European Commission

ESAC Opinion

on the

Scientific Validity of the Bioelution Test Method

ESAC Opinion No. 2019-03
of 2 December 2019

Joint Research Centre

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The independent scientific peer review of the Bioelution *in vitro* test method described in this report was organised by the Joint Research Centre's EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) and conducted by the EURL ECVAM Scientific Advisory Committee (ESAC).

The ESAC peer review was coordinated by João Barroso on behalf of JRC / EURL ECVAM.

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EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM
SCIENTIFIC
ADVISORY
COMMITTEE
(ESAC)

ESAC OPINION

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ESAC Opinion Nr.	2019-03
Relevant ESAC Request Nr.	2019-01
Date of Opinion	02/12/2019

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Abstract

ESAC, the EURL ECVAM Scientific Advisory Committee, advises EURL ECVAM on scientific issues. Its main role is to conduct independent peer review of validation studies of alternative test methods and to assess their scientific validity for a given purpose. The committee reviews the appropriateness of study design and management, the quality of results obtained and the plausibility of the conclusions drawn. ESAC peer reviews are formally initiated with a EURL ECVAM Request for ESAC Advice, which provides the necessary background for the peer-review and establishes its objectives, timelines and the questions to be addressed. The peer review is normally prepared by specialised ESAC Working Groups. ESAC's advice to EURL ECVAM is formally provided as 'ESAC Opinions' and 'Working Group Reports' at the end of the peer review. ESAC may also issue Opinions on other scientific issues of relevance to the work and mission of EURL ECVAM but not directly related to a specific alternative test method.

The ESAC Opinion expressed in this report relates to the peer-review of the Bioelution *in vitro* test method.

Ispra, 2 December 2019

ESAC Opinion

In March 2019, the EURL ECVAM Scientific Advisory Committee (ESAC) (Annex 1) was formally asked by EURL ECVAM to review the available proof of the scientific validity of the Bioelution test method to assess the relative *in vitro* bioaccessibility (IVBA) of metals and metalloids in inorganic metal compounds and metal (metalloid)-containing materials as compared to reference materials using a simulated gastric fluid. An ESAC Working Group (WG) was established for this purpose (Annex 1), which delivered an ESAC WG report (Annex 2) to support the development of this opinion. The analysis and conclusions of the ESAC WG were based primarily on the Bioelution test method files submitted to EURL ECVAM, including all the relevant study Annexes and supporting documents. The assessment also included direct requests from the ESAC WG to the test method submitter for supporting information.

At its 46th meeting, held on 2-3 December 2019 at EURL ECVAM, Ispra, Italy, the non-Commission members of ESAC unanimously endorsed this opinion.

The Bioelution test method generates relative IVBA data that can be used to (i) support grouping and read across of inorganic metal compounds and metal (metalloid)-containing materials (e.g. UVCBs, pigments and alloys) and (ii) establish the presence or absence of a matrix effect to inform alloy classification based on the relative bioaccessible concentration (%RBC) of a metal in an alloy versus a reference material (e.g. a pure metal).

To assess the scientific validity of the Bioelution test method, the ESAC evaluated the biological relevance of the method, the relevance of the measurement of %RBC, the robustness of the protocol and the reproducibility of the %RBC measurement.

Based on the available information, the existing scientific literature and the experts' own extensive experience as detailed in the ESAC WG report, the ESAC unanimously concluded the following:

The Bioelution test method is biologically relevant

In vivo oral bioavailability refers to the fraction of the total amount of a substance that is released in the gastrointestinal tract and absorbed into the blood stream. This can be estimated *in vitro* by measuring the dissolved quantity of a metal ion release under surrogate physiological conditions in a bioaccessibility test. IVBA testing is a well-established approach in use for several years. Bioaccessibility tests for the oral route of exposure (including gastric compartment) have been applied to the assessments of human

exposures to metal compounds and minerals in soils and dust. US EPA Validated Test Method 1340 assesses the bioaccessibility for lead (Pb) in soil. Validation studies of IVBA test methods for estimation of bioavailability of arsenic (As) from soil and sediment have been conducted. Moreover, the proposed Bioelution test method is similar to the Bioaccessibility Research Group of Europe (BARGE) test method, which has been in use in Europe for several years to assess As, Pb, Antimony (Sb) and Cadmium (Cd) in soil samples and was accepted as an ISO standard in 2018 (ISO 17924).

The Bioelution test method is representative of the gastric compartment. Publications in the peer-reviewed literature show that additional components (e.g., ascorbic acid, pepsin, and glycine) could be added to the gastric solution to increase solubility of certain metals (e.g., Whitacre et al. 2017). However, these additional components add complexity to the method and are unlikely to affect the relative measurement that the method produces. The selection of the 2-hour extraction time is consistent with other IVBA methods using simulated gastric fluid and with the time for complete emptying of the human stomach. The ESAC supports the current Bioelution test method protocol and acknowledges that the simple gastric solution is fit-for-purpose considering the proposed context of use.

A comparison of the IVBA data with *in vivo* oral relative bioavailability data was not conducted in this ESAC review. Relative bioavailability is the ratio of the oral absorption fraction of the chemical present in some target material to the oral absorption fraction of the chemical in an appropriate reference material. It accounts for differences in absorption between the chemical in the reference material and in the test material. Such comparison is not necessary and does not compromise the assessment of the scientific validity of the method given that relative measurements between target and reference materials are used both for grouping and read across, and classification of alloys.

The Bioelution test method is appropriate to assess if a matrix effect occurs in alloys

The Bioelution test method can be used to assess the matrix effect of an alloy by comparing the release of metal ions between a reference material (e.g. pure metal) and an alloy. When a matrix effect occurs, there is an increased or decreased relative release of the metal ions from the alloy in comparison to the reference material. In the absence of a matrix effect (e.g. simple metal mixture), a linear relationship between the fraction of metal ion release and the total content of that metal in the material is expected and this has been confirmed by the method developers. Therefore, the ESAC considers that this is an appropriate use of the Bioelution test method.

The Bioelution test method is a simple, reproducible approach and is applicable to many metals

The ESAC considers that the within- and between-laboratory reproducibility of absolute bioaccessibility measurements for the six metals tested (i.e. Co, Cu, Fe, Ni Pb, Zn tested in the round robin trial as published by Henderson et al., 2014) were acceptable. The ESAC

considers that this should hold true also for other metals; however, data have not been provided to support this assumption. The current standard operating procedures (SOP) further ensures reproducibility across laboratories by ensuring high consistency in the preparation of the simulated gastric fluid. Reproducibility and international harmonisation are also ensured by clearly defining the reference materials for each metal that must be run in parallel to test materials for alloy classification purposes. While for the read across and grouping application it is not possible to predefine the reference materials, the SOP still requires these to be run concurrently to the target material so as to guarantee the robustness and consistency of the relative measurement. The applicability domain is clearly described in the SOP in terms of technical limitations and context of use and is considered appropriate. In conclusion, the ESAC considers that the SOP is well described and robust, thus ensuring reproducibility of data and easy implementation of the assay by naïve laboratories.

Conclusion and Recommendation

The ESAC concludes that the Bioelution test method is scientifically valid for determining the relative IVBA of metals and metalloids in inorganic metal compounds and metal (metalloid)-containing materials to be used in the context of their classification. Thus, the ESAC recommends that the Bioelution test method progresses to Test Guideline development at OECD level to achieve international harmonisation.

