Proposed responses to questions raised in the Test Pre-Submission Assessment Report

1. Explain and justify that the experimental conditions in the proposed protocol (only gastric fluid) represent the worst case scenario for the oral route of exposure. Is this the case for most metals? Please list the exceptions.

The proposed protocol is relevant for systemic health effects of metals and not for local effects that may occur after inhalation or dermal exposure to inorganic metals and metal compounds. Health effects secondary to the systemically bioavailable metal species of concern (e.g. reproductive effects) can manifest after exposures via dermal, inhalation or oral routes of exposure, as long as these exposures result in bioaccessible (first) and bioavailable (second) metal levels above the threshold values for these effects. In general, the dermal absorption of inorganic metals and metal compounds is very low <1% and will not contribute significantly to systemic health effects (see more information about dermal absorption under Q14). Inhalation exposure can contribute to systemic health effects via direct absorption of released metal ions from particles deposited in the alveolar-pulmonary region of the lung or via gastrointestinal (oral) absorption of particles (e.g. nanos) or metal ions from particles deposited in other respiratory tract areas (tracheobronchial and nose). Therefore, the oral route of exposure covers not just the ingestion of metals but also a good fraction of the inhalation of metal particulates.

The following artificial fluids have been considered for the oral route: saliva, gastric and intestinal, separate or in sequence, with variations in the composition of these fluids. In general, the gastric fluid has been found to result in the higher metal releases from soils, metal compounds and alloys[1].
For the inhalation route of exposure, the following artificial fluids have been generally tested: interstitial or Gamble fluid mimicking the liquid on the surface of the lung and relevant to the dissolution of particles upon lung deposition; and artificial lysosomal fluid ('ALF') mimicking the lysosomal fluid within the macrophages and relevant to the subfraction of particles that are cleared from the pulmonary region of the respiratory tract by a macrophage-mediated process.

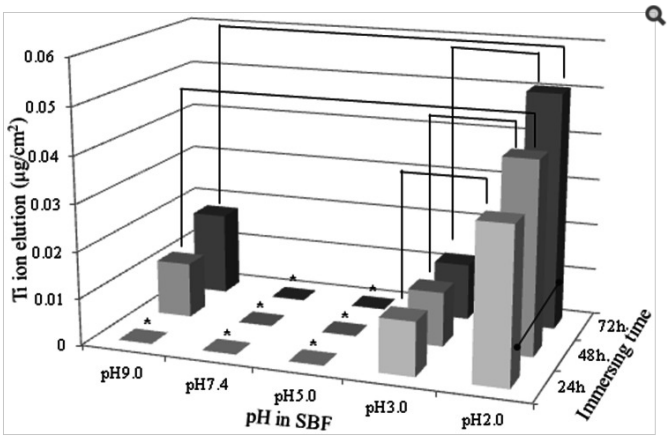
In general, the metal release in gastric fluid is higher than metal release in any other of the fluids mentioned above, with a few exceptions. The following metals have shown releases in gastric fluid to be higher than releases in other oral fluids (nickel, cobalt, lead, arsenic, cadmium, chromium, titanium, copper, iron, indium, and zinc). This can be explained by the very low pH of the gastric fluid; most metals and metal compounds show highest dissolution at lower pH. This is further documented in Annex 1, Tables 1-3 for metals like **Cd, Cr, Zn, Pb, Ni, Cu and Co.**

Titanium (Ti):

The effect of pH on the release of Titanium (Ti) is illustrated in Figure 1 based on data from Suito et al. 2013. A similar higher ion release from commercially pure (CP) Ti at low pH compared to higher pH is observed in Barao et al. 2011, with further supportive evidence from Cai et al. 1999.

[1] There are some exceptions such as Sb, W and Mo (see below).

Figure 1



Effect of pH on Ti ion elution from specimens of CP Ti immersed in SBF solutions for 24 to 72 h (*: below detection).

Figure 1: Effect of pH on Ti ion elution from specimens of CP Ti immersed in simulated body fluids solutions for 24, 48 or 72 h (corresponds to Figure 1 of Suito et al. 2013).

Indium (In):

Releases in gastric fluid (lower pH) can be compared to releases in sweat (higher pH). Bioelution test results showed higher releases in gastric fluid from In metal, chloride, nitrate, hydroxide, oxide, and sulfide than in sweat. In sweat, measured values were < LOD as In precipitates at neutral, slightly alkali pH (6-8) (Chrysikopoulos and Kruger 1986; Wang et al. 1998).

Boron (B):

For boron, the ECHA dissemination website has an entry for bioelution testing (under toxicokinetics) (https://echa.europa.eu/registration-dossier/-/registered-dossier/14776/7/2/2), where it is indicated that B is basically rather inert, with very low releases (0.1-0.2% release in 5 different media) and thus there appears to be hardly any effect of pH on B release.

Iron (Fe):

The releases of Fe from Fe metal and FeMo alloy in gastric fluid (GST, pH 1.5) were compared to other fluids of different composition and higher pHs such as lysosomal fluid (ALF), sweat (ASW), phosphate buffer (PBS) and Gamble (GMB) (Mörsdorf et al. 2015). The 2 and 24 hour results are shown in Figure 2 below. For iron metal, releases are the highest in GST after either time period. For FeMo, Fe releases are higher in GST than in ALF (pH 4.5), PBS (pH 7.4) and GMB (pH 7.4) after 2 hs, and as high or higher after 24 hours; by contrast, ASW (pH 6.5) yielder higher releases of Fe from FeMo than any other fluid at each time period. Results are consistent with those reported by Hedberg et al 2010a for Fe releases from stainless steel particles.

