. Embedding: for embedding the samples one can use EpoFix Kit from Struers or equivalent. The polymerisation process is done as directed by the resin supplier, e.g. Struers, as follows: to prepare the resin fluid an amount of hardener is mixed into the correct amount of resin (25 parts of resin / 3 parts of hardener by weight). The mixture is mixed well for approximately 2 minutes without introducing too many air bubbles. The mixture is left to rest for 2 minutes (pot life of this mixture: about 30 minutes). The epoxy is carefully poured over the specimen in an achieved mould. A vacuum system can be used to reduce air bubbles. The embedded samples are left at room temperature for hardening during at least 12 hours.

c. Surface preparation: the surface preparation may depend on the nature and hardness of the test material. This is best followed by the instructions of the manufacturer of the used polishing machine (e.g. Struers). Ultimately, a uniform and scratch-free surface should be obtained. The followed polishing step must be well documented and subsequently reported. The following procedure was for example well applicable for medium hard materials:

   Each epoxy sample is wet ground with 400 grit, 800 grit, 1200 grit and finally 2000 grit SiC paper in sequence. In order to avoid maintaining a memory imprint of the grinding scratches deeper into the bulk material matrix, it is important to apply only a very gentle pressure during the grinding. Each sample is ground only for approximately one minute on each grit size paper with very gentle pressure, recognizing that hard and soft surfaces may require different grinding times. After wet grinding, the samples are rinsed with ethanol and dried with nitrogen gas to avoid corrosion during the sample preparation. Polishing is performed using 3, 1 and 0.25 µm diamond paste (DP-Paste M) in turn with ethanol as a lubricant, and with utmost care. To obtain smooth scratch-free surfaces, the samples are held very gently against the polishing cloth with very little applied pressure. Plenty of ethanol is used as a lubricant to obtain a well-defined surface. Each polishing step with diamond paste takes about 5 to 10 minutes, depending on the type of material. After polishing the samples are cleaned with ethanol and quickly dried with cold nitrogen gas. To remove any residual particles left on the surface after grinding and polishing, all samples are cleaned ultrasonically first in ethanol for approximately 6 minutes followed by ultrasonic cleaning in isopropyl alcohol for another 6 minutes, and a final rinsing with isopropyl alcohol. Isopropyl alcohol is used as the last cleaning medium. After the ultrasonic cleaning, all samples are dried quickly again with cold nitrogen gas.

The polished surface is checked by means of a microscope. The metal surfaces shall be free from scratches and spots, otherwise, re-polishing is necessary.

The samples are stored for a minimum of 168 hours in a desiccator before testing.

While in most cases the average free metal surface is about 100 mm², the exact exposed surface should always be measured and reported.

Figure 1. Example of polished surfaces embedded in epoxy resins



4. Test system

The test system is the whole of the chosen test vessel (usually 250 mL Erlenmeyer flask) with the appropriate volume of test medium (a minimum of 50 mL).

4.1. Test vessels

Inert, chemical-resistant Erlenmeyer flasks (e.g. Polyethylene Terephthalate Glycol (PETG) or borosilicate glass) of 250 mL or larger vessels in case of testing massives should be used for testing[4]. The vessels shall be properly covered (e.g. screw caps, silicon or rubber stoppers) to avoid (cross)contamination or evaporation of the test medium[5].

All test vessels, whether new or used, shall be cleaned, to reduce possible background levels for the elements of interest before use and to be suitable for working with metals at trace levels. Cleaning procedure: acid soak for 24h in 10% HNO_3 then rinse at least 4x in ultrapure water (e.g. MilliQ water 18.2MΩ·cm) and dry (by air at room temperature or in low temperature, < 50 ºC oven). This applies to new and re-used test vessels.

For the handling of powders, prior to each use, spatulas or spoons shall, as a minimum, be carefully cleaned in ultrapure water and isopropyl alcohol or, preferably, acid-cleaned in the manner described above. Do not

[4] Care shall be taken to ensure that the test vessels do not interact with the test material, either by causing abrasion or releasing metal ions to the test medium. Abrasion has been observed with some materials like slags in borosilicate glass. Adsorption to vessel walls can also occur (e.g. Ag). Borosilicate can also contribute Si and B to the test medium and should be avoided when Si or B are elements of interest (e.g. silicates).

[5] In case where release of gas can be expected (e.g. carbonate salts brought in an acidic environment), care needs to be taken that no pressure builds up in the vessel (avoid too tight-fitting caps).

use the same spatula or spoon on different test samples unless it has undergone cleaning after the previous transfer process.

4.2. Test Medium.

The test medium is simulated gastric fluid (0.07N HCl solution brought at 37 ± 1 °C and pH 1.5 ± 0.1). The chemical composition is shown in Table 1 and follows the Standards ASTM D5517 (2014), ASTM F963 (2017), and BS EN 71-3 (2013, 2017).