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Annex 1

COMPOSITION OF THE ESAC AND ESAC WORKING GROUP

Composition of the ESAC and ESAC Working Group

EURL ECVAM Scientific Advisory Committee (ESAC)

Core Members

- Dr. Chantra ESKES (ESAC Chair)
- Prof. Paula M. ALVES
- Dr. Rebecca CLEWELL
- Prof. Emanuela CORSINI
- Prof. Ian COTGREAVE
- Prof. Annette KOPP-SCHNEIDER
- Dr. José Maria NAVAS ANTÓN
- Prof. Aldert PIERSMA
- Dr. Carl WESTMORELAND

Ad-hoc Members

- Dr. Valerie HANLEY
- Prof. Irina KARADJOVA
- Dr. Mina SUH

ESAC Working Group (WG)

- Dr. Chantra ESKES (WG Chair)
- Prof. Emanuela CORSINI
- Prof. Annette KOPP-SCHNEIDER
- Dr. Valerie HANLEY
- Prof. Irina KARADJOVA
- Dr. Mina SUH (WG Rapporteur)

EURL ECVAM (Secretariat)

- Dr. João BARROSO (ESAC Coordinator)
- Dr. Pilar PRIETO
- Prof. Maurice WHELAN (Head of Unit)

Annex 2

ESAC WORKING GROUP REPORT

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM
SCIENTIFIC
ADVISORY
COMMITTEE
(ESAC)

ESAC WORKING GROUP REPORT

on the

Scientific Validity of the Bioelution Test Method

Title page information	
File name	ESAC_WG_Report_Bioelution.doc
Abbreviated title of ESAC request	Bioelution
Relating to ESAC REQUEST Nr.	2019-01
Request discussed through	Written procedure following ESAC44 (December 2018)
Report to be handed over to ESAC Chair and EURL ECVAM Coordinator by	Chantra Eskes (Working Group Chair)

Version tracking

Date	Version	Author(s)	Description
20 November 2019	V1.0	ESAC WG	First agreed draft of ESAC WG Report
30 January 2020	V2.0	ESAC WG	Second revised draft after commenting
6 February 2020	V3.0	ESAC WG	Third revised draft after commenting
13 February 2020	V4.0	ESAC WG	Fourth revised ESAC WG Report draft after commenting
26 February 2020	V5.0	ESAC WG	Final approved draft of ESAC WG Report sent to ESAC for endorsement

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ESAC Working Group

Full title: ESAC Working Group on the Bioelution Test Method

Abbreviated title: ESAC WG Bioelution

The ESAC WG was established in April 2019 by written procedure to assist in the production of an ESAC Opinion on the scientific validity of the Bioelution test method to assess the relative *in vitro* bioaccessibility (IVBA) of metals and metalloids in inorganic metal compounds and complex metal (metalloid)-containing materials using a simulated gastric fluid.

This report was prepared at the request of EURL ECVAM by the "ESAC Working Group Bioelution" (ESAC WG), which was charged with conducting a detailed scientific peer review of the Bioelution test method. The basis for the scientific peer review was the EURL ECVAM Request for ESAC Advice approved by the ESAC by written procedure following the ESAC44 plenary meeting of December 2018 (ESAC request 2019-01).

The ESAC WG met at EURL ECVAM on 02-03/05/2019 and 25-26/09/2019 to conduct its peer review. This ESAC WG Report was endorsed by the ESAC WG on 26/02/2020 and represents its consensus view. The Report was endorsed by the ESAC on 13/03/2020.

The ESAC WG had the following members:

- Dr. Chantra ESKES (ESAC Core Member, WG Chair)
- Prof. Emanuela CORSINI (ESAC Core Member)
- Prof. Annette KOPP-SCHNEIDER (ESAC Core Member)
- Dr. Valerie HANLEY (ESAC Ad-hoc Member)
- Prof. Irina KARADJOVA (ESAC Ad-hoc Member)
- Dr. Mina SUH (ESAC Ad-hoc Member, WG Rapporteur)

EURL ECVAM (Secretariat):

- Dr. João BARROSO (ESAC Coordinator)
- Dr. Pilar PRIETO

ABBREVIATIONS USED IN THE DOCUMENT

- **BLR** Between-laboratory reproducibility
- **CV** Coefficient of Variation
- **ESAC** EURL ECVAM Scientific Advisory Committee
- **ESAC WG** ESAC Working Group
- **EU CLP** European Union Regulation on Classification, Labelling and Packaging of Substances and Mixtures
- **EURL ECVAM** European Union Reference Laboratory for Alternatives to Animal Testing
- **GLP** Good Laboratory Practice
- **IVBA** *In Vitro* Bioaccessibility
- **ICP-MS** Inductively Couple Plasma-Mass Spectrometry
- **LOD** Limit of Detection
- **LOQ** Limit of Quantification
- **OECD** Organisation for Economic Co-operation and Development
- **PC** Positive Control
- **QA** Quality Assurance
- **RBC (%)** Relative Bioaccessible Concentration
- **RRT** Round Robin Trial
- **RSD** Relative Standard Deviation
- **SOP** Standard Operating Procedure
- **Sr** Repeatability Standard deviation
- **S_R** Reproducibility Standard deviation
- **TST** Test Submission Template
- **UN GHS** United Nations Globally Harmonized System for the Classification and Labelling of Chemicals
- **VMG** Validation Management Group
- **VSR** Validation Study Report
- **WLR** Within-laboratory reproducibility

1. Study objective and design

1.1 Analysis of the clarity of the study objective's definition

(a) ESAC WG summary of the study objective as outlined in the Test Submission

The Bioelution method is proposed to generate relative bioaccessibility data by comparing the *in vitro* metal ion releases from target and reference material in simulated gastric fluid for the oral route of exposure.

In 2016, EURL ECVAM received a pre-submission of the Bioelution method by the test submitter, which led to launch of a consultation within the PARERE network. On the basis of the evaluation of the pre-submission and feedback received from the PARERE members, EURL ECVAM invited the test submitter to submit a full submission and provide clarifications and detailed information to address the comments from PARERE and EURL ECVAM. In 2018, EURL ECVAM received the full submission of the Bioelution method for testing of metals, inorganic metal compounds, and complex metal-containing materials in simulated gastric fluid.

The ESAC WG was formed and met in May and September 2019. Several teleconferences were also held between October 2019 and February 2020. The ESAC WG was requested to review the available proof of the scientific validity of the Bioelution method to assess relative bioaccessibility of metals in inorganic metal compounds and metal-containing materials as compared to reference materials.

The ESAC WG was asked by EURL ECVAM to deliberate on the following issues concerning the Bioelution method:

1) The number and quality of the test items used in view of the study objectives;
2) The reliability (reproducibility within and between laboratories) of the results obtained *in vitro*;
3) The biological relevance with regard to the *in vitro* system and experimental conditions used as being:
    a. Representative of the gastric compartment,
    b. Worst case scenario for the oral route of exposure,
    c. Relevant in relation to the dermal and inhalation routes of exposure;
4) Appropriateness of identification of applicability and limitations of the method;
5) The relationship between *in vitro* bioaccessibility (IVBA) in simulated gastric fluids and relative *in vivo* bioavailability (e.g. from contaminated soil studies) and its relevance in the context of the two regulatory applications proposed (1-grouping and read across and 2-establish the presence or absence of a matrix effect to inform alloy classification);
6) Appropriateness of the relative measurements of bioaccessibility between pure metal and alloy used to calculate the relative bioaccessible concentration (%RBC) to show metal releases affected by matrix effects;
7) Appropriateness of the experiments carried out to demonstrate that there is a linear relationship between the fraction of metal ion release in the absence of a matrix effect (simple mixture) and the total content of that metal in the material;
8) Clarity and completeness of the protocol and the prediction model;
9) The readiness of the method for regulatory use.