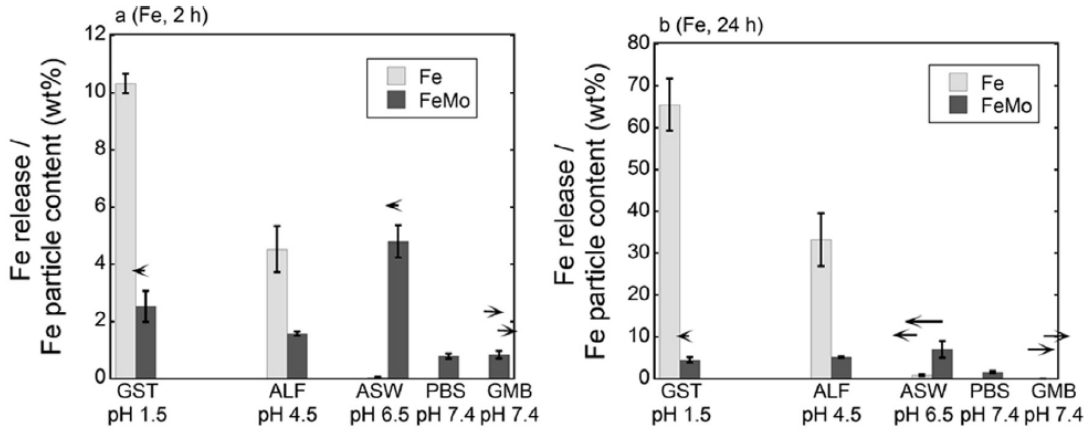


Figure 2: Influence of biological media and pH on the release rate of iron from stainless steel particles (corresponds to Figure 4 of Mörsdorf et al. 2015).

Information for other metals (silver, molybdenum, silicon, antimony, tungsten, and mercury) that require some further attention is reported below:

Silver (Ag). The release rate of silver from substances like Ag metal powder, Ag metal nanoparticles, disilver oxide (all three practically insoluble in water) and Ag nitrate (water solubility up to 2 kg/L) decreased according to the following sequence for all samples investigated after 2 hours of exposure: PBS > GMB > ASW > ALF >> GST (PMC, unpublished data). The presence of Ag+ complexing species such as chlorides or thiols in experimental media is known to favor dissolution thermodynamically. In the context of bioaccessibility testing, the presence of chloride ions in the different media and formation of poorly soluble silver chloride or silver chloride complexes can reasonably be expected to limit the concentration of free silver ions in physiological media (e.g. Zhang et al. 2013). Indeed, the silver release rate in the different test media corresponded with the total Cl concentrations in the various media: PBS (5.35 g/L) > GMB (3.97 g/L) > ASW (3.05 g/L) > ALF (2.02 g/L) > GST (0.97g/L)[2]. Less than 0.5% of the particle mass was dissolved after 24 hours of exposure in all media for all silver test materials investigated. Furthermore, the dissolved concentrations of silver in various artificial physiological media were very similar and seem independent of material type. The concentrations measured after 2 hours and 24 hours of exposure were practically constant. There was no kinetic behavior observed from the measured silver concentrations. It may be hypothesized that the complex ionic environment and the likely formation of poorly soluble silver chloride leads to very similar equilibrium concentrations of dissolved silver, independent of the originating substance. ***Thus, for silver containing substances, measuring metal release in fluids such as PBS or GMB that have higher chloride content than GST 2.5 g/L should be considered.***

Molybdenum (Mo) and Silicon (Si): Higher releases may be observed at neutral than acidic pH (Herting et al. 2014; Mörsdorf et al. 2015).

Herting et al. 2014 found that the release of Si from two metallurgical silicon grades was strongly dependent on both pH and media composition with the highest release in pH neutral media (see

[2] Please note that many other gastric fluid compositions (including the one proposed in the bioelution gastric test method) include 2.5 g Cl/L instead of 0.97 g Cl/L.

Figure 3 below) but did not test specifically other oral media like saliva or intestinal fluid. No similar effect was observed for a FeSi alloy or an aluminium silicate mineral.

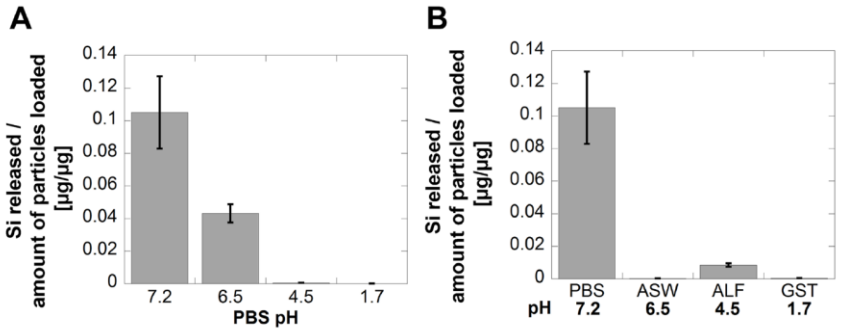


Figure 4. Released fractions [µg Si/µg] of Si from SiHG immersed in PBS of pH 7.2 and pH-adjusted PBS solutions (A), and corresponding released fractions of Si after 168 h of exposure in synthetic biological fluids of similar pH (GST, ALF and ASW) (B).
doi:10.1371/journal.pone.0107668.g004

Figure 3: Released fractions [µg Si/µg] of Si from SiHG immersed in PBS of pH 7.2 and pH-adjusted PBS solutions (A) and corresponding released fractions of Si after 168 h of exposure in synthetic biological fluids of similar pH (GST, ALF and ASW) (B) (corresponds to Figure 4 of Herting et al. 2014)

Mörsdorf et al. 2015 investigated the release of molybdenum and iron from powder particles of molybdenum metal (Mo), a ferromolybdenum alloy (FeMo), MoO_2, and MoO_3 in different synthetic body fluids of pH ranging from 1.5 to 7.4 and of different composition (see Figure 4). The release of molybdenum from the Mo powder after 24 hours generally increased with pH and was influenced by the fluid composition; at the 2-hour time point, gastric fluid showed the highest Mo releases from Mo metal and MoO_2 but not from FeMo or MoO_3.