The solution of the simulated gastric fluid has to be prepared freshly every day.

Table 1. Chemical composition of simulated gastric fluid (0.07N HCl).

Chemical	Amount
HCl (analytical grade or better)	2.55 g (e.g. 7.41 mL HCl 30 vol.-% or 5.9 mL HCl 37 vol.-%)
Ultrapure water (18.2 MΩ cm)	Add ultrapure water up to about 900-950 mL
Temperature	Bring the temperature of the test system to 37 ± 1 °C (e.g. water bath)
NaOH solution	Adjust pH to pH 1.5 (± 0.1)
Ultrapure water	Add ultrapure water (37 °C) up to 1 L

5. Equipment

5.1 Agitation equipment

Thermostated linear (horizontal) or orbital shaker (37 ±1°C, 100 rpm; stroke length=1 inch) can be used to provide agitation.

Agitation at 100 rpm is selected as a means to provide a gentle agitation that is sufficient to maintain the flow of the aqueous medium over the test materials, while maintaining the integrity of the surface of the test materials and any solid reaction product coatings formed during the test. Abrasion of particles or sample surfaces will in this way be be minimized.

5.2 Thermometer

Controlled thermometer, readable to 0.1 °C.

5.3 pH meter

Calibrated pH meter readable to 0.01 pH unit.

5.4 Balance

A calibrated microbalance at least readable to 0.01 mg; controlled with standard weights daily before and after each use. Two decimal numbers should be reported.

5.5 Filtration equipment

The use of filtration to separate undissolved from dissolved metal ions is in line with ASTM 5517.

Single use 0.2 µm membrane filters, e.g. Whatman UNIFLO syringe filters, Pall Acrodisc syringe filters, or equivalent filter system should be used, as these filters do not absorb or release metals in any significant manner.

Note: The effectiveness of the filter to separate dissolved from undissolved metal for a particular test material can be confirmed by visual inspection and/or light scattering or equivalent method. Any particles <0.2 µm can in theory pass the filter and will be counted as soluble ions. This could result in overestimating the bioaccessibility of the metal from the sample (conservative approach).

Appropriate single-use latex- and oil-free (e.g. disposable polypropylene) syringes (volume depending on the amount required for the analysis technique used) shall be used.

Appropriate high-density polypropylene sample tubes known not to release or adsorb significant amounts of the metals of interest shall be used.

6. Experimental Procedures

6.1 Sample loadings

Two loadings (0.2 and 2 g /L) are included so that materials that release low amounts of ions can be reliably assessed. The physical form of the test material (i.e. powders, non epoxy embedded massive samples, epoxy embedded massive samples) determines how loading is defined, see further instructions under 6.2 Test setup).

Note: it is up to the sponsor to provide a representative sample of the test material with a complete characterisation (as described in section 6.8 Reporting and Record-keeping). For powders, if there is a small amount of sample, the test material should be mixed in between weighing the three replicates.

6.2 Test setup

At least ten pre-heated test vessels shall be set up. The size of the vessels (minimum volume 250 mL) or the amount of sample and volume of solution added to these vessels will be determined for each of the 2 loadings by the sample characteristics and analytical needs. A minimum of 50 mL of gastric fluid is needed with a liquid volume to headspace volume ratio of about 1:4. This flexibility is needed to make sure that enough fluid is available to cover all the surfaces of massive samples that come in different shapes (whether epoxy embedded or not). The test set up depends on the physical form of the test material.

Note: At the request of the sponsor, additional vessels can be added to the standard test set up as described below for various reasons (additional negative, positive control and replica test material vessels).

6.2.1 Powder test materials

- Ten pre-heated Erlenmeyer flasks (250 mL) shall be set up as follows:
  - ✓ **1 negative control vessel**
    contains only the test medium
  - ✓ **1 positive control vessel**
    contains a solution of the element(s) of interest to be added to the test medium, (preferably) at a concentration that can be expected to be released from the test material
  - ✓ **4 test material vessels at 0.2 g/L loading:**
    approximately 10 mg (± 0.5 mg) test material shall be weighed into each of the 3 replica test material vessels and 1 extra test material vessel (for pH measurement at the start of the test). The exact weight shall be noted.
  - ✓ **4 test material vessels at 2 g/L loading:**
    approximately 100 mg (± 0.5 mg) test material shall be weighed into each of the 3 replica test material vessels and 1 extra test material vessel (for pH measurement at the start of the test).

- A volume of simulated gastric fluid consistent with desired loading (e.g. 50 mL in above examples) at pH of 1.5, preheated to 37 ± 1°C shall be added to the negative control vessel, to each of the eight test material vessels and to the positive control vessel.

- Swirling is not recommended for powders, as they may adhere to the vessel walls.