(b) Appraisal of clarity of study objective as outlined in the Test Submission

The ESAC WG agrees that the study aimed to demonstrate the reproducibility and robustness of the proposed protocol to assess the relative IVBA of metals, inorganic metal compounds, and complex metal-containing materials using a simulated gastric fluid.

1.2 Quality of the background provided concerning the purpose of the test method

The ESAC WG is of the understanding that the objective of the test method is not to predict the actual *in vivo* bioavailability values, but rather to allow comparisons of bioaccessibility of a given metal between target and reference materials.

The relative IVBA generated by the Bioelution method can be used to i) support grouping and read across and ii) establish the presence or absence of a matrix effect to inform alloy classification. Under grouping and read across, relative IVBA data may be used, in a weight-of-evidence (WoE) approach, to support grouping and read across of metal-containing substances and mixtures. Under alloy classification, IVBA data may be used to calculate the %RBC of a metal ingredient in an alloy, which is compared to its bulk concentration.

The ESAC WG considers that the information provided is of sufficient quality to evaluate this context of use.

(a) Analysis of the scientific rationale provided in the Test Submission

The systemic toxicity of most metals and metalloids is associated with the release of metal ions and their uptake by the body and/or interaction at their target organ sites (i.e., bioavailability). The bioavailability of the released metal at the site of action in the organism is relevant for determining toxicity of metals and minerals. Currently, there are many relative IVBA methods that evaluated metal releases in simulated gastric fluid from various materials. These methods have been tested in soils (e.g. ASTM D5517 2007; BS EN71-3 2013; BARGE UBM 2016). However, there are no internationally recognized (e.g. EU or OECD) bioelution test methods specifically designed to assess metal releases relevant to systemic health effects after oral exposure.

Bioelution method is an *in vitro* method used to measure metal releases in simulated gastric fluid (0.032M HCl, pH 1.5). It does not address a biological effect. Bioelution method provides data regarding *in vitro* estimates of relative bioaccessibility, which is related to systemic bioavailability of metal ions.

The test submission makes sufficient reference to the relevant body of the scientific literature. IVBA tests for the oral route of exposure (including gastric compartment) have been applied to the assessments of human exposures to metal compounds and minerals in soils and dust (e.g. Ruby et al. 1999, Suh et al. 2019, Whitacre et al. 2017, Wragg et al. 2011). USEPA Validated Test Method 1340 (one of SW-846 guidance methods) assesses the bioaccessibility assay for lead (Pb) in soil. Validation of an *In Vitro* Bioaccessibility Test Method for Estimation of Bioavailability of Arsenic from Soil and Sediment has been conducted (ESTCP Project ER-200916 2012; Brattin et al. 2013). Moreover, the proposed Bioelution method is similar to the Bioaccessibility Research Group of Europe (BARGE) method, which has been in use in Europe for several years to assess arsenic (As), Pb, antimony (Sb) and cadmium (Cd) in soil samples and was accepted as an ISO standard in 2018 (ISO 17924).

(b) Analysis of the regulatory rationale provided in the Test Submission

The relative IVBA data generated by the Bioelution method can be used to i) support grouping and read across and ii) establish the presence or absence of a matrix effect to inform alloy classification. Under grouping and read across, relative IVBA data may be used to group metal compounds or metal-containing materials that release similar amounts of metal ion and to use this information in a WoE approach to read across oral systemic effects. Under alloy classification, the %RBC of the metal ion between the alloy and the pure metal ingredient is calculated and compared to the bulk concentration of that metal ingredient in the alloy in order to assess the presence of a matrix effect.

The ESAC WG is of the understanding, however, that while the Bioelution method addresses the generation of IVBA in simulated gastric fluid and its use for the calculation of %RBC of metals in alloys, it is not to be used to predict relative bioavailability values for application in a risk assessment context without further evaluation.

The application of the Bioelution assay in a regulatory context is not part of the present assessment.

1.3 Appraisal of the appropriateness of the study design

Henderson et al., 2014 Round Robin study

Number of laboratories: Five laboratories were used for the round robin trial (RRT), and the results were published in Henderson et al. (2014).

Organisation of study: The method validation study was initiated in 2010 by a group of representatives from various metal commodities seeking to standardize fluid compositions and testing protocols for the basic bioelution methods being used across the industry. To investigate the within-laboratory repeatability and between-laboratory reproducibility of bioaccessibility testing in different simulated fluids (including gastric fluid), five laboratories measured metal release from six metal-containing materials following one defined protocol (standard operating procedure, SOP) issued by the study sponsors. The study was funded by five commodity groups, with each organization having a key point person that participated in steering team and technical discussions (Henderson et al., 2014).

Chemical selection: Six different metals and metal-containing materials following the SOP were evaluated in the RRT. These test items represent water soluble metal compounds, insoluble metal compounds, pure metals, and complex materials (alloys and ore concentrates). They cover the various types of metals and materials the proposed test method is intended to be applied to. They also represent a variety of physicochemical properties that impact the metal releases. Samples of each of the six materials (pre-characterized for particle size, metal content, and surface area) were distributed to each of the five laboratories from two independent repositories (Particle Technologies Laboratory (Downers Grove, IL, USA) and Outotec (Finland)). Given the nature of the test materials and the bulk metal content, the procedure for chemical selection was deemed appropriate by the ESAC WG.

Quality Assurance (QA) of data: Each laboratory generated a comprehensive report, which underwent a QA exercise. A detailed review and comparison between the SOP and the five laboratory reports was performed. An external inter-laboratory comparison was conducted to investigate the within-laboratory repeatability and between-laboratory reproducibility of bioaccessibility testing in different simulated fluids, including gastric fluid. The outcome of the study was published in Henderson et al. (2014). The QA of the gastric fluid data was evaluated by the ESAC WG and considered appropriate.

Statistical independence: The statistical analysis was conducted by two independent biostatisticians.

Additional studies provided

Appendix 1 provides a detailed list of all additional studies that have been provided to the ESAC WG between 2017 and 2019. These additional studies included data to demonstrate calculations of %RBC and evaluation of matrix effect. The test submitter included data on the metal ion release in the absence of a matrix effect (simple mixture) versus metal ion release in the presence of matrix effect (metal alloy) for various metals and metal-containing compounds. The data also included comparisons of the different methods used to prepare the gastric fluid as well as showing the proof-of-concept for the SOP. Additionally, reproducibility of %RBC values was also demonstrated.

1.4 Appropriateness of the statistical evaluation

Two independent statistical analyses were conducted. One was based on the international standard ISO 5725-2 [Accuracy (Trueness and Precision) of Measurement Methods and Results-Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method, 1994] and used Mandel's k and h statistics to evaluate within- and between laboratory consistency, respectively. Cochran test and Grubbs test were used to identify outliers during the analysis of within - and between laboratory consistency, respectively.

The second approach used to assess within- and between-laboratory reproducibility was based on the relative standard deviation (RSD) analysis of the log concentration and was expressed in percentages. RSD < 10% was the criteria used for acceptable repeatability and RSD < 20% for acceptable laboratory-to-laboratory variability. Repeatability standard deviation (Sr; within-laboratory) and reproducibility standard deviation (S_R; between-laboratories) were used as measurements of precision. RSD was used to assess the fluctuations in the data relative to the data mean. The ratio S_R:Sr of the log-concentration was used to compare the mean results of the laboratories. Good, fair or poor agreement between laboratories was indicated by ratios up to 3, between 3 and 6, and above 6, respectively.

