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# Inter-laboratory validation of bioaccessibility testing for metals

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ABSTRACT

Bioelution assays are fast, simple alternatives to *in vivo* testing. In this study, the intra- and inter-laboratory variability in bioaccessibility data generated by bioelution tests were evaluated in synthetic fluids relevant to oral, inhalation, and dermal exposure. Using one defined protocol, five laboratories measured metal release from cobalt oxide, cobalt powder, copper concentrate, Inconel alloy, leaded brass alloy, and nickel sulfate hexahydrate. Standard deviations of repeatability ($s_r$) and reproducibility ($s_R$) were used to evaluate the intra- and inter-laboratory variability, respectively. Examination of the $s_R$:$s_r$ ratios demonstrated that, while gastric and lysosomal fluids had reasonably good reproducibility, other fluids did not show as good concordance between laboratories. Relative standard deviation (RSD) analysis showed more favorable reproducibility outcomes for some data sets; overall results varied more between- than within-laboratories. RSD analysis of $s_r$ showed good within-laboratory variability for all conditions except some metals in interstitial fluid. In general, these findings indicate that absolute bioaccessibility results in some biological fluids may vary between different laboratories. However, for most applications, measures of relative bioaccessibility are needed, diminishing the requirement for high inter-laboratory reproducibility in absolute metal releases. The inter-laboratory exercise suggests that the degrees of freedom within the protocol need to be addressed.



## 1. Introduction

As the demand for understanding the potential hazard and risk of chemicals to human health continues to grow, the data required for elucidating these concerns continues to expand as well. Meeting the new and evolving demands of regulatory programs such as the Registration, Evaluation, and Authorization of Chemicals (REACH) regulation in Europe (Regulation (EC) No 1907/2006, 2006) necessitates the generation of new and scientifically robust data on chemical substances, including metals. The *in vivo* testing that would be required to fill these needs is often cost-prohibitive and time-consuming, and also raises concerns with regards to

*Abbreviations:* CEN, European Committee for Standardization; CLP, classification, labeling and packaging of substances and mixtures regulation; RBA, relative bioavailability; ECHA, European Chemicals Agency; RBALP, relative bioaccessibility leaching procedure; REACH, Registration Evaluation and Authorization of Chemicals; RSD, relative standard deviation; $s_r$, repeatability standard deviation; $s_R$, reproducibility standard deviation; UBM, unified BARGE method.

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animal welfare due to the extent of testing potentially required. As such, alternative approaches such as read-across (extrapolation of known data from one substance to another substance) based on structure activity relationships or bioavailability are often encouraged to perform hazard and risk assessment while reducing animal testing (ECHA, 2008, 2013). For most routes of exposure and health endpoints, it is indeed the bioavailability of the metal at the target site in an organism that is the most important factor determining its potential toxicity. Bioaccessibility, referring in this context to the amount of metals released from a given material in fluids designed to mimic those of the human body that may become available for uptake (e.g., synthetic gastric fluid to simulate oral exposure) (Ruby et al., 1999; Henderson et al., 2012), provides a conservative estimate of bioavailability. Bioaccessibility is measured in *in vitro* bioelution assays, whose application to hazard and risk assessment has been increasingly used as an alternative to *in vivo* testing in recent years. Bioaccessibility is a conservative concept because not all metals available will be absorbed or induce damage (effects will depend on dose and metal speciation). Bioaccessibility data are particularly informative, as the presence of a metal does not always impart its biological properties on a given material, for example when the release of the metals and their absorption may be limited due to surface and material properties (e.g., for alloys).

The comparison of bioaccessibility data for two or more forms of the same metal (e.g., a pure metal and an alloy with the same metal constituent) enables an estimate of their relative *in vivo* bioavailability. This type of information can be used in a variety of ways for metals assessment, including: as a tool in determining hazard classification (e.g., using relative bioavailability to determine classification or justifying a derogation because of a lack of bioavailability; ECHA, 2013), to aid in establishing categories of metal substances (grouping; ECHA, 2008), as part of the weight of evidence approach applied in performing read-across (e.g., Henderson et al., 2012), and for risk assessments for exposure to metals required by some consumer product safety regulations (Brock and Stopford, 2003). In addition, relative bioaccessibility can be used to estimate the effective concentration (defined as the bioaccessible concentration of a constituent substance in a complex material) of a metal in a complex material where matrix effects may occur (e.g., alloys) and enable read-across between these materials (Stockmann-Juvala et al., 2013; Hedberg et al., 2013).

The bioaccessibility concept is already incorporated in some standard bioelution test methods and regulatory frameworks, such as the European standard for release of nickel in artificial sweat (BS EN 1811, 2011), ASTM D5517 (2007) for metals in art materials, and BS EN 71-3 (2013) that specifies safety requirements for metals in toys. Bioaccessibility has been listed as a possible approach for complying with information requirements of REACH as part of the chapter on grouping of chemicals (ECHA, 2008).

Method development for – and utilization of – bioelution testing by independent and government research groups have increased. The bioaccessibility approach to estimate metal bioavailability has been applied in recent years to human exposures to metals and minerals in soils, consumer products, and to the evaluation of metal substances (Hillwalker and Anderson, 2014; Henderson et al., 2012; Stopford et al., 2003; Herting et al., 2008; Hedberg et al., 2010; Mazinanian et al., 2013; Oller et al., 2009; Hamel et al., 1998; Vasiluk et al., 2011; Drexler and Brattin, 2007; Wragg et al., 2011; Ellickson et al., 2001; Turner, 2011; Gray et al., 2010; Twining et al., 2005; Hedberg et al., 2013, 2012; Hedberg and Odnevall Wallinder, 2013; Jiang et al., 2012; Guney and Zagury, 2014). In addition, some groups have developed research programs to perform inter-laboratory validation of bioelution methods for specific systems and metals. For example, Drexler and Brattin (2007) reported the outcome of a validation exercise for a method to estimate *in vivo* bioavailability of lead (Pb) from soils. Additionally, a separate group also performed a round-robin study for a different physiologically-based method for estimating the bioaccessibility of Pb, as well as cadmium (Cd) and As, from soils (Wragg et al., 2011). Cordeiro et al. (2012) reported the results of an inter-laboratory comparison of 8 metal releases in comminuted flakes from alkyd resin paints simulating a toy coating using BS EN 71-3 (2013).

Although some groups have sought to standardize specific methods (Drexler and Brattin, 2007; Wragg et al., 2011; Ashley et al., 2012; Cordeiro et al., 2012), generally standardized fluid compositions and testing protocols for the basic bioelution method are lacking. In addition, there are no reference standards to ensure the accuracy of these bioaccessibility results and existing studies have demonstrated that sample characteristics and methodological differences (e.g., temperature, pH, sample loading) can affect the amount of metals released (Stopford et al., 2003; Midander et al., 2006; Hedberg et al., 2013).

The aim of the current study, therefore, was to perform a cross-laboratory testing of different metal-containing materials in select simulated biological fluids that are relevant to characterizing key routes of human exposure, using a defined protocol. To do so, five laboratories measured the release of metal from six different metals and metal-containing materials in synthetic gastric, lysosomal/interstitial, and perspiration fluids (representing oral, inhalation, and dermal routes of exposure, respectively). The results of these bioelution analyses were evaluated by characterizing within-laboratory repeatability and between-laboratory reproducibility measures.

## 2. Materials and methods

### 2.1. General study design

The five laboratories participating in the inter-laboratory validation study were Center of Ecotoxicology and Chemistry of Metals, Universidad Adolfo Ibañez (Santiago, Chile), ECTX-Consult (Hasselt, Belgium) with analytical work conducted at Labtium Oy (Finland), Kirby Memorial Health Center (Wilkes-Barre, PA, USA), Oregon State University (Corvallis, Oregon, USA) and KTH Royal Institute of Technology (Stockholm, Sweden). Each laboratory was assigned an identification code of A–E in no specific order and is referred to by its respective coding throughout this manuscript. All labs performed bioaccessibility testing in the following four simulated biological fluids: gastric, lysosomal, interstitial, and perspiration. Labs were asked to follow a Standard Operating Procedure (SOP; dated November 2010) provided and discussed prior to study initiation. In brief, test materials were added to simulated fluids and extracted for a set period of time under standard conditions (e.g., pH, temperature). Following a filtration step, extracts were analyzed and the amounts of metals released into solution were reported. Laboratories measured the release of seven different metals (Cr, Co, Cu, Fe, Ni, Pb and Zn) depending on the composition of the test materials.

### 2.2. Test materials

The six materials tested are listed in Table 1 with their respective chemical formula, CAS number, metal content, mean particle size, surface area, and supplier. The materials were Co oxide, Co powder, Cu concentrate, Inconel alloy, leaded brass alloy, and Ni sulfate hexahydrate. All test materials were powders with a median particle size <60 μm in diameter representing a size range relevant for oral and dermal exposures. However, although the SOP