- All test vessels shall then be covered as soon as possible (within a minute of mixing) and placed in a thermostatic shaker (37 ± 1°C) for 2 hours: 1 hour at an agitation rate of 100 rpm followed by 1 hour without agitation. The test should be performed in the dark.

- The temperature and pH of the extra vessels (with the test material for pH measurement) shall be measured and recorded (t= 0 hour values). These flasks and their content can then be discarded.

6.2.2 Massive non epoxy embedded test materials

These materials include e.g. non-embedded coupons or sheets, or massive materials placed on the market as coarse pellets or granules (≥1 mm).

- Ten pre-heated Erlenmeyer flasks (250 mL) shall be set up as follows:
  - ✓ **1 negative control vessel**
    contains only the test medium
  - ✓ **1 positive control vessel**
    contains a solution of the element(s) of interest to be added to the test medium at a (preferably) concentration that can be expected to be released with the test material
  - ✓ **4 test material vessels at 0.2 g/L loading:**
    one or more pieces of test material shall be weighed into each of the 3 replica test material vessels and 1 extra vessel (for pH measurement at the start of the test)
  - ✓ **4 test material vessels at 2 g/L loading:**
    one or more pieces of test material shall be weighed into each of the 3 replica test material vessels and 1 extra vessel (for pH measurement at the start of the test).

Note: for non epoxy embedded massive samples (e.g. sheets, ≥ 1 mm pellets or granules), the weight of the sample can be used to modify the volume of fluid and comply with the desired loading. [A surface equivalent loading can also be calculated for M^o and alloy samples using the geometric exposed surface area of the sample.]

- A volume of simulated gastric fluid consistent with desired loading (e.g. 50 mL in above examples) at pH of 1.5, preheated to 37 ± 1°C shall be added to the negative control vessel, to each of the eight test material vessels and to the positive control vessel.

- Gently swirl and visually inspect the vessels to ensure that the sample is covered by fluid and/or the coarse pellets are dispersed in the solution.

- All test vessels shall then be covered as soon as possible and placed in a thermostatic shaker (37 ± 1°C) for 2 hours: 1 hour at an agitation rate of 100 rpm followed by 1 hour without agitation. The test should be performed in the dark.

- The temperature and pH of the additional vessels (with the test material) shall be measured and recorded (t= 0 hours values). These flasks and their content can then be discarded.

6.2.3 Massive epoxy embedded test materials (surface loaded)

- Ten pre-heated suitable sized vessels shall be set up shall be set up as follows:
  - ✓ **1 negative control vessel**
    a blank piece of epoxy resin to be added to the test medium
  - ✓ **1 positive control vessel**
    a blank piece of epoxy resin + a solution of the element(s) of interest to be added to the test medium at a (preferably) concentration that can be expected to be released with the test material
  - ✓ **4 test material vessels at 0.2 g/L surface equivalent loading**:
    pieces of epoxy resin with test material with a known exposed metal surface shall be introduced into each of the 3 test material vessels and 1 extra vessel for pH measurement
  - ✓ **4 test material vessels at 2 g/L surface equivalent loading**:
    pieces of epoxy resin with test material with a known exposed metal surface shall be introduced into each of the 3 test material vessels and 1 extra vessel for pH measurement.

  Note: The loading of epoxy-embedded massive samples (2g/L or 0.2g/L) can be expressed as surface equivalent. The metal release from these materials will be reported in 2 ways: 1) expressed per unit of volume of liquid or per unit of mass, and 2) expressed per unit of surface.

- An appropriate amount of simulated gastric fluid (pH 1.5, preheated to 37 ± 1°C, and no less than 50 mL) shall be added to the negative control vessel and to each of the eight test material vessels to achieve the desired "surface" loading[6]. The positive control vessel with the solution of the element(s) of interest. will be filled with the same volume.

- Swirl and visually inspect the vessels to ensure that the sample is facing up and is well covered by fluid;

- All test vessels shall then be covered as soon as possible and placed in a thermostatic shaker (37 ± 1°C) for 2 hours: 1hour at an agitation rate of 100 rpm followed by 1 hour without agitation. The test should be performed in the dark.