The statistical approaches used to evaluate within- and between-laboratory reproducibility, although different from the traditional ones used in validation studies, follow international standards for the determination of repeatability and reproducibility. The ESAC WG considers these methods to be appropriate and sufficiently justified.

2. Collection of existing data

2.1 Existing data used as <u>reference</u> data

The Bioelution method is not to be used for predicting relative bioavailability for application in a risk assessment context. As such, the absence of *in vivo* relative bioavailability data to make a direct comparison to relative IVBA data does not compromise the assessment of the scientific validity of the method. However, there is evidence that IVBA is a conservative assumption of *in vivo* bioavailability, i.e., higher RBCs than those provided by *in vivo* data (Whitacre et al. 2017., Suh et al. 2019). Additionally, the comparative nature of the approach is inherent to both proposed applications with relative measures that are being calculated. This provides further support for use of the Bioelution method and mitigates the concern that *in vivo* bioavailability reference data associated with the test chemicals are absent.

As part of the existing information provided to help understand the relationship between IVBA and *in vivo* bioavailability, the submitter provided a review performed by Alloy, LLC (Attachment 18 of the submission). This review evaluated 38 peer-reviewed studies (and 1 unpublished study), of which 22 contained data for evaluations conducted both *in vivo* and *in vitro* and an additional 17 presented data from *in vitro* extraction testing of soils. The metals evaluated in these studies included Sb, As, Cd, cobalt (Co), Pb, mercury (Hg), and nickel (Ni). This review nicely captures how IVBA relates to *in vivo* bioavailability. Importantly, the review stresses that the IVBA data may not be used directly to predict *in vivo* relative bioavailability, but rather the equation describing the regression line in paired *in vivo* and *in vitro* data is used to "translate" IVBA into a prediction of relative bioavailability. For the majority of the studies evaluated the slope of the regression equation was <1, indicating that the IVBA was higher than the *in vivo* relative bioavailability and that the IVBA is typically a conservative measurement for *in vivo* relative bioavailability. The review further evaluated various components of the simulated gastric fluid used in the various studies and discussed that pH was the major determining factor regarding extraction of soluble metals. The

Alloy, LLC review was thorough and beneficial among the submission materials reviewed by the ESAC WG.

However, the ESAC WG did not consider Diamond et al., 2016 publication mentioned in the Alloy, LLC review as appropriate evidence regarding the uncertainty associated with IVBA/*in vivo* bioavailability correlations. Two flaws were identified in the statistical analysis. First, instead of performing a (random-effects) meta-analysis, the authors fitted separate regression models for each data set and subsequently evaluated the pooled data in a weighted regression analysis based on the fact that the slopes in the separate analyses were not significantly different. Second, even more importantly, IVBA data are observed with measurement error and hence can only be used as explanatory variable in a regression analysis when an error-in-variables model is applied. The consequence of using the standard regression model instead of an error-in-variables model is that the estimated slope underestimates the true value. Furthermore, the ESAC WG considers that the combination of data from two species (swine and mouse) into a single regression as presented in Diamond et al. (2016) is not appropriate.

2.2 Existing data used as testing data

Several sources were used as the testing data for validation of the Bioelution method including the RRT published by Henderson et al. (2014), and the additional studies that were submitted from June 2017 through October 2019 to ESAC as listed in Appendix 1.

ESAC WG evaluated all data and information provided by the test submitter and EURL ECVAM. The data are pertaining to the Bioelution method, and thus the WG considers them to be appropriate. Additionally, in the SOP, the test submitter specify the data pertaining to each metal that are applicable to the Bioelution method.

2.3 Search strategy for retrieving existing data

Not applicable.

2.4 Selection criteria applied to existing data

No selection criteria were applied to the existing data because all data that have been provided were generated and/or sponsored by the test submitter. All data were evaluated and pertained to the Bioelution method, and thus the WG considers them to be appropriate.

3. Quality aspects relating to data generated during the study

3.1 Quality assurance systems used when generating the data

The study reports mentioned above specify that the delegated analytic phase has been performed according to the criteria of the ISO 17025 standard. Moreover, the study reports indicate that each study has been audited by the QA Unit to assure compliance with the study plan, SOPs, and according to the OECD Principles of GLP. The ESAC WG endorses the QA measures taken in these studies used in support of the Bioelution method.

3.2 Quality check of the generated data prior to analysis

See section 3.1. In-addition to in-house QA procedures, QA checks were performed and presented in Henderson et al. (2014) RRT as well as in an independent statistical analysis.

4. Quality of data used for the purpose of the study (existing and newly generated)

4.1 Overall quality of the evaluated testing data (newly generated or existing)

The assessment of within-laboratory reproducibility was done with 6 inorganic metal-containing materials, tested in five laboratories, as reported in Henderson et al. (2014). Two independent statistical analyses were carried out as described in section 1.4.

The between-laboratory reproducibility was assessed during the inter-laboratory RRT reported by Henderson et al. (2014). The data available in this paper is not very extensive, but the test submitter calculated, based on an analysis of coefficients of variation (CVs) and negative control values, the percentages of disqualified tests. Furthermore, limits of quantification (LOQs) were not reported, but calculated from limits of detection (LODs) as 2xLOD. Out of 43 measurements 5 (12%) had CVs that did not meet the acceptance criteria. The quality of testing data is therefore very high. All laboratories included in testing and validation studies are accredited (Henderson et al. 2014). Reagents, standards and reference materials used are of very high quality, and the most sensitive method Inductively Couple Plasma-Mass Spectrometry (ICP-MS) is used for measurements. The ESAC WG considers these data to be acceptable.

At the request of ESAC WG, the test submitter provided additional studies in form of laboratory reports in August 2019 and October 2019 (see Appendix 1). The data were requested by ESAC WG to demonstrate that the different methods used to prepare simulated gastric fluid would not significantly impact the %RBC, which is based on the metal ion release in the reference material vs. metal ion release in the test material, and that %RBC calculations would be reproducible. The additional generated data was used as a proof-of-concept for the proposed SOP revisions. The ESAC WG determined that data were robust and of sufficient quality for evaluation.

4.2 Quality of the reference data for evaluating relevance[1]

Not applicable. See section 2.1.

4.3 Sufficiency of the evaluated data in view of the study objective

The ESAC WG requested for additional data on the different methods used to prepare the simulated gastric fluid and whether the preparation of simulated gastric fluid made any difference in the metal ion release and the %RBC calculations. These additional data demonstrated the reproducibility of the %RBC calculations based on the different methods used to prepare the simulated gastric fluid. The ESAC WG considers the quality of the entire data set sufficient to draw robust conclusions.

[1] OECD guidance document Nr. 34 on validation defines relevance as follows: "Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of accuracy (concordance) of a test method."

5. Test definition (Module 1)

5.1 Quality and completeness of the overall test definition

The Bioelution method is well-defined as an *in vitro* physico-chemical test that measures metal releases in simulated gastric fluid. The test provides IVBA of metals and metalloids from a variety of materials. This method is always used to compare a test material to a reference material so the output is always a relative measurement.

The test system is well-defined and simple. Typically a 250 mL Erlenmeyer flask with the chosen volume (minimum of 50 mL) of the simulated gastric fluid (0.032N HCl solution at 37 ± 1 °C and pH 1.5 ± 0.1) is used.

There are two distinct applications defined for the test method: 1. grouping and read-across of substances and 2. establishing the presence or absence of a matrix effect to inform alloy classification.

Mechanistic Relevance for Application 1:
It is well-recognised that the systemic toxicity effects of metal-containing materials are related to the bioavailability of the metal ion after systemic absorption. The bioelution method measures relative bioaccessiblity and is used as a tool to establish categories of metal-containing materials and is part of the WoE approach applied to perform read-across (extrapolation of known data from a reference material to a test material based on the assumption that the two materials will cause similar biological responses) and grouping.

Mechanistic Relevance for Application 2:
Within the EU CLP alloys are considered as mixtures and classified based on their content of classified metal (metalloid) ingredients (i.e. the concentration of classified ingredient is compared to its classification cut-off value, such as Generic Concentration Limit or Specific Concentration Limit). Yet, alloys (composed of more than one metal ingredient) have metallic bonds that can affect metal releases in such ways that the bulk concentration of the metal ingredients is not reflected in the bioavailability or bioaccessibility of that metal from the alloy (matrix effect), as evidenced by the unique physico-chemical properties of alloys. Since the systemic toxicity effects of alloys are related to the bioavailable metal ions, it follows that the bioavailability of metals from alloys is not expected to match that of a simple mixture. IVBA data from alloy and pure ingredients can be used to calculate the %RBC of the metal in the alloy and determine a possible matrix effect of the alloy.

The bioelution method is representative of the gastric compartment :
Publications in the peer-reviewed literature show that additional components (e.g., ascorbic acid, pepsin, and glycine) could be added to the gastric solution to increase solubility of certain metals (e.g., Whitacre et al. 2017). However, these additional components have varying effects on solubility for the different metals and add complexity to the method that may lead to extra variability. Therefore, the ESAC WG supports keeping the method as simple as possible and acknowledges that the simple simulated gastric solution is fit-for-purpose considering the proposed context of use. The selection of the 2-hour extraction time is consistent with other IVBA methods using simulated gastric fluid and with the time for complete emptying of the human stomach.

5.2 Quality and completeness of the documentation concerning SOPs and prediction models

The SOP is detailed and presents an easy to follow and reproducible method for evaluating the relative IVBA of metals in inorganic metal compounds and metal-containing materials such as alloys. Test material and reference materials are always to be run in parallel and the SOP outlines which metals are in scope for each of the two applications and how to analyse and make use of the relative IVBA (See Section 11.2).

6. Test materials

6.1 Sufficiency of the number of evaluated test items in view of the study objective

The ESAC Working group is satisfied with the number of test items submitted to support the use of the Bioelution method for both intended applications. Section 2.2 summarises the studies that were conducted with various test materials to validate the method. Testing was conducted on a limited number of metals e.g. Co, copper (Cu), iron (Fe), Ni, Pb, zinc (Zn). However, the ESAC WG considers that the Bioelution method should also work for other metals (within the applicability domain of the method), especially taking into account that a reference material should always be tested concurrently with the tested material.

6.2 Representativeness of the test items with respect to applicability

The test items were suitable for demonstrating proof-of-concept of both applications of the method. Test materials were evaluated at both suggested loadings (0.2 and 2 g/L). The SOP was amended in response to the comments by the ESAC WG. In order to demonstrate that the change in protocol did not change the applicability of the method a subset of test materials were evaluated using both preparations of the gastric fluid solution and the results were within the inherent variability of the method (as demonstrated by the RRT analysis of the 6 materials within Henderson et al., 2014). Annex 2 of the SOP further provides references for each metal included in the applicability domain to demonstrate that the various listed metals can be evaluated for bioaccessibility under the test parameters (i.e. not anticipated to precipitate, etc.).

7. Within-laboratory reproducibility (WLR) (Module 2)

7.1 Assessment of repeatability and reproducibility in the same laboratory

The proposed procedure is very simple and easily reproducible. The procedure could be performed in the frame of routine laboratory practice of accredited laboratories. The variability between replicates is always in the range of introduced criterion for acceptance of the results from the test. ESAC agrees that the procedure is fit-for-purpose and the intrinsic data variability is very low.

7.2 Conclusion on within-laboratory reproducibility as assessed by the study

a) In the RRT study the within-laboratory precision was evaluated calculating the Sr. The lowest Sr value was 0.009 (Ni compound) and the highest 0.083 (Fe-Inconel alloy 718). The second approach used to assess within-laboratory reproducibility was based on the RSD analysis of the log concentration and was expressed in percentages. RSD < 10% was the criteria used for acceptable repeatability. All RSD values were below 4%. The highest and lowest variability was obtained with Fe-Inconel alloy 718 (3.1%) and Ni compound (0.2%) (Henderson et al., 2014). This outcome is consistent with the above Sr approach. The ESAC WG considers this to be excellent.

b) Various experiments with different types of samples (pure metals and alloys) have been performed in an accredited laboratory (ECTX) and the within-laboratory repeatability assessed is very good with CVs in the range 2-5% for all types of samples, depending only on the concentration range. For example, 3% CV for Ni in 0.2 g/L loading and 2% CV in 2 g/L loading for Ni (FR X0a-214); 2% CV for Pb and 4% CV for Zn for 0.2 g/L loading for leaded

brass (FR X02a-216); 2% CV for Pb in Pb metal for both 0.2 and 2 g/L loadings (FR X0a-217).

c) Parallel samples of pure metals and alloys have been analysed in a period of 5 years in the same accredited laboratory 2014-2019 (ECTX) [Henderson et al., 2014; (FR X02a-216); (FR X0a-217); (FR X02a-229 (ECTX, 2019)]. The calculated RSDs, representing within-laboratory reproducibility is up to 10% even after small changes of analytical protocol. These values confirmed the sustainability of developed method.

d) The accredited laboratory ECTX showed consistent results of %RBC for Co in cobalt alloy (2 g/L loading) and for Pb in leaded brass alloy (0.2 and 2 g/L loadings) using two methods of HCl pH 1.5 preparation[2]. This confirms good within-laboratory reproducibility of the proposed method for %RBC determination (Summary bioelution tests, 2019).

8. Transferability (Module 3)

8.1 Quality of design and analysis of the transfer phase

A transferability study was not conducted but the method was implemented in multiple laboratories during the RRT and between-laboratory reproducibility data are available. The proposed procedure is very simple and easily reproducible. As the procedure will be performed in the frame of routine laboratory practice of accredited laboratories, the ESAC WG considers that a transferability study is unnecessary.

8.2 Conclusion on transferability to a naïve laboratory / naïve laboratories as assessed by the study

Not applicable.

9. Between-laboratory reproducibility (BLR) (Module 4)

9.1 Assessment of reproducibility in different laboratories

Five laboratories were used for the RRT, and the results were published in Henderson et al. (2014).

9.2 Conclusion on between-laboratory reproducibility as assessed by the study

The between-laboratory reproducibility with respect to both, the intrinsic data variability and the agreement between laboratories, assessed by RSD and S_R:Sr, was considered to be adequate by the ESAC WG.

[2] i) A 0.07N HCl solution was prepared and the pH adjusted to 1.5 with NaOH; ii) A HCl dilution to pH 1.5 was prepared from a 0.1M HCl titrated solution.

10. Predictive capacity and overall relevance (Module 5)

10.1 Adequacy of the assessment of the predictive capacity in view of the purpose

The Bioelution method is a useful approach for determining the relative IVBA of metal released from inorganic metal compounds and metal-containing materials compared to metal released from a reference material. The relative IVBA data generated by the Bioelution method can be used to i) support grouping and read across and ii) establish the presence or absence of a matrix effect to inform alloy classification. Importantly, the Bioelution method is not to be used for predicting relative bioavailability for application in a risk assessment context.

IVBA testing is a well-established approach in use for several years. Bioaccessibility tests for the oral route of exposure (including gastric compartment) have been applied to the assessments of human exposures to metal compounds and minerals in soils and dust. "It is important to consider that while the proposed SOP addresses the generation of IVBA data in simulated gastric fluid and its use for the calculation of the %RBC of metals in alloys, how this will be used in a regulatory context is not part of the SOP." A comparison of the relative IVBA data with *in vivo* relative bioavailability data was not conducted in this ESAC review. Relative bioavailability is the ratio of the oral absorption fraction of the chemical present in some target material to the oral absorption fraction of the chemical in an appropriate reference material as measured in an animal model. It accounts for differences in absorption between the chemical in the reference material and in the test material. Such comparison is not necessary and does not compromise the assessment of the scientific validity of the method given that relative measurements between target and reference materials are used both for grouping and read across, and classification of alloys. The lack of *in vivo* bioavailability to support the results of the IVBA analysis is considered a significant limitation of the method and prevents the ESAC WG from recommending the direct use of bioelution data in human health risk assessments.

10.2 Overall relevance (biological relevance and accuracy) of the test method in view of the purpose

The Bioelution method is an *in vitro* method used to measure metal releases in simulated gastric fluid (pH 1.5). It does not address a biological effect. The Bioelution method provides data regarding relative IVBA, and it is important to consider that bioaccessibility is related to systemic bioavailability of metal ions (see section 2.1).

11. Applicability domain (Module 6)

11.1 Appropriateness of study design to conclude on applicability domain, limitations and exclusions

ESAC WG considers that the information provided on the applicability domain and the limitations of the test method is appropriate.

11.2 Quality of the description of applicability domain, limitations, exclusions

The applicability domain is clearly described in the SOP in terms of technical limitations and context of use and is considered appropriate. Reproducibility and international harmonisation are ensured by clearly defining the reference materials for each metal that must be run in parallel to test materials

for alloy classification purposes. While for the read across and grouping application it is not possible to predefine the reference materials, the SOP still requires these to be run concurrently to the target material so as to guarantee the robustness and consistency of the relative measurement. Table 1 outlines which metals/metalloids are within scope for each of the two applications; the references for this table are provided in Annex 2 of the SOP. In conclusion, the ESAC considers that the SOP is well described and robust, thus ensuring reproducibility of data and easy implementation of the assay by naïve laboratories.

Table 1. Metal/Metalloid within and out of scope of main two applications

Metal/metalloid	**Simulated gastric fluid technical applicability[a]**	**Application 1[b]**	**Application 2[c]**
Ag	NA	NA	NA
Ag compounds	NA	NA	NA
As	✓	✓	✓
As compounds	✓	✓	NR
Au	✓	✓	NC
B	✓	✓	NC
B compounds	✓	✓	NR
Be	*pending* [d]	✓ [d]	✓ [d]
Cd	✓	✓	✓
Cd compounds	✓	✓	NR
Co	✓	✓	✓
Co compounds	✓	✓	NR
Cr	✓	✓	NC
Cr compounds	✓	✓	NR
Cu	✓	✓	NC
Cu compounds	✓	✓	NR
Fe	✓	✓ [e]	NC
Fe compounds	✓	✓	NR
Ge	✓	✓	NC
Ge compounds	✓	✓	NR
Hg	NA	NA	NA
Hg compounds	NA	NA	NA
In	✓	✓	NC
In compounds	✓	✓	NR
Mn	✓	✓	NC
Mn compounds	✓	✓	NR
Mo	✓	✓	NC
Mo compounds	✓	✓[e]	NR
Ni	✓	✓	✓
Ni compounds	✓	✓	NR
Pb	✓	✓	✓
Pb compounds	✓	✓	NR
Pd	✓	✓	NC
Pd compounds	✓	✓	NR
Pt	✓	✓	NC
Re	✓	✓	NC
Rh	✓	✓	NC
Rh compounds	✓	✓	NR
Ru	✓	✓	NC
Sb	✓	✓	NC
Sb compounds	✓ [f]	✓[e]	NR
Se	*pending* [d]	✓ [d]	✓ [d]
Si	✓	✓[e]	NC

Si compounds	✓	✓	NR
Ti	✓	✓	NC
Ti compounds	✓	✓	NR
V	✓	✓	NC
V compounds	✓	✓	NR
W	✓	✓[e]	NC
W compounds	✓	✓	NR
Zn	✓	✓	NC
Zn compounds	✓	✓	NR
Zr	✓	✓	NC

[a] A check mark means that the element can be measured in simulated gastric fluid without technical limitations (e.g. no precipitation, no complexation). [b] A check mark means that the element is within scope of grouping and read across. [c] A check mark means that the element is within scope of calculating %RBC to inform alloy classification for oral route. [d] Testing their applicability in simulated gastric fluid will be addressed as part of the proposed testing of Reference Materials, see Annex 1 of the SOP. [e] Element release in simulated gastric fluid is not a worst-case scenario for the oral route and additional information is needed. See examples in text above table. [f] Except Antimony trichloride (ATC) and Antimony pentachloride (APC) and any other corrosive Sb compounds.

NA: not applicable; NR: Not relevant as metal compounds are not the primary references for the calculation of relative bioaccessible concentration (%RBC) in alloys; NC: the metal/metalloid (Metal^{0}) is not currently classified for systemic effects and therefore it is outside of the scope of application .

12. Performance standards (Module 7)

Performance Standards are not considered necessary due to the fact that the test method has no proprietary elements.

12.1 Adequacy of the proposed Essential Test Method Components

Not applicable.

12.2 Adequacy of the proposed Reference Chemicals

Not applicable.

12.3 Adequacy of the proposed performance target values

Not applicable.

13. Readiness for standardised use

13.1 Assessment of the readiness <u>for regulatory purposes</u>

The ESAC WG considers the Bioelution method to be ready for standardised use in a regulatory context to i) support grouping and read across and ii) establish the presence or absence of a matrix effect to inform alloy classification. However, the exact manner in which the relative IVBA data generated with the simulated gastric fluid will be used in a regulatory context was not part of the present assessment.

13.2 Assessment of the readiness for other uses

The available information did not permit the assessment of the validity of the Bioelution method to predict relative bioavailability values for application in a risk assessment context.

13.3 Critical aspects impacting on standardised use

The following factors should be taken into consideration to guarantee a standardised use of the Bioelution test method:

- Standardised materials (i.e. proficiency materials and reference materials) should be available for testing by laboratories using the gastric Bioelution test SOP.
- The test material should always be run in parallel to a reference material.
- Same reference materials need to be used across laboratories to establish the presence or absence of a matrix effect in alloys and to calculate the RBC% of the metal in the alloy.
- For the alloys, the reference material and the test material should have similar physical form.
- Acceptance criteria for the proficiency materials need to be established.
- A physical repository of proficiency materials and the reference materials for the alloys and a publically available database to housing measurements of the samples present in the repository need to be established.

13.4 Gap analysis

All gaps identified were addressed with the test method submitter during the peer-review process. These were considered to be addressed before issuing the present assessment report and the ESAC Opinion. The main points addressed are listed here below.

General:

- Clarify the proposed applications i) grouping and read-across and ii) alloy classification.
- Ensure using the wording 'relative' bioaccessible concentration throughout the documents.
- Clarify the rationale for the proposed gastric fluid composition.
- Clarify the applicability domain of the test method.

Procedural:

- Make use of a simplified preparation of the gastric fluid.
- Clarify in the SOP the need to run in parallel the testing reference materials and indicate the selection criteria of these reference chemicals.
- Commitment from test submitter to set up a repository of reference materials.
- Proof-of-concept testing that demonstrate the revised SOP provide reproducible results.

Standard Operating Procedure:

- Clarifications regarding homogeneity of the test samples.
- Clarifications regarding the data interpretation procedure.
- Use of a confidence interval for the %RBC.
- Clarifications on how to select the release data from high or low loading for each application.
- Clarifications on when epoxy embedded sample can be used.
- Clarify the procedures to be undertaken in case of pH drift.

14. Other considerations

No other considerations were addressed by the ESAC WG.

15. Conclusions on the study

15.1 ESAC WG summary of the results and conclusions of the study

In vivo oral bioavailability refers to the fraction of the total amount of a substance that is released in the gastrointestinal tract and absorbed into the blood stream. This can be estimated *in vitro* by measuring the dissolved quantity of a metal ion release under surrogate physiological condition in a bioaccessibility test.

The Bioelution method generates relative IVBA data that may be used as supportive information for (i) classification of inorganic metal compounds and metal-containing materials (e.g. UVCBs, pigments and alloys) by grouping and read across and (ii) classification of alloys, where the %RBC of a metal in an alloy versus a reference material (e.g. a pure metal) is used to establish the presence or absence of a matrix effect.

A comparison of the IVBA data with *in vivo* oral relative bioavailability data was not conducted in this ESAC review. Relative bioavailability is the ratio of the oral absorption fraction of the chemical present in some target material to the oral absorption fraction of the chemical in an appropriate reference material. It accounts for differences in absorption between the chemical in the reference material and in the test material. Such comparison is not necessary and does not compromise the assessment of the scientific validity of the method given that relative measurements between target and reference materials are used both for grouping and read across, and classification of alloys.

15.2 Extent to which study conclusions are justified by the study results alone

The Bioelution method can be used to assess the matrix effect of an alloy by comparing the release of metal ions between a reference material (e.g. pure metal) and an alloy. When a matrix effect occurs, there is an increased or decreased relative release of the metal ions from the alloy in comparison to the reference material. In the absence of a matrix effect (e.g. simple metal mixture), a linear relationship between the fraction of metal ion release and the total content of that metal in the material is expected and this has been confirmed by the method submitter. Therefore, the ESAC considers that this is an appropriate use of the Bioelution method.

ESAC considers that the within- and between-laboratory reproducibility of absolute bioaccessibility measurements for the six metals tested (i.e. Co, Cu, Fe, Ni, Pb, Zn tested in the RRT as published by Henderson et al., 2014) were acceptable. The ESAC considers that this should hold true also for other metals; however, data have not been provided to support this assumption. The current SOP further ensures reproducibility across laboratories by ensuring high consistency in the preparation of the simulated gastric fluid. Reproducibility and international harmonisation are also ensured by clearly defining the reference materials for each metal that must be run in parallel to test materials for alloy classification purposes. While for the read across and grouping application it is not possible to predefine the reference materials, the SOP still requires these to be run concurrently to the target material so as to guarantee the robustness and consistency of the relative measurement.

The applicability domain is clearly described in the SOP in terms of technical limitations and context of use and is considered appropriate. In conclusion, the ESAC considers that the Bioelution method is well described and robust, thus ensuring reproducibility of data and easy implementation of the assay by naïve laboratories.

15.3 Extent to which conclusions are plausible in the context of existing information

The Bioelution method is representative of the gastric compartment. Publications in the peer-reviewed literature show that additional components (e.g., ascorbic acid, pepsin, and glycine) could be added to the gastric solution to increase solubility of certain metals (e.g., Whitacre et al. 2017). However, these additional components add complexity to the method and are unlikely to affect the relative measurement that the method produces. The selection of the 2-hour extraction time is consistent with other IVBA methods using simulated gastric fluid and with the time for complete emptying of the human stomach. The ESAC supports the current Bioelution method protocol and acknowledges that the simple gastric solution is fit-for-purpose considering the proposed context of use.

IVBA testing is a well-established approach in use for several years. Bioaccessibility tests for the oral route of exposure (including gastric compartment) have been applied to the assessments of human exposures to metal compounds and minerals in soils and dust. US EPA Validated Test Method 1340 assesses the bioaccessibility for Pb in soil. Validation studies of IVBA test methods for estimation of bioavailability of As from soil and sediment have been conducted. Moreover, the proposed Bioelution method is similar to the Bioaccessibility Research Group of Europe (BARGE) method, which has been in use in Europe for several years to assess As, Pb, Sb and Cd in soil samples and was accepted as an ISO standard in 2018 (ISO 17924).

16. Recommendations

16.1 General recommendations

The ESAC WG concludes that the Bioelution method is scientifically valid for determining the relative IVBA of metals in inorganic metal compounds and metal-containing materials. Furthermore, the ESAC WG recommends that the Bioelution method progresses to Test Guideline development at OECD level to achieve international harmonisation. The development of a standardized and agreed test guideline will be helpful to ensure the protocol of the Bioelution method is applied consistently across jurisdictions.

16.2 Specific recommendations (e.g. concerning improvement of SOPs)

Specific recommendations were addressed during the peer-review process and are described in section 13.4. The only pending action remains the set-up of a repository of reference materials.

17. References

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Appendix 1. Overview of the tests performed at ECTX laboratories during Submission/review of the Bioelution Test Method

FR X02a-081 (18/06/2017)	Exp. starting date: **11-11-2016** Exp. completion date: **05-12-2016**	2 hours Bio-elution Study on **Stainless Steel 316L Massive** *(Epoxy resin embedded)* at a 2 g/L loading (surface equivalent) in a simulated gastric fluid	No reference materials tested in parallel	**Co release:** 0.063 µg/g ± 0.003 **Ni release:** 1.55 µg/g ± 0.08
FR X02a-084 (18/06/17)	Exp. starting date: **11-11-2016** Exp. completion date: **05-12-2016**	2 hours Bio-elution Study on **Stainless Steel 316L Powder** at a 2 g/L loading in a simulated gastric. Cobalt (content: ≤0.214 %), nickel (content: 10.65 %)	No reference materials tested in parallel	**Co release:** 2.91 µg/g ± 0.04 **Ni release:** 61.4 µg/g ± 0.3
FR X02a-130 (19/12/17)	Exp. starting date: **19-09-2017** Exp. completion date: **22-09-2017**	2 hours Bio-elution Study on **Fe-Cr-Ni Powder Mixture** (68.6/16.4/10.7 mass fraction) at a 0.2, 1 and 2 g/L loading in a simulated gastric fluid	Reference materials tested: Cr, Fe, Ni powders tested at 0.2g/L loading	• To demonstrate presence of a **matrix effect** in alloys. *See Table 7 in "Responses to questions in EURL ECVAM Test Presubmission Assessment Report".* • To examine the **influence of sample mass**, either as inert material (SiO_2) or as alloy material on the release of Ni. *See Table 8 in "Responses to questions in EURL ECVAM Test Presubmission Assessment Report".*
FR X02a-144 (21/04/18)	Exp. starting date: **24-01-2018** Exp. completion date: **02-02-2018**	2 hours Bio-elution Study on **Stainless Steel 316L Powder** at a 2 g/L loading in a simulated gastric fluid. Cobalt (content: ≤0.214 %), nickel (content: 10.65 %)	Three reference materials tested: Nickel powder with a comparable amount of nickel(nominal loading 0.2g/L) Nickel powder (nominal loading 0.2g/L) supplemented with an inert material (silicon dioxide) up to a final loading of 2g/L Silicon dioxide (1.79 g/L)	

FR X02a-145 (21/04/18)	Exp. starting date: **24-01-2018** Exp. completion date: **02-02-2018**	4 hours Bio-elution Kinetics Study on **Stainless Steel 316L Powder** at a 2 g/L loading in a simulated gastric fluid. Nickel (content 10.65 % Iron (content 68.61 %) An alloy (SS316L), two of its pure ingredients (Fe^0 and Ni^0) and two water soluble compounds of these metal ingredients ($FeSO_4$ $7H_2O$ and $NiCl_2$ $6H_2O$) were tested	Reference materials: 2 g/L nickel powder 2 g/L loading of nickel(II)chloride hexahydrate 2 g/L iron powder 2 g/L loading of iron(II) sulfate heptahydrate	To **examine the kinetics of metal release** in gastric fluid. *See Figure 7 in "Responses to questions in EURL ECVAM Test Presubmission Assessment Report".*
FR X02a-146 (21/04/18)	Exp. starting date: **24-01-2018** Exp. completion date: **02-02-2018**	4 hours Bio-elution Kinetics Study on **Z45 (massive)** at a 2 g/L loading (surface equivalent) in a simulated gastric fluid An alloy (Z45, 62.176% copper) and its pure ingredient (Cu^0, 100% Cu), both as disks, were tested	Reference material: copper **massive**	To **examine the kinetics of metal release** in gastric fluid (massive sample). *See Figure 8 in "Responses to questions in EURL ECVAM Test Presubmission Assessment Report".*
FR X02a-213 (09/10/19)	Exp. starting date: **22-07-2019** Exp. completion date: **02-08-2019**	2 hours Bioelution Study on **Nickel sulfate hexahydrate** at a 0.2 and 2 g/L loading in a simulated gastric fluid		**Comparability of bioelution results** obtained with three nickel compounds tested in 2009 (reported in Henderson et al., 2012) and with current SOP (pH simulated gastric fluid adjusted with NaOH) *See Table 3 in Annex 1 to responses to ESAC comments – "Annex 1 RCOM 24092019" (Summary of bioelution tests performed to address questions/concerns raised by ESAC).*
FR X02a-214 (09/10/19)	Exp. starting date: **22-07-2019** Exp. completion date: **02-08-2019**	2 hours Bioelution Study on **Nickel subsulfide** at a 0.2 and 2 g/L loading in a simulated gastric fluid		
FR X02a-215 (09/10/19)	Exp. starting date: **22-07-2019** Exp. completion date: **02-08-2019**	2 hours Bioelution Study on **Nickel oxide** at a 0.2 and 2 g/L loading in a simulated gastric fluid		

FR X02a-216 09/10/19)	Exp. starting date: **22-07-2019** Exp. completion date: **02-08-2019**	2 hours Bioelution Study on **Leaded Brass Alloy** at a 0.2 and 2 g/L loading in a simulated gastric fluid (58.45% **Cu**, 3.22% **Pb**, 37.75 % **Zn**)	Leaded brass alloy used in Henderson et al., 2014	**Comparability of bioelution results** with those obtained in round robin (Henderson et al., 2014). *See Table 2 in Annex 1 to responses to ESAC comments – "Annex 1 RCOM 24092019" (Summary of bioelution tests performed to address questions/concerns raised by ESAC).* **Calculate the RBC%** of Pb in leaded brass alloy. Consider data from **both loadings.** *See Table 5 in Annex 1 to responses to ESAC comments – "Annex 1 RCOM 24092019" (Summary of bioelution tests performed to address questions/concerns raised by ESAC).*
FR X02a-217 (09/10/19)	Exp. starting date: **22-07-2019** Exp. completion date: **02-08-2019**	2 hours Bioelution Study on **Lead (powder)** at a 0.2 and 2 g/L loading in a simulated gastric fluid		

FR X02a-218 (09/10/19)	Exp. starting date: **22-07-2019** Exp. completion date: **02-08-2019**	2 hours Bioelution Study on **Cobalt (powder)** at a 0.2 and 2 g/L loading in a simulated gastric fluid	Co metal powder used in Henderson et al., 2014	**Comparability of bioelution results** with those obtained in round robin (Henderson et al., 2014). *See Table 2 in Annex 1 to responses to ESAC comments – "Annex 1 RCOM 24092019" (Summary of bioelution tests performed to address questions/concerns raised by ESAC).*
FR X02a-219 (09/10/19)	Exp. starting date: **22-07-2019** Exp. completion date: **02-08-2019**	2 hours Bioelution Study on **Cobalt (powder)** at a 0.2 and 2 g/L loading in an *alternative prepared simulated gastric fluid*		**Comparison of metal release using simulated gastric fluid prepared in two different ways**: a) from 0.07 N HCl adjusted to pH 1.5 with NaOH or b) as a diluted of a concentrated HCl solution. *See Table 4 in Annex 1 to responses to ESAC comments – "Annex 1 RCOM 24092019" (Summary of bioelution tests performed to address questions/concerns raised by ESAC).*
FR X02a-220 (09/10/19)	Exp. starting date: **11-08-2019** Exp. completion date: **16-08-2019**	2 hours Bioelution Study on **Lead (powder)** at a 0.2 and 2 g/L loading in an alternative prepared simulated gastric fluid		
FR X02a-229 (11/12/19)	Exp. starting date: **10-10-2019** Exp. completion date: **22-10-2019**	2 hours Bioelution Study on **Leaded Brass Alloy** at a 0.2 and 2 g/L loading in a simulated gastric fluid Leaded brass alloy powder used in *Henderson et al 2014* and in X02a-2016	Leaded brass alloy powder used in Round Robin (Henderson et al., 2014) and in previous studies (X02a-216)	**Calculation of %RBC of lead in leaded brass**, at two loadings, and with two methods of HCl pH 1.5 preparation. *See Tables 2 and 3 in "Results of Oct*

FR X02a-230 (11/12/19)	Exp. starting date: **10-10-2019** Exp. completion date: **22-10-2019**	2 hours Bioelution Study on **Lead (powder)** at a 0.2 and 2 g/L loading in a simulated gastric fluid	Pb metal powder used in previous studies (X02a-220)	*2019 testing" (Summary of bioelution tests performed to address questions/concerns raised by ESAC).*
FR X02a-231 (11/12/19)	Exp. starting date: **10-10-2019** Exp. completion date: **22-10-2019**	2 hours Bioelution Study on **Cobalt (powder)** at a 2 g/L loading in a simulated gastric fluid	Cobalt metal powder	**Calculation of %RBC of cobalt in a cobalt alloy**, at the high loading of 2 g/L, and with two methods of HCl pH 1.5 preparation. *See Tables 4 and 5 in "Results of Oct 2019 testing" (Summary of bioelution tests performed to address questions/concerns raised by ESAC).*
FR X02a-232 (11/12/19)	Exp. starting date: **10-10-2019** Exp. completion date: **22-10-2019**	2 hours Bioelution Study on **Cobalt alloy** (powder) at a 2 g/L loading in a simulated gastric fluid	Cobalt alloy	

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