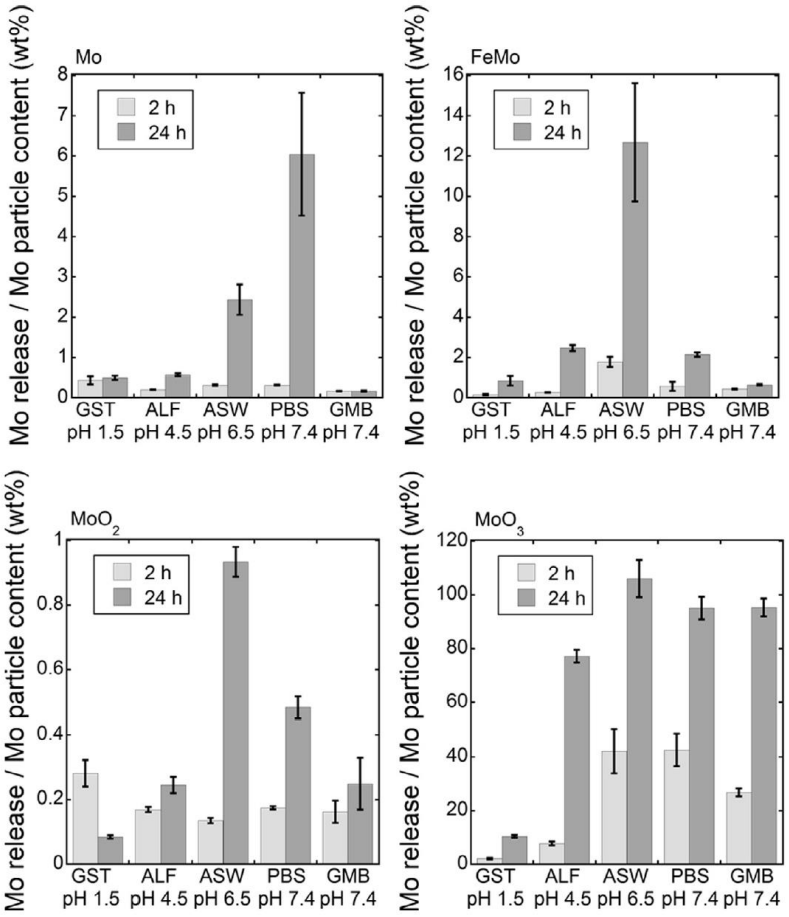


Fig. 2. Released amounts of molybdenum (Mo) normalized to the powder particle content of molybdenum (wt%) for the Mo, FeMo, MoO_2, and MoO_3 powders exposed for 2 and 24 h in different synthetic body fluids (GST – artificial gastric solution; ALF – artificial lysosomal fluid; ASW – artificial sweat; PBS – phosphate buffered saline; GMB – Gamble's solution).

Figure 4: Released amounts of molybdenum (Mo) normalized to the powder particle content of molybdenum (wt%) for the Mo, FeMo, MoO2 and MoO3 powders exposed for 2 and 24 hours in different synthetic body fluids (GST-artificial gastric solution; ALF-artificial lysosomal fluid; ASW- artificial sweat; PBS-Phosphate Buffered Saline; GMB-Gamble's solution) (corresponds to Figure 2 of Mörsdorf et al. 2015).

In the cases described above, bioelution tests in artificial intestinal or saliva fluids would be required to predict systemic bioavailability by the oral route.

Antimony (Sb). The table below shows examples of antimony substances tested in various artificial fluids. The metal releases (mg/cm^2/h) are shown. For antimony, release in gastric seems to be similar or higher than release in neutral pH solutions such as Gamble (GMB) or PBS (Phosphate Buffered Saline) that have same pH as intestinal fluid.

Test item	Exposure time (h)	PBS pH 7.4	GMB pH 7.4	ALF pH 4.5	GST pH 1.6	ASW pH 6.5
Sb	**2**	24.5	19.8	44.2	20.0	31.1
	24	4.1	5.6	8.1	2.0	6.0
$NaSb(OH)_6$	**2**	8.6	3.1	19.6	71.7	15.0
	24	2.5	2.7	5.9	8.0	5.1
Sb_2S_3	**2**	1.3	0.7	1.9	2.0	1.3
	24	0.6	0.3	0.4	0.3	0.3
Sb_2O_3	**2**	0.8	2.1	30.6	2.4	
	24	0.1	0.2	6.2	0.3	

Table 1: releases of antimony compounds in various artificial fluids (Hedberg et al. 2010b)

Tungsten (W). The dissolution of tungsten carbide (WC) and W-metal in three types of gastric juice (all with pH 1.4-1.5 but differing in complexity) were assessed by Stefaniak et al. 2010. Very low metal releases were observed in all gastric fluids. The findings of Stefaniak et al. 2010 were confirmed by an unpublished study (IITRI 2010) conducted under GLP guidelines. This study used several artificial human fluids (sweat, and lung interstitial, alveolar, and lysosomal fluids), in addition to gastric (pH=2). Neither WC nor W metal were bioaccessible to any significant extent (all <0.5%) in any of the fluids tested (IITRI 2010). Hedberg et al. 2017 examined the dissolution of nanoparticles of WC. They conclude that significantly higher W releases can be found at pH-neutral and alkaline conditions than at acidic ones.

Mercury (Hg). Mercury is not very reactive when exposed to hydrochloric acid and mercury chloride is insoluble in aqueous solutions; higher mercury releases from soil found at neutral than at acidic pHs (Safruk et al. 2015; Welfringer and Zagury 2009).

Conclusion: In addition to Ag, there are other elements (Mo, Sb, Si, W, and Hg) for which there is insufficient evidence to conclude that gastric fluid will yield the highest metal release values for the oral route. For these metals and metal compounds it is also recommended that in addition to the gastric fluid (current SOP) bioelution tests be conducted in saliva and/or intestinal fluid for oral route assessments related to applications such as Grouping and Alloy Classification.

When comparing oral to inhalation fluids, the metals have often shown releases in gastric fluid to be higher or within 2-fold of releases in lysosomal fluid (ALF). For some metals, however, ALF may yield comparable or even higher bioaccessible metals than gastric fluid (after the same test duration) due to the presence of a metal complexing agent (citric acid) added to the fluid to mimic the presence of proteins.

For antimony (Table 1 above), gastric fluid results in the highest releases for only half of the samples with ALF yielding the highest releases for the other half. For silver, ALF always yielded higher values than gastric due to higher chloride content and presence of other complexing agents. When comparing metal releases between fluids, analytical uncertainties or metal specificities (e.g. silver) need to be considered.

For nickel, Table 2 shows examples of % metal release of total metal content after 24 h in gastric or ALF for a variety of nickel substances (KMHC 2010). The metal releases are comparable for both fluids for the nickel compounds, although not for the nickel metal that released considerably more ions in gastric than in ALF after equal test time (the metal release in this case is driven by corrosion rather than simple dissolution).

	24 gastric	24 ALF
carbonate	100	100
sulfide	22.7	24.4
subsulfide	33.3	29.6
metal	79	5.8
sulfate	100	91.5
oxide g	1.1	0.57
oxide b	15.3	5.4

Table 2: Percent nickel release of available nickel from various nickel-containing substances after 24 hours in gastric or ALF (lysosomal fluid) (KMHC 2010).

In the study of Latvala et al. 2016, the dissolution of metal particles inside cells' lysosomes was predicted to be very rapid based on 24 h bioelution results in ALF, but this was found to not be the case. Cells exposed to nickel particles showed internalization of these particles in lysosomes; yet, 24 h later the particles remained undissolved (Latvala et al. 2016). Therefore, given that ALF can over predict intracellular dissolution of particles, that the differences in metal release between gastric and lysosomal are usually small (and often not statistically significant), and the fact that dissolution in macrophage lysosomes is a minor contributor to the systemic absorption of particulate metals after inhalation, the use of gastric fluid for assessing systemic health effects from oral and inhalation exposure can be justified. This is true for most metals with the exceptions noted above.

2. Justify the relevance of the composition of the gastric fluid and the pH in relation to the human situation.

The method and simulated gastric fluid composition in the validation study were selected as an outcome of previous exercises; in general, gastric protocols previously published by Stopford et al. 2003 and ASTM 2007 served as the basis for developing the SOP for the validation study. This method measures release of metal ions in synthetic gastric fluid (pH 1.5) after two hours. Metal solubility/release has generally been found to be higher with decreasing pH.

The human stomach pH varies considerably (from 1.2−6.4) among individuals due to prandial state, type, and volume of food consumed (Mudie et al. 2010; Kalantzi et al. 2006). The typical pH range for fasted gastric fluid is 1.2−2.7, and a median value of pH 2.4 has been reported (Axson et al. 2015). The pH for fed gastric fluid can range from 3−6.4 depending on the stage of digestion (e.g. Kalantzi et al. 2006; Jantratid et al. 2009). Therefore, gastric fluid at pH 1.5 can be considered representative of worst case fasted state.

There are many studies that have looked at the composition of gastric fluid and the influence of its components on absolute metal release. In general, the simple fluid composition (HCl at pH 1.2-1.5) is considered as a predictive fluid for the oral route. Details on the composition of gastric fluid are provided by earlier standards (e.g. BS EN 71- 3; 2013; ASTM D5517 2007) and in Figure 6 of Annex 1 (reproduced below as Table 3).

Table 6. Composition and parameters utilized in common in vitro bioaccessibility assays

Method / phase	ASTM D5517-4	BARGE UBM	DIN	EN 71-3	IVG	PBET	RBALP	SBRC
		Saliva–gastric Saliva (pH 6.5±0.5):		"Maceration" for oily or waxy samples				
In vitro parameters								
Composition (g l−1)		0.896 g KCl, 0.888 g NaH2PO4, 0.2 g KSCN, 0.57 g Na2SO4, 0.298 g NaCl, 1.8 ml of 1 M NaOH, 0.2 g urea, 0.145 g amylase, 0.05 g mucin, 0.015 g uric acid		water				
pH		1.2		not specified				
Soil/ solution ratio		0.6:22.5		1:25				
Extraction time (h)		1		1 min, follow by gastric				
	Gastric	Gastric phase	Gastric	Gastric	Gastric	Gastric	Gastric	Gastric
In vitro parameters								
Composition (g l−1)	0.7N HCl	2.752 g NaCl, 0.266 g NaH2PO4, 0.824 g KCl, 0.4 g CaCl2, 0.306 g NH4Cl, 8.3 ml of 37 % HCl, 0.65 g glucose, 0.02 mg glucuronic acid, 0.085 g urea, 0.33 g glucosaminehydrochloride, 1 g bovine serum albumin, 3 g mucin, 1 g pepsin	1 g pepsin, 3 g mucin, 2.9 g NaCl, 0.7 g KCl, 0.27 g KH2PO4	0.7N HCl	10 g pepsin, 8.77 g NaCl	1.25 g pepsin, 0.5 g sodium malate, 0.5 g sodium citrate, 420 µl lactic acid, 500 µl acetic acid	0.4M Glycine	0.4M Glycine
pH	1.0 to 1.5	1.2	2.0	1.0 to 1.5	1.8	2.5	1.5	1.5
Soil/ solution ratio	1:50	0.6:22.5	1:50	1:50	1:150	1:100	1:100	1:100
Extraction time (h)	1shaken, 1 stationary	1	2	1shaken, 1 stationary	1	1	1	1
		Gastric–intestinal Duodenal phase (pH 7.4±0.2)	Intestinal		Intestinal	Intestinal	Intestinal	Intestinal
In vitro parameters								
Composition (g l−1)		7.012 g NaCl, 5.607 g NaHCO3, 0.08 g KH2PO4, 0.564 mg KCl, 0.05 g MgCl2, 0.18 ml of 37 % HCl, 0.1 g urea, 0.2 g CaCl2, 1 g bovine serum albumin, 3 g pancreatin, 0.5 g lipase	9.0 g bile, 9.0 g pancreatin, 0.3 g trypsin, 0.3 g urea, 0.3 g KCl, 0.5 g CaCl2, 0.2 g MgCl2		3.5 g bile, 0.35 g pancreatin	1.75 g bile, 0.5 g pancreatin		1.75 g bile, 0.5 g pancreatin
pH		6.5	7.5		5.5	7.0		7.0
Soil/ solution ratio		0.6:58:5	1:100		1:150	1:100		1:100
Extraction time (h)		4	6		1	4		4
		Bile phase (pH 8.0±0.2)						
In vitro parameters								
Composition (g l−1)		5.259 g NaCl, 5.785 g NaHCO3, 0.376 g KCl, 0.18 ml of 37 % HCl, 0.25 g urea, 0.222 CaCl2, 1.8 g bovine serum albumin, 6 g bile						
pH		6.5						
Soil/ solution ratio		0.6:58:5						
Extraction time (h)		4						

ASTM: International Association for Testing Materials
BARGE UBM: Bioaccessibility Research Group Europe, Unified BARGE Method
DIN: Deutsches Institut fur Normunge (2000)
EN 71-3: European Committee for Standarization
IVG: In Vitro Gastrointestinal (Rodriquez 1999)
PBET: Physiologically-Based Extraction Test (Ruby 1996)
RBALP: Relative Bioavailability Leaching Procedure (Drexler & Brattin, 2007)
SBRC: Solubility Bioavailability Research Consortium (Ruby 1999)

Table 3: Composition and parameters utilized in common in vitro bioaccessibility assays (Annex 1)

The composition of gastric fluid employed in a variety of studies is generally uniform, with HCl acid (0.07N) and pH in 1- 2 range being the most common fluid composition (Brandon et al. 2006; Brattin et al. 2013; Denys et al. 2012; Drexler and Brattin 2007; Juhasz et al. 2009; 2014; Maltby et al. 1991; Molina et al. 2013; OSWER 2007; Ruby et al. 1999; Schroder et al. 2004; Walsh et al. 2015). The pH is generally reported as 1.5 (typical interval found: 1.2 to 1.8, with a pH of 1.5- 1.6 being more biologically relevant). Studies that compare the metal release in gastric fluid at different pHs in the range 3.0-8.0, consistently demonstrate higher metal ions release at the lower pH for Fe, Ca, Cu and Zn) (e.g. Eckert et al. 2014). Another example for As, Cd, Cu, Pb and Zn is provided in Figure 5.

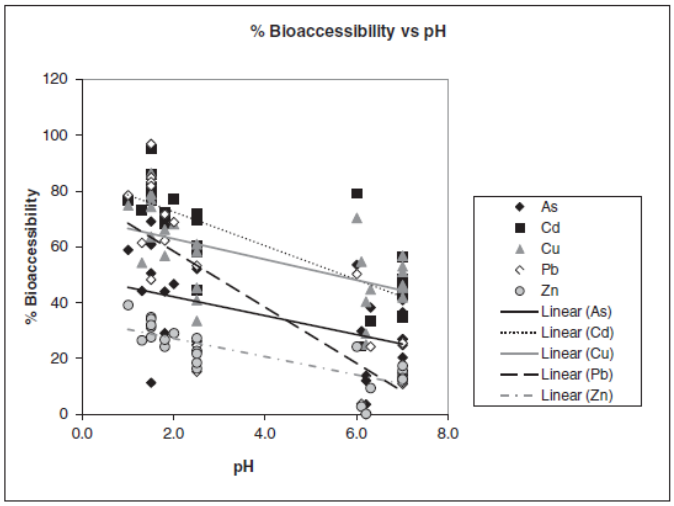


Fig. 3. Percent bioaccessibility vs. pH (gastric and gastric + intestinal phases) for some trace elements of interest.

Figure 5: Percent bioaccessibility vs. pH (corresponds to Figure 3 of Koch et al. 2013).

In some instances, pepsin, surfactants, or glycine have been added to the fluids (e.g. Oliver et al. 1999) but these additional components do not increase metal dissolution. A complete and realistic sequential testing would introduce more technical problems and a lot of variability and does not seem to improve predictivity over the simpler (gastric fluid) approach. The selection of a simple HCl acid fluid for the validation of this protocol (round robin) seems therefore justified and providing a reasonable worst-case estimate (Galia et al. 1998; Hillwalker et al. 2014). The selection of the gastric fluid composition in the validation study seems therefore justified for the metals measured (Co, Cr, Fe, Cu, Ni, Pb and Zn) and consistent with existing standards and available research data.

3. Provide the justification and rationale behind the time of exposure proposed (i.e. 2 hours). Is this the most appropriate/representative of the in vivo situation?

Extractions in gastric fluid are to be conducted for two hours based on an average half time for gastric emptying of 17.7 min and complete emptying of the stomach of 91 min in human volunteers (Tomlin et al. 1993; Wang et al. 2001). In addition, this duration has been used in other existing standards (ASTM D5517, 2007) and shown to be correlated with acute oral toxicity of nickel compounds in a recent study by Henderson et al. 2012. Sampling times of 1 to 2 hours have also been used in the other studies investigating bioaccessibility in surrogate gastric fluid (Brandon et al. 2006; Brattin et al. 2013; Denys et al. 2012; Drexler and Brattin 2007; Hedberg et al. 2012, 2016; Herting et al. 2014; Hedberg and Wallinder 2015; Hedberg and Midander 2014; Hillwalker and Anderson 2014 ; Midander et al. 2010; Molina et al. 2013; Mörsdorf et al. 2015; OSWER 2007; Schroder et al. 2004; Rodriguez et al. 1999; Vasiluk et al. 2011; Walsh et al. 2015) and reported in the review performed by Alloy LLC (attached as Annex 1).

The selection of the 2-hour extraction time in the validation (round robin) study seems therefore justified and consistent with existing standards and available research data. In addition, these bioaccessibility methods do not take into account absorption, and therefore a simple extraction step is all that is necessary, as the bioaccessibility methods are generally considered to overestimate absolute bioavailability and toxicity. For the metals considered in Annex 1, we compiled information on in vivo absorption for the most water soluble of the metal compounds which are the ones typically used as “reference compounds” in the RBA soil studies (Annex 2). The data show that even for the most bioavailable of the metal compounds, absolute absorption and bioavailability are always below 100%.

4. In vivo the process of dissolution is dynamic [as fluid carrying the dissolved metals gets removed and replenished] while the proposed in vitro method is static and the ions released and solubilised will remain in the solution for the full duration of the experiment. Could it be that in vivo more ions will be solubilised compared to in vitro because they are produced and continuously removed? Can saturation happen in vitro?

The few studies where dynamic bioelution tests for oral route have been compared to static tests have not shown the outcome to be significantly different (Annex 1). While it is true that in a static system there is a possibility that the release of metals may be limited because released metals are not removed from the system, resulting in an underestimate of bioaccessibility, this is mitigated by the

following factors: 1) lower loading, 2) short duration of the assay, and 3) relative estimates. To address possible artefacts related to loading, the proposed gastric protocol includes two loadings: 2 g/L and 0.2 g/L. For substances that are highly soluble at pH 1.5 and release a lot of metal, the lower loading will avoid limiting the dissolution reaction. For substances with very low releases, the higher loading will maximize the possibilities that the measured values are > LOQ (e.g. some alloys or very water insoluble metals or metal compounds). The study duration is short (2 hours), and that combined with a lower loading would make it highly improbable that saturation of dissolution would be achieved within this time period. In addition, the solution is stirred at 100 rpm. This agitation action will assist removal of the released metals from e.g. the metal or alloy surfaces and reduce the likelihood of mechanisms that would inhibit further dissolution. Furthermore, the stirring action would also assist in maintaining the level of dissolved oxygen in the test solution and, thus, maintain an important cathodic component of the dissolution process. The results from the bioelution test are not used in isolation to provide accurate estimates of in vivo bioavailability but are rather considered to provide information on relative bioavailability of target and reference samples. As indicated above, side-by side in vivo and in vitro studies do not suggest that dynamic bioelution tests perform any better than static ones for gastric fluid.

In a study carried out by ECTX (2018b), the kinetics of metal release in gastric fluid were examined. The objective of the study was to assess if saturation of metal release from alloys and their pure metal ingredients could be observed at the time period of 2 hours which has been selected for the gastric fluid bioelution test. For this purpose, both powders and massive forms of alloys and ingredients were tested. The following powder samples were tested: an alloy (SS316L), two of its pure ingredients (Fe^o and Ni^o) and two water soluble compounds of these metal ingredients ($FeSO_4$ $7H_2O$ and $NiCl_2$ $6H_2O$). Measurements were carried out after 1, 2 and 4 hours in gastric fluid (pH 1.5) at a loading of 2g/L. It was reasoned that if no saturation of metal release was observed at the higher loading of 2 g/L, no saturation would be expected to occur at the lower loading of 0.2 g/L. Single sample vessels were included at each time point, except for SS316L were duplicate samples were tested; each vessel was sampled twice. The results for the pure ingredients (Fe^o and Ni^o) and the two water soluble compounds ($FeSO_4$ $7H_2O$ and $NiCl_2$ $6H_2O$) are shown in Figure 6; the results for SS316L are shown in Figure 7.

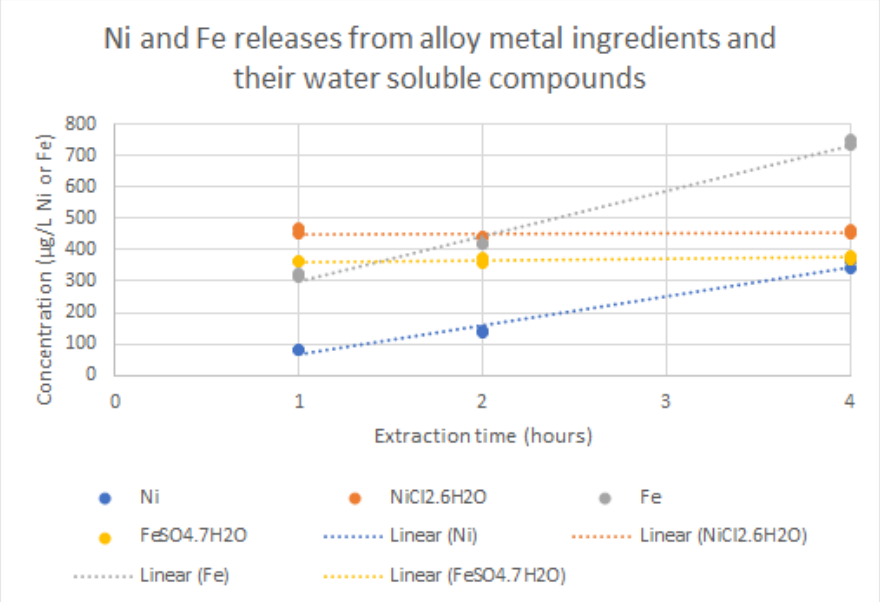


Figure 6: Time-dependent metal release in gastric fluid for pure metals and compounds (powder forms).

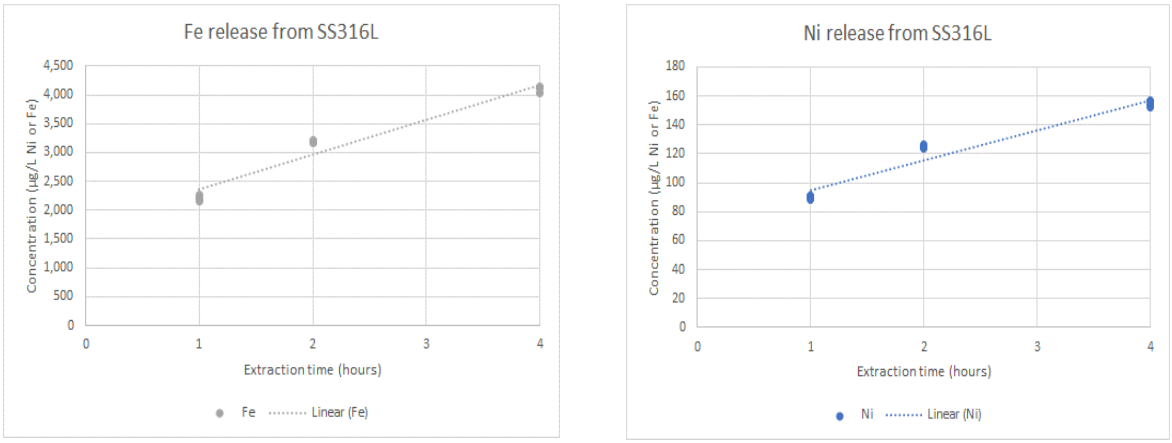


Figure 7: Time-dependent metal release in gastric fluid for SS316L (powder form) (ECTX 2018b).

As could be expected, Figure 6 indicates that the two water soluble metal compounds were fully dissolved within the first hour in gastric fluid. By contrast the release of Fe and Ni from the metal samples was reasonably linear with time between 1 and 4 hours. No saturation of metal release is observed at the 2 h extraction time. Likewise, Figure 7 indicates that no saturation is observed for metal releases from the alloy SS316L.

The following massive samples (as received, representative of materials on the market) were tested: an alloy (Z45, 62.176% copper) and its pure ingredient (Cu°, 100% Cu), both as disks. Loadings of 2 g/L surface equivalent were tested (ECTX 2018c). Triplicate sample vessels with duplicate sampling from each vessel were included. Figure 8 shows the copper releases as a function of time.

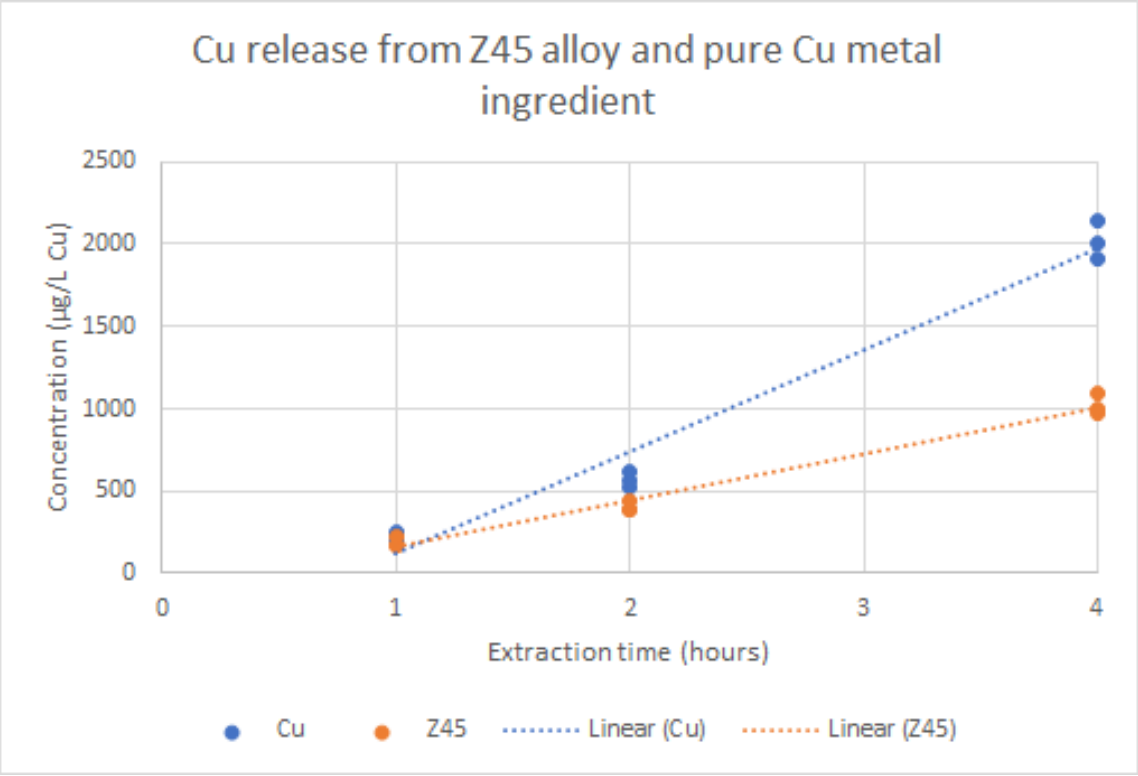


Figure 8: Time-dependent Cu release in gastric fluid from Z45 and pure copper metal (massive forms) (ECTX 2018c).

The results indicate that for massive forms of alloys, as for powders, no saturation of metal release after 2 hours in gastric fluid is evident for the alloy examples tested.

For **silver**, dissolution is similar after 2 and 24 hours, as discussed under question 1, and this is likely driven by the rapid formation of insoluble silver chlorides.

For **antimony**, release of ions in gastric fluid (pH 1.6) from the metal and various Sb compounds (as % of antimony mass dissolved) shows similar levels after 2 and 24 hours at 0.1 g/L loading (see Figure 9, right panel). These results are consistent with the release rates (mg $Sb/cm^2/h$) being much higher during the first 2 hours of the test (Figure 9, left panel).

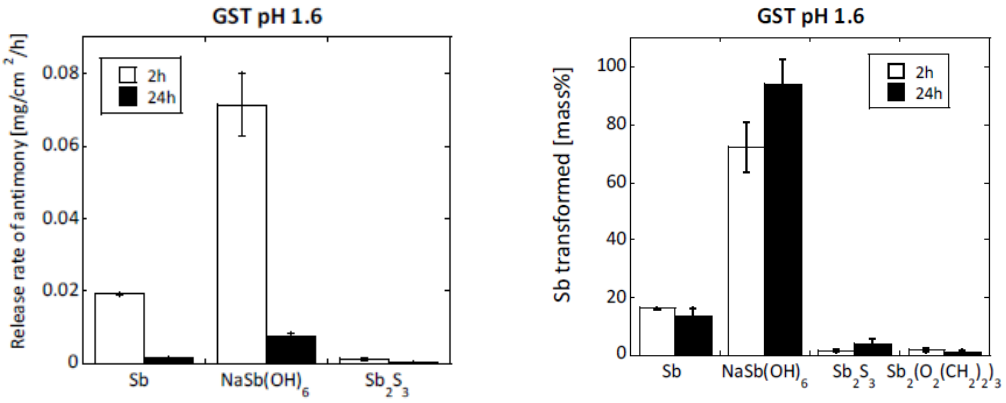


Figure 9: Sb releases from various Sb-containing substances in gastric fluid (corresponds to Figure 8 of Hedberg et al. 2010b, unpublished). Antimony transformed [mass%], equivalent to percentage of the amount particles dissolved since normalized to the antimony content of the test item. Data presented as average of values of triplicate samples in different media. Blank values for each individual media and exposure period have been subtracted. Abbreviations: GMB-Gamble's solution, PBS-phosphate buffered saline, ASW- artificial sweat, ALF-artificial lysosomal fluid, GST-gastric fluid.

Work published by Mörsdorf et al. 2015 using a loading of 0.1 g/L, demonstrates that **molybdenum** releases in gastric fluid (GST) from FeMo alloy and MoO3 increase between 2 and 24 hours, and the same is true for **iron** (Fe) releases from FeMo alloy and Fe metal (Figures 10 and 11 below). By contrast, Mo release from Mo metal stays the same and it decreases with time for MoO_2 (Figure 10).

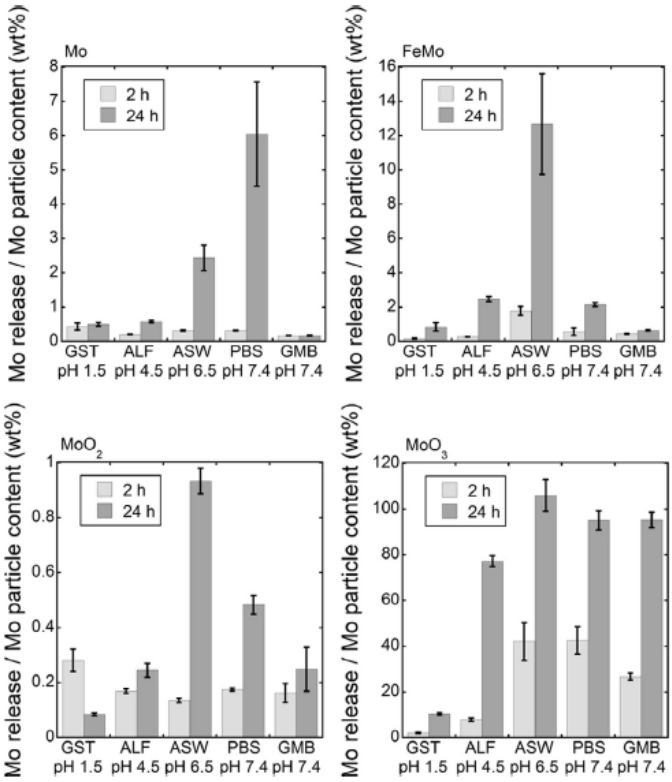


Fig. 2. Released amounts of molybdenum (Mo) normalized to the powder particle content of molybdenum (wt%) for the Mo, FeMo, MoO_2, and MoO_3 powders exposed for 2 and 24 h in different synthetic body fluids (GST – artificial gastric solution; ALF – artificial lysosomal fluid; ASW – artificial sweat; PBS – phosphate buffered saline; GMB – Gamble's solution).

Figure 10 (identical to Figure 4): Released amounts of molybdenum (Mo) normalized to the powder particle content of molybdenum (wt%) for the Mo, FeMo, MoO2 and MoO3 powders exposed for 2 and 24 hours in different synthetic body fluids (GST-artificial gastric solution; ALF-artificial lysosomal fluid; ASW- artificial sweat; PBS-Phosphate Buffered Saline; GMB-Gamble's solution) (corresponds to Figure 2 of Mörsdorf et al. 2015)

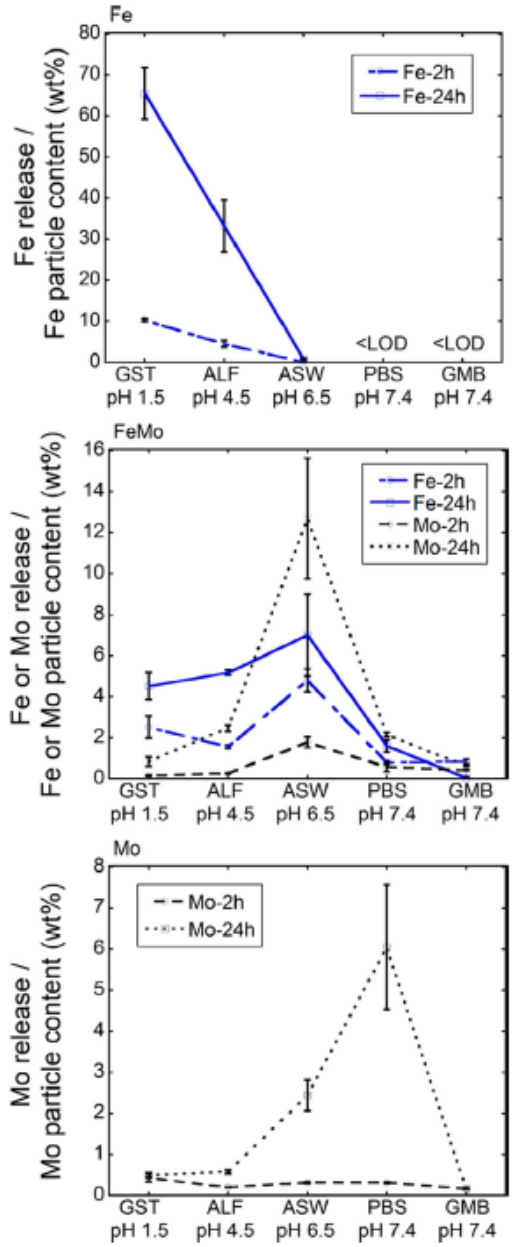


Fig. 3. Release of molybdenum or iron normalized to the corresponding metal particle content (wt%) for the Fe, FeMo, and Mo powders exposed for 2 and 24 h in different synthetic body fluids (GST – artificial gastric solution; ALF – artificial lysosomal fluid; ASW – artificial sweat; PBS – phosphate buffered saline; GMB – Gamble's solution). The lines are only guidance for the eye. <LOD – measured concentrations below the limit of detection. The measured concentration of iron was similar to the limit of detection in the case of released iron from the Fe metal powder after 2 h in ASW.

Figure 11: Release of molybdenum or iