DB-ALM Protocol

Template for Data Content

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EURL ECVAM DATABASE SERVICE ON ALTERNATIVE METHODS TO ANIMAL EXPERIMENTATION

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Part A. Protocol Introduction

Protocol Name: Bioelution Testing of Metals, Inorganic Metal Compounds, and Complex Metal-Containing Materials: Simulated Gastric Fluid

Abstract: The proposed gastric fluid 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).

Résumé

Bioaccessibility data has been directly compared to reference values for hazard identification ad classification (e.g. EU Nickel Directive, BS EN 1811 2011), to reference release rates (BS EN 71-3 2013 for toys and REACH Annex XVII restriction on lead and its compounds in articles supplied to the general public that can be mouthed by children) or to predict relative bioavailability after oral exposure (ASTM D5517 2014). Further experience was gained with the testing of various metal compounds and alloys using variations of the ASTM D5517. The method and simulated gastric fluid composition in the validation study (Henderson et al. 2014) were finally selected based on the outcome of these previous exercises and served as basis to develop the test method.

Experimental Description

Biological Endpoint and Endpoint Measurement:

The proposed test method is an in vitro physico-chemical test that measures metal releases in artificial gastric fluid. This type of test, commonly referred to as a bioelution test, does not directly address a biological effect, but rather provides an in vitro estimate of oral bioaccessibility of metals from a variety of materials. This test can be used to compare the metal releases from reference and target substances[1] and mixtures. Bioelution test data are used to group substances of a given metal or mixtures containing the metal and to predict if they will have similar or different 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. The test can also be used to compare metal releases from alloys to those from their pure metal ingredients.

The concentration of all elements of interest shall be measured for each sample using appropriate analytical equipment (e.g. ICP-MS) and calibration solutions. The analyses shall be carried out according to an accepted quality system which assures properly qualified personnel and validated analytical instrumentation (e.g. according to ISO 17025 or equivalent). The analytical methods shall be described, and a reporting limit should be provided for each element of interest (limit of quantification, LOQ or at minimum the limit of detection LOD).

Endpoint Value:

Amount of metal ions or complexes released after 2 h of extraction.

Experimental System:

Artificial gastric fluid (0.07N, pH 1.5) at 37 °C.

[1] reference substances are those substances, which are usually well characterised and for which toxicological data and/or harmonized classifications exist, target substances are the substances lacking toxicological data for one or more health endpointscheck consistency with SOP

Discussion

This test is an *in vitro* phys-chem test and does not use animal or human cells or tissues.

The method is applicable with standard laboratory **equipment**. However, if the test materials need to be prepared (epoxy embedding and polishing to prepare massive samples) additional specialised equipment is required like e.g. a polishing machine.

The materials that are used do not require special **handling** or restricted access facilities. However, as some metals or metal-containing materials are classified as carcinogenic, their storage and handling should carefully follow the **instructions** provided by the manufacturer or supplier.

No special **training** is needed for testing of powders and/or as received (unmodified) or epoxy-embedded massives from the sponsor. The test can be performed based on the detailed instructions recorded in the test protocol. Estimation of the testing cost per test item is on average 950€ (test vessels, chemicals for cleaning, testing, sampling filters, syringes, sample tubes, labels, analyses, waste disposal).

If the embedding of massive forms in epoxy needs to be conducted at the receiving lab prior to bioelution testing, the testing cost per test item would be 1750€ (epoxy resin, grinding and polishing consumables, test vessels, chemicals for cleaning, testing, sampling filters, syringes, sample tubes, labels, analyses, waste disposal) and some **experience** is needed to carry out the grinding and the polishing.

The **time** required to complete data acquisition for a run is estimated to be between 5-7 days. 10 samples can be included in a typical run (total cost ranging from 9500€ to 17500€).

The analyses of the metals in solution shall be carried out according to an accepted quality system which assures properly qualified personnel and validated analytical instrumentation (e.g. according to ISO 17025 or equivalent).

Special attention should be given to possible (cross) contaminations. Contaminations by easily soluble metal compounds or present as trace elements in chemicals, or in non-acid cleaned test vessels can adversely affect the results of sparingly or insoluble test items.

This protocol can be combined with other assays providing information to be used in the weight of evidence in the assessment of metals, inorganic metal compounds, and metal-containing complex materials (e.g. comprehensive phys-chem tests and other sample information relevant for the interpretation of bioelution test results) and the refinement of the hazard classification of metal-containing complex materials.

The current protocol is applicable to powder (defined as ≥0.1 µm- < 1 mm) and massive (defined as ≥1 mm) forms of metal-containing materials but not to nanoforms.

Status

In Development:
A completed EURL ECVAM Test Submission Template (TST) for the gastric bioelution test method was submitted to ECVAM (Directorate General Joint Research Centre, EC) on February 16, 2018

Known Laboratory Use:
A synthetic gastric fluid similar to that proposed in this test has been used extensively, for example by the Comité Européen de Normalisation standard, Safety of Toys (BS EN 71-3 2013, 2017), adopted in the United States as ASTM D5517 (2014, Standard Method for Determining

the Solubility of Metals in Art Materials) to measure the release of elements from toys and materials.

The ASTM D5517 method served as the basis to assess the bioaccessibility of various cobalt and nickel compounds from arts materials was published by Stopford et al. 2003 and Henderson et al. 2012. Variations of the ASTM D5517 method have been used to measure Cu, Cr, Fe, Mn, Mo, Si, Ti, Zn, Zr releases from compounds or alloys by for example the KTH Royal Institute of Technology (Sweden). Several unpublished datasets for other metals or metal-containing samples (Rh, Ru, Pt, Pd, Ag, Au, Re, Cu, In, Ge, Cd, Pb, Si, Al, Zr, Sb, V, Sn) were generated to gather more information to fulfil REACH data requirements and perform read-across/grouping under REACH and CLP.

Tests have also been carried out to compare the releases of metal ions from alloys and their reference metal ingredients under the same test conditions to assess their relative bioavailability in the context of CLP Article12(b).

The learnings from the development and validation of the OECD N° 29 Guidance note (OECD 2001) have also been helpful in building up the experience for the development of the SOP and the setup of the validation for the bioelution gastric test method.

Participation in Evaluation Study:
The predictive capacity of the bioelution test can be surmised from studies assessing the relationship between gastric metal bioaccessibility and oral metal bioavailability. Test items include simple metal compounds (e.g. nickel, cobalt) and complex samples like soils.

Participation in Validation Study:
An inter-laboratory validation exercise using 6 test materials, 4 fluids and 5 labs was published in Henderson et al. 2014.

Regulatory Accepted:
ASTM D5517 is the closest standard to the proposed gastric bioelution test method Standard Operating Procedure (SOP).

Proprietary and/or Confidentiality Issues

No component of the test method is copyright protected.

Abbreviations and Definitions

- **Bioavailability**: Refers to the portion of the total quantity of a chemical present that is absorbed by a living organism and reaches the central (blood) compartment, whether exposures occur via the GI tract, skin, or lungs.

- **Bioaccessibility**: refers to the solubility of a chemical under physiological conditions. It most commonly is used to express solubility of a chemical under simulated physiological conditions (e.g. simulated gastric fluid) and referred to interchangeably in the published literature as “bioaccessibility,” “bioelution,” or “in vitro bioaccessibility.” It is most commonly reported as the fraction of the total mass of chemical present in the test material that is soluble under the test conditions (i.e., [mass soluble]/[total mass in test material] and cannot be higher than 100%).

- **Bioelution**: Bioelution refers to the **in vitro** extraction methods used to measure the **degree to which a substance/metal is released into simulated biological fluids** (e.g.

gastric fluid). A bioelution test is a physico-chemical test.

Version Number	Type of change	Author	Date
2.0		V Verougstraete A Oller	09/11/2018

Part B. Procedure Details, Latest Version: November 2018.

- **Protocol Name:** Bioelution Testing of Metals, Inorganic Metal Compounds, and Metal-Containing Complex Materials: Simulated Gastric Fluid.

- **Status:** Assessment of available data, improvement and validation of an existing protocol (Annex 1) for which information and data is already available

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Health and Safety Issues

General precautions:
Personnel can be exposed to common lab chemicals and test materials containing metals. The usual personal protective equipment (laboratory clothing, gloves, safety glasses and a dusk mask) should suffice to face most potential risks. The MSDS of the material provides information about its hazard profile and could trigger additional precautions for handling and disposing of the samples.

MSDS Information:
Material Safety Data Sheets (MSDS) or equivalent should accompany the test materials.

Materials and Preparations

CELL OR EXPERIMENTAL 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).
- Test medium corresponds to simulated gastric fluid (0.07N HCl), pH adjusted to pH 1.5 (37 °C ± 1 °C) in compliance with the ASTM 5517 standard.

EQUIPMENT

Fixed Equipment

- Usual laboratory equipment
- Balance with a graduated scale of at least 0.01 mg; calibrated with standard weights

daily before and after each use.

- Controlled thermometer, readable to 0.1°C.
- Calibrated pH meter readable to 0.01 unit.
- Thermostated linear (horizontal) or orbital shaker (37 ± 1°C, 100 rpm; stroke length=1 inch).
- Automatic pipettes
- The concentrations of released elements of interest shall be measured with an appropriate (validated) measurement method (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

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

Consumables

- 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
- Appropriate single-use latex- and oil-free (e.g. disposable polypropylene) syringes (volume depending on the amount required for the analysis technique used)
- Single use 0.2 µm membrane filters. Whatman UNIFLO syringe filters, Pall Acrodisc syringe filters, or equivalent filter system that do not adsorb or release the elements of interest in a significant manner
- Appropriate high-density polypropylene sample tubes known not to release or absorb significant amounts of the metals of interest

MEDIA, REAGENTS, SERA, OTHERS

- Ultrapure water: 18.2 MΩ.cm
- Concentrated hydrochloric acid, HCl
- Sodium hydroxide, NaOH (to adjust the pH of the test system to pH 1.5 (± 1) if necessary)
- Concentrated Nitric acid, HNO_3 (preservation of the samples)
- Except where indicated, all reagents shall be of recognized analytical grade (p.a.) or better.

Certified standard metal solutions containing the metal(s) of interest (for analytical calibration and to prepare the positive controls).

PREPARATIONS

Media and Endpoint Assay Solutions

- Simulated gastric fluid (0.07N HCl), 37 °C ± 1 °C, pH adjusted to pH 1.5 ± 1, in compliance with the ASTM D5517 standard.

Test Materials

- Test materials shall be metals, inorganic metal compounds, or metal-containing complex materials like e.g. alloys, UVCBs, pigments. Materials can be in powder or in 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. Depending on the type of test material and physical form, specific sample preparation and test execution applies:
- When materials on the market are present in micron-size **powder** form (defined as particles < 1 mm diameter but above 100 nm), additionally testing the subfraction of particles <100 µm 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. Massive material can be tested as it is or tested as an epoxy embedded sample. For epoxy embedded samples, starting from the mass loadings, 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
- 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.

Positive Control(s)

- 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.

Negative Control(s)

- 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.

Method

EXPERIMENTAL SYSTEM PROCUREMENT

Simulated gastric fluid (0.07N HCl) shall be freshly prepared. Its composition, shown in the table below follows the and follows the Standards ASTM D5517 (2014), ASTM F963 (2017), and BS EN 71-3 (2013, 2017).

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

ROUTINE PROCEDURES

- All “glassware” shall be cleaned, and free of elements of interest before use and suitable for working with metals at trace levels. Cleaning procedure: acid soak for 24h in 10% HNO3 then rinse at least 4x in ultrapure water (e.g. MilliQ water 18.2 MΩ. cm) and dry (by air at room temperature or in low temperature <50°C oven). This applies to new and re-used test vessels. The vessels shall be properly covered (e.g. screw caps, silicon or rubber stoppers) to avoid (cross) contamination and evaporation of the test medium.
- Agitation speed: 100 rpm on a temperature-controlled laboratory shaker (type should

be recorded). 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 minimized.

- Sampling time: at 2 h (i.e. 1 hour at an agitation rate of 100 rpm followed by 1 hour without agitation.), and
- The test should be performed in the dark.
- The size of the vessels (minimum volume of 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 effectiveness of the filter to separate dissolved from undissolved metal shall be confirmed by visual inspection and/or light scattering. If separation is not achieved for all triplicate samples (indicating an inherent issue with sample itself), centrifugation can be applied; the exact conditions needed to obtain optimal separation will depend on the sample. This deviation should be properly recorded.

TEST MATERIAL EXPOSURE PROCEDURES

The test set up depends on the physical form of the test material.

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.

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).

- 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.

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.

- 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[2]. 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

SAMPLING

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

1. At the end of the incubation period (2 h), 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 (e.g. syringe) filter, 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
2. After sampling, the pH of the test materials vessels shall be measured and recorded. The temperature and pH of the negative control vessel shall be measured and recorded.
3. 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 (avoid refrigeration as it can result in errors by e.g. condensation) in the dark until analysis; up to 1 month after sampling.

Note to sampling protocol: 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.

ENDPOINT MEASUREMENT

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

The concentrations of released elements of interest are measured with an appropriate (validated) measurement method like ICP-MS or ICP-AES. However other validated analysis methods can also be applied (such as AAS (graphite furnace or flame -atomic absorption spectrophotometry). 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, ...).

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

The analytical methods shall be described, and the following information reported: limits of quantification and the limits of detection for the elements of interest.

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).

Other analytical performance characteristics of the analysis (measuring range, repeatability, reproducibility, accuracy, measurement uncertainty) should be reported.

ACCEPTANCE CRITERIA

A **test can b**e 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.

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

- A LOQ is available for each metal of interest (or at 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

When the coefficient 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 blank value) before deciding whether the study should be repeated.

Data Analysis

- Results shall be reported as measured element release (mg or µg element per L solution).
- 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.

Prediction Model

- The relationship between gastric bioaccessibility and bioavailability needs to be considered when translating bioelution test data into predictions of similar bioavailability. Once this has been established for a particular metal, based on oral ingestion of metal-containing substances (e.g. compounds or soils), this relationship is valid for other substances containing that same metal (e.g. alloys).
- The relationships between oral bioaccessibility (including gastric fluid) and relative oral bioavailability of metals and metalloids from soils have been extensively studied (e.g. Denys et al. 2012, Diamond et al. 2016, Koch et al. 2013). Likewise, some data exist on the relationship between gastric bioaccessibility of metal compounds and oral toxicity (e.g., Henderson et al. 2012). A review of these data is provided in Alloys LCC 2017 (Annex 2). The review was limited to As, Pb, Be, Se, Sb, Cd, Co, Ni, and Hg (i.e. focusing on metals for which exposure is associated with systemic effects via the oral route).
- Strong predictive relationships between relative bioavailability and in vitro bioaccessibility emerged for lead and arsenic, with fewer available data for Sb, Cd, Co, Ni, and Hg, and no data for Be and Se. Among the oral fluids tested, gastric fluid was found to yield the higher metal releases, with a few exceptions. For several metals (As, Cd, Co, Cu, Mn, Ni, Pb, Sb, Zn) there is good statistical evidence of a relationship between the in vitro bioaccessibility of metals in gastric fluid and their in vivo systemic relative bioavailability. The review concludes that in general, in vitro

bioaccessibility methods provide an efficient and effective method for predicting the relative bioavailability of metals.

Annexes

- Annex 1 (TSTAttachment1arev): SOP Bioelution Testing of Metals, Inorganic Metal Compounds, and Metal-Containing Complex Materials: Simulated Gastric Fluid
- Annex 2 (TSTAttachment18): Review on bioavailability of metals and in vitro methods to predict RBA by Alloy, LLC (December 2017) and summary of response to questions prepared by Eurometaux

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