- The temperature and pH of the additional vessels (with the test material) shall be measured and recorded (t= 0 hours values). These flasks and their content can then be discarded

Note: Surface equivalent loading calculation:

a) Starting from a selected mass loading (e.g. 2 g/L) and with knowledge of the test material's density, a surface equivalent loading can be calculated by considering the massive material as a 1 mm diameter sphere. 1 mm is the particle diameter which is the generally accepted to be the cut-off between powders

[6] It is important to record the surface area to volume ratio for massive materials.

and massives (e.g. for aquatic toxicity classification under ECHA CLP Guidance section IV.5.5 rev. 5 2017 or in Annex 9 of the UN GHS A9.7.5.4.3 GHS rev. 6 2015).

b) A spherical particle with a diameter of 1 mm has a surface area of 3.14 mm^2 ($4 \cdot \pi \cdot r^2$) and a volume of 0.524 mm^3 ($\frac{4}{3} \cdot \pi \cdot r^3$). Using the information on density of the material (e.g. 8.38 mg/mm^3), it can be calculated that a Ø 1 mm spherical reference particle has a mass (mg) = density (mg/mm^3) x volume (mm^3). In the example: 8.38 mg/mm^3 x 0.524 mm^3 = 4.39 mg per particle.
c) A loading of e.g. 2 g/L means 2000 mg divided by 4.39 mg (the weight of a particle) to calculate the number of particles needed per liter of fluid (i.e. 456 particles/L in the example above).
d) Since each particle has a surface area of 3.14 mm^2, a surface loading (e.g. in mm^2/L) can be calculated. In the example, a 2 g/L loading corresponds to 456 reference spheres and a total free metal surface loading of 1433 mm^2/L (456 particles x 3.14 mm^2).
e) So, to test a massive material with a density of 8.38 mg/mm^3 according to the embedding process, an exposed metal surface of 1433 mm^2/L is required. For a standard test volume of 50 mL this means an exposed metal surface area of 71.65 mm^2 (1433 mm^2/L x 0.05 L = 71.65 mm^2). The loading for other available surface areas can be adjusted by adding the corresponding amount of test medium.

6.3 Observations, sampling and measurements at the end of the test

Any visual observations of e.g. precipitation, colouration, decolouration that differ between replicate test vessels or are consistent for all three replicates, should be recorded as they can help explain the bioelution results.

The following sampling procedure shall be used to collect and preserve the samples for elemental analysis.

- At the end of the incubation period (2 hours), two samples (e.g. 15 mL, dependent upon the analytical set-up of each laboratory) shall be pipetted from each test vessel at a depth of 2/3 up from the bottom of the liquid, filtered through a 0.2 µm filter (e.g. syringe), and transferred to uniquely labelled (e.g. 15 mL polypropylene) sample tubes for analysis. The filter should be prewetted with 1 mL of test solution before filtration of test material to avoid metal adsorption to the filter.
- The samples will be preserved with 1% HNO_3 (100µL $HNO_{3(concentrated)}$/10mL sample to keep the pH of the samples <pH 2.
- The samples shall be covered (to avoid evaporation and increased concentration) and stored at room temperature in the dark until analysis; up to 1 month after sampling.
- After sampling, the pH of the test material vessels shall be measured and recorded.
- After sampling, the temperature and pH of the negative control vessel shall be measured and recorded.

Note: sampling procedures are expected to be conducted in a timely manner (no longer than 10 minutes per test material to process the samples at the end of the incubation period) in order to e.g. keep variation between replica test vessels as low as possible.

6.4 Sample analysis

The concentrations of released elements of interest are measured with an appropriate (validated) measurement method. In order to guarantee the basic quality (use of a validated measuring method, involvement of appropriately trained staff, traceability of the measurements, data processing and archiving,…) the sample analyses are preferably performed in a laboratory that works according to a standardised quality system (e.g. GLP, GMP, ISO17025, ...).

Examples of universal and commonly used method for measuring dissolved element concentrations (and thus recommended) are:

- ICP-MS (inductively coupled plasma-mass spectrometry); well documented in ISO 17294-1:2004 Water quality - Application of inductively coupled plasma mass spectrometry (ICP-MS) - Part 1: General guidelines and ISO 17294-2:2016 Water quality - Application of inductively coupled plasma mass spectrometry (ICP-MS) - Part 2: Determination of selected elements including uranium isotopes.
- ICP-AES (inductively coupled plasma-atomic emission spectrometry); well documented in ISO 11885:2007 Water quality - Determination of selected elements by inductively coupled plasma optical emission spectrometry (ICP-OES)

However, other validated analysis methods can also be applied (such as AAS (graphite furnace or flame - atomic absorption spectrophotometry).

Unless otherwise agreed, only 1 measurement result is reported for each analytical sample and element (in µg/L or mg/L).

In addition to the measurement results of the test samples, a reporting limit is always provided for each element of interest (limit of quantification, LOQ or at minimum the limit of detection LOD, see glossary for definitions). Other analytical performance characteristics of the analysis (measuring range, repeatability, reproducibility, accuracy, measurement uncertainty) should be reported.

6.5 Data analysis

Results shall be reported as measured element release (µg/L or mg/L). The absolute releases need to be corrected by subtracting the negative control values in the case where they exceeded the limit of

quantification (LOQ). All data should be corrected based on the actual loading for each test run. Results should also be presented as:

- absolute release per g test material (mg/g);
- percent of total element released (%);

The element released per unit surface area (mg/m^2) should be reported in the case of embedded massive material and should also be reported for other samples of metals and alloys. A calculation of the average release and the between vessel standard deviation and coefficient of variation should also be provided.

The two applications considered in this SOP (application 1: grouping an read-across and application 2: classification of alloys) require to calculate the relative bioaccessibility and the bioaccessible concentration. These calculations use the measured values generated by this SOP for different materials, as further explained in Annex 1.

6.6 Negative Controls, Positive Controls, Proficiency materials & Certified Standard Metal Solutions

Negative control: A test vessel with test medium only, with or without a clear resin in the case of embedded massive samples. A negative control run in parallel helps to detect background values of the elements of interest in the test fluid and helps rule out contamination. The measurements of the elements of interest in the negative control should generally be < 20 x SD of negative controls for the lab (based on 10 or more replicates). Higher values would indicate possible contaminations of the test system, glassware, etc.

Positive control: A test vessel with test medium that contains a certain amount of a well-soluble form of the element(s) of interest that is (are) measured. The positive control can easily be prepared, for example, by spiking certain amounts of the Certified Standard Metal solution into the test medium. Its purpose is to assure that the gastric test produces repeatable results. It provides evidence that the test system is responsive under the actual conditions of the assay. The positive control is run in parallel with the test materials of that metal or element. The positive control is independent of the application of the test data. The positive control does not need to match the physical form of the test materials. The positive control is predicted to give reproducible results over time. The positive control can also be used to assess the recovery of the metal ions through the test (mixing with test medium, incubation, filtering, sampling, etc). This solution shall be run at the time when the SOP for one or several metals of interest is established in a lab. Using this solution, high and low metal ion concentration would be added to the test medium at time = 0 and run through the protocol. Recovery of 100 ± 10% would be acceptable.

Proficiency materials: are well characterized and defined materials (e.g. regarding elemental composition and particle size) that may or may not include the metal of interest but need to match the physical form of the test materials (e.g. samples of powder metals or metal compounds, embedded and non-embedded massive forms of alloys). These materials are tested initially by the lab when establishing the SOP and are

run periodically afterwards (at least once a year). Results corresponding to ± 20% of reported values are deemed acceptable. Proficiency materials are independent of the application of the test data.

Such proficiency materials should be:

- easily (and foreseeably) available in the appropriate physical form as the test material and quality at a low cost
- have well characterized metal release data
- stable under ambient conditions
- not extremely toxic so that no special measures need be taken beside the usual protective clothing, dust mask, glasses and gloves

In powder form, they should be characterised in terms of particle size distribution or particle size range; specific surface area (m^2/g) should be measured using BET analysis or an equivalent technique.

A list of possible proficiency materials is provided in Annex. For these materials, it is proposed to set up both a physical repository and a publicly available database to house the repeated measurements of these materials (with Shewart charts). Ideally, as tests are conducted, and more measurements are added, the upper and lower confidence interval for each proficiency material can be accurately determined in the Shewart charts. However, initially, the database will have at least 10 measurements for each proficiency material collected by at least 2 different labs.

Certified Standard Metal solutions. These are the standard solutions containing the metal(s) of interest that are used for analytical calibration (see Section 6.4 on Sample Analysis) and may be used to prepare the positive controls.

6.7 Data Acceptance Criteria

A **test can be assumed valid (i.e. satisfies quality criteria of test system) if:**

- The values of pH and temperature (T) of the test medium at the start of the test fall within the set boundary conditions: i.e. pH 1.5 ± 0.1 and temperature at 37 °C ± 1 °C.
- The values of pH and T of the negative control at the end of the test fall within the set boundary conditions: i.e. pH 1.5 ± 0.1 and temperature at 37 °C ± 1 °C.

Notes:

- At the end of the test, the pH of the triplicate test material vessels may deviate significantly from 1.5, depending on the nature of the test material (e.g. tin) and should be recorded. In such a situation, the pH of the triplicate samples test material deviating significantly, it is recommended to compare the changes in pH between the high and low loadings. If the pH is above 4.5 and there is a difference between the low and high loadings, it shall be considered to use the results from the loading (expected to be the lower one) at which the pH is closer to 1.5.

- If the pH among the 3 test material replicates at the end of the test differ by more than 10%, contamination could be suspected and the test should be repeated.

The test results are considered acceptable if the following criteria are met :

- **A LOQ is available for each metal of interest (or at a minimum the LOD)**
- **the between test material vessels' Coefficient of Variation (CV) is ≤ 40% for each metal of interest in ≤ LOQ – 10x LOQ range, or CV ≤20% for measurements in 10x LOQ – end of measuring range,**
- **accurate metal measurements (± 10% of reported values) are obtained from a positive control solution that contains the metal of interest**

Notes:
When the coefficients of variation values of test materials exceed the recommended values (leading to data rejection), the proficiency material should be run. If the values for the proficiency materials are met but not for test material, a thorough review of the sample characteristics should be conducted before it is attempted to repeat the study; in some instances, the large variability in metal release could be linked to the intrinsic nature of the sample. Some materials (e.g. UVCBs) are very complex and can, despite being from the same batch, have a wide range in the composition and also in particle size which will result in significant variations within a sample and between "identical" samples. Large variations will therefore provide important information on the test material but this information needs to be combined with proper physical-chemical characterization of the test material, e.g. SEM-EDS, XPS, IR, BET, size distribution, etc.

The average negative control measurements that exceed the LOQ should be subtracted from test sample values. Contamination is suspected when negative control values are > 20 x SD of the mean negative control value for the lab (based on 10 or more replicates). In the latter case, a thorough review of all the test data should be conducted (including ratio of test sample value to negative control value) before deciding whether the study should be repeated.

6.8. Reporting and Record-keeping

A test report is prepared for each test material and shall include (but is not limited to) the following information:

- **Information on the lab/study**
    - Identification of the study, the sponsors, the test facility, and study director;
- **Description of the test material**:
    - Name of the test material, including identifiers such as CAS/EINECS numbers when relevant
    - Batch number and/or sampling date of the test material
    - Expiration date of the test material when applicable

- Details of supplier(s)/manufacturer/importer (e.g. address, contact information)
- Information available on composition (e.g., Certificate of Analysis), at least all substances > 0.1 wt% to assess purity of the sample and nature of impurities (% or ppm)
- Information available on method of manufacture (particularly for powder forms) indicating if atomization (water or air), chemical or grinding method was used
- Information on processing, preparation, surface finish, transport and storage, if available (e.g. heat treatment/fabrication history; metallurgical microstructure particularly for massive forms)
- Information on specific surface area, surface charge (particles), surface composition, and other surface properties (e.g. surface oxidation for both massive and powder forms; surface finish particularly for massive forms), if available.
- Particle size distribution (powders only) using parameters such as $d_{0.1}$, $d_{0.5}$, and $d_{0.9.}$ Report method of assessment and if values are based on volume or number distribution and in which media (e.g. composition, ionic strength, pH) the size distribution was measured (e.g. air, water).
    - *For example, size distribution may be determined by sieve analysis or laser diffraction.*
- If surface charge information is available, include the medium used to measure the surface charge (e.g. composition, ionic strength, pH)
- Information on morphology (shape of the particles) and metallurgical structure
    - *For example, the morphology of particles may be determined by examination using a Scanning Electron Microscope (SEM).*
- Information on particle agglomeration behaviour in solution
    - *For example, agglomeration can be assessed by comparing size distribution measurements (using laser diffraction techniques) in Phosphate Buffered Saline (PBS) to the size distribution in air.*
- Specific surface area in m^2/g (powders only)
    - *For example, particle surface area can be determined by Brunauer-Emmett-Teller (BET) analysis (absorption of nitrogen or krypton at cryogenic conditions) method (Brunauer et al. 1938).*
- Surface area *(massive only)*
    - *Measure the geometric exposed area of massive materials and embedded test samples with a Vernier caliper or other suitable graduated measuring device e.g. measuring projector.*
- If the sample (powder) was sieved, information about the original sample could also be provided.

- **Description of the test system and loading:** the following information should be provided
  - Test material weight, final loading and final volume;
  - pH and temperature at the start and the end of the test;
  - Detailed descriptions of the test apparatus and procedure, including analytical instruments used;

    Surface area to volume ratio for massive materials

  Results from the analyses of metal concentrations:

    - Date of test
    - LOQ (or at a minimum the LOD)
    - metal release (µg/L or mg/L) (measured element concentrations in the samples, including negative and positive controls, test material samples, and/or proficiency material samples),
  - metal release per g test material (calculated mg/g),
  - percent of total element released (calculated %),
  - element release per unit surface area for metals and alloys (calculated mg/m^2)
  - description of quality assurance program applied;

- **Deviations from this Standard Operating Procedure, if any and reason(s) for such;**
- **Any circumstances that may have affected the results;**
- **Reference to the records and all raw data necessary to reconstruct the study.**

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8. Glossary

- Bioavailability: In vivo bioavailability is defined as the extent to which a substance is taken up by an organism and is available for metabolism and interaction at target organ/sites (e.g. kidney, skin).
- Bioaccessible concentration: in the context of the classification of alloys, the bioaccessible concentration is calculated according to the formula below and compared to the bulk concentration in order to assess the presence of a matrix effect.

$$BC\% = \frac{\mu g\ metal\ ion\ released\ (measured\ in\ extract)/g\ alloy\ sample\ tested}{\mu g\ metal\ ion\ released\ (measured\ in\ extract)/g\ pure\ metal\ sample\ tested}\ x\ 100$$

- Bioaccessibility: In vivo oral bioaccessibility is defined as the fraction of a substance that is released in the gastrointestinal tract and becomes available for absorption. This can be estimated in vitro by measuring the dissolved quantity of a metal ion released under surrogate physiological conditions (in vitro bioaccessibility) in bioelution tests
- Bioelution: in vitro physico-chemical test that measures metal releases in artificial physiological fluids (e.g. artificial gastric juice)
- BET: Brunauer, Emmett and Teller method to measure the specific surface area of a sample
- Bridging: bridging principles allow deriving health or environmental classifications of mixtures based on available data on similar tested mixtures and on the ingredient substances.
- CAS number: Chemical Abstracts Service number
- Certified Standard Metal solution: standard solutions containing the metal(s) of interest that are used for analytical calibration and may be used to prepare the positive controls.
- EINECS number: European Inventory of Existing Commercial Chemical Substances number
- GLP: Good laboratory Practices
- GMP: Good Manufacturing Practices
- ICP AES: inductively coupled plasma-atomic emission spectrometry
- ICP MS: inductively coupled plasma-mass spectrometry
- IR: Infrared
- LOD (Limit of detection): can be determined as the smallest amount or concentration of an analyte in the test sample that can be reliably distinguished from zero (Thompson et al. 2002; ECHA 2016). The LOD for each metal can be calculated as three times the standard deviation of the mean measured concentration of a negative control test medium (e.g. average of 10 measurements).
- LOQ (limit of quantification): is determined as the concentration below which the analytical method cannot operate with acceptable precision for the given matrix (Thompson et al. 2002). Alternatively, the LOQ for each metal can also be calculated as six times the standard deviation of the mean measured concentration from the negative control test medium (e.g. average of 10 measurements).
- Massive: metals with a particle size ≥ 1 mm diameter
- MSDS: Material Safety Data Sheets
- Positive control: The positive control can easily be prepared, for example, by spiking certain amounts of the Certified Standard Metal solution into the test medium. Its purpose is to assure that the gastric test produces repeatable results. It provides evidence that the test system is responsive under the actual conditions of the assay. The positive control is run in parallel with the test materials of that metal or

element. The positive control is independent of the application of the test data. The positive control does not need to match the physical form of the test materials. The positive control is predicted to give reproducible results over time. The positive control can also be used to assess the recovery of the metal ions through the test (mixing with test medium, incubation, filtering, sampling, etc). This solution shall be run at the time when the SOP for one or several metals of interest is established in a lab. Using this solution, high and low metal ion concentration would be added to the test medium at time = 0 and run through the protocol. Recovery of 100 ± 10% would be acceptable.

- Proficiency materials: are well characterized and defined materials (e.g. regarding elemental composition and particle size) that may or may not include the metal of interest but need to match the physical form of the test materials (e.g. samples of powder metals or metal compounds, embedded form and non-embedded massive forms of alloys). These materials are tested initially by the lab when establishing the SOP and are run periodically afterwards (at least once a year). Proficiency materials are independent of the application of the test data. A physical repository and Shewart charts with test results will be made available.
- Reference materials: materials containing the same metal or element as the test material; the reference material test results are used to calculate relative bioaccessibility (in the case of the grouping application) or the bioaccessible concentration (in the case of the alloy classification application). For the latter case, the reference material and the test material should have similar physical form. The selection of the exact reference material to use in each case, depends on the application and on the characteristics of the test materials.
- Read-across: extrapolation of known data from one material (e.g. reference material) to another substance (target material) based on the assumption that the two materials (e.g. substances) will cause similar biological responses
- Relative metal bioaccessibility: results from the comparison of the bioaccessibility results of a given metal released from different compounds of the same metal or from different materials containing this metal
- SEM-EDS: Scanning Electron Microscopy / Energy Dispersive X-Ray Spectroscopy
- SiC: silicon carbide
- Target material: materials (e.g. substances) lacking toxicological data for one or more health endpoints but that have sufficient physico-chemical data. Their bioaccessibility is usually compared to the bioaccessibility of a reference (source) material (substance) (see also relative bioaccessibility and read-across)
- Test material: Test materials shall be metals, inorganic metal compounds, or complex metal-containing materials like e.g. alloys, UVCBs, pigments.
- Test medium: simulated gastric fluid (0.07N HCl solution brought at 37±1°C and pH 1.5±0.1).
- Test system: test medium + test vessels.
- UVCB: Substance of Unknown or Variable composition, Complex reaction products or Biological materials
- XPS: X-ray photoelectron spectroscopy

Annex 1: How are data generated by this SOP used in the two proposed applications?

Application 1: Grouping and read-across.

In this application, the bioaccessibility results of a given metal released from different compounds of the same metal or from different materials containing this metal are compared. The measured values[7] (expressed as µg metal/L) or the calculated values (µg metal/g sample or as percent of metal content) can be compared to those from one or more reference materials. The resulting relative bioaccessibility is used in a subsequent step (independent of this SOP) to group metal compounds or metal-containing materials that release similar amounts of metal ion and to use this information in a weight-of-evidence approach to determine if target and reference substances can be predicted to have similar or different oral systemic effects.

In practice:

- When reference and target samples are tested at equal loadings and times in the same solutions, metal releases per volume of solution (e.g. µg metal/L) can be directly compared.
- In some cases, when very high or very low releases are expected, different loadings may be applied to the reference and target samples. In this case, each loading has to be considered to express the results as ($\frac{\text{mass of released metal}}{\text{mass of sample}}$). Metal release per mass of sample (e.g. mg metal/g) from reference and target substances and mixtures can then be compared.
- With knowledge of the bulk concentration of a metal in a sample, it is also possible to calculate the percent of metal content that is released.

Table 2: example of use of relative bioaccessibility for grouping (all samples tested at same loading)

Samples	**Results (average mg metal/L ± S.D.)[a]**	**Relative bioaccessibility to reference 1 (%)**
1 - Reference material 1	1000 ± 90	100
2 - Reference material 2	10 ± 0.5	1
3 - Target material	900 ± 100	90
4 - Target material	800 ± 75	80
5 - Target material	100 ± 9	10
6 - Target material	9 ± 1	0.9

a After subtracting negative control values.

While the SOP details just how to generate the results presented in Table 2, the information could subsequently be considered for the purposes of grouping as follows: samples 3 and 4 have metal release properties comparable to sample 1 (reference material 1) and could be grouped with sample 1. Sample 6 has

[7] See section 6.5: The absolute releases need to be corrected by subtracting the negative control values in the case where they exceeded the limit of quantification (LOQ).

similar metal release as sample 2 (reference material 2) and could be grouped with sample 2. For sample 5, additional information may be needed to decide on the best reference material to use (e.g. phys-chem, dissolution kinetics).

Application 2: Alloy classification

When data on ($\frac{\text{released amount of metal}}{\text{mass of sample}}$) from alloys and reference metal ingredients, generated in the conditions described above are considered, the bioaccessible concentration (BC) of the metal in the mixture can be calculated using the following equation.

$$BC\% = \frac{\mu g\ metal\ ion\ released\ (measured\ in\ extract)/g\ alloy\ sample\ tested}{\mu g\ metal\ ion\ released\ (measured\ in\ extract)/g\ pure\ metal\ sample\ tested}\ x\ 100$$

Table 3: example of calculation of bioaccessible concentration for classification of alloys

Samples	**Results (µg metal X/g sample ± SD)**	**Bioaccessible metal X concentration in alloy 1 (%)**
Alloy 1 (containing classified metal X as an ingredient)	61 ± 6	0.038
Metal X	160,000 ± 1000	

Calculation of BC% for metal X in Alloy 1: $BC = \frac{61\ \mu g\ X/g\ alloy}{160000\ \mu gX/g\ metal\ X}\ x\ 100$ = 0.038%.

Annex 2. Proposed proficiency materials

See also attachment 19 to the TST. The proficiency materials should include at least one powder, one massive non-embedded and one massive embedded, to match the physical forms of the test materials. The list should be short (N=6-10). These materials will be stable over time and include examples of high and low metal releasing materials. A physical repository of proficiency materials will be set up. Full characterisation of the samples will be sought from sample provider or by performing appropriate physico-chemical tests. Once the SOP is approved, the materials will be initially tested at least 10 times and in at least two separate labs using the gastric fluid SOP. Shewart charts will be created and made publicly available. Examples of possible materials are provided below.

Massive non-embedded

Lead shot (CAS 7439-92-1): 99.9995% trace metals basis ; 1 mm,

Nickel metal (CAS 7440-02-0): 99.9% Ni (Materion Advanced Chemicals Inc. [formerly Cerac]; 1.1 ppm Co, Cylinders: 3.1 mm diameter x 3.35 mm long; surface area: 47.7 mm^2 per pellet

Massive embedded

SS316 sheets: Outokumpu Pte (Finland) 68.1% Fe (balance), 16.2% Cr, 11.2% Ni, 2.27% Mo, 1.10% Mn, 0.65% Si, 0.25% Cu, ≤0.18% Co, 0.12% Al. Squares: 10 mm x 10 mm x 2 mm.

Brass rod CW602N: Wieland (Germany)

Powders

Cobalt metal (CAS 7440-48-4)

Copper slag (to be further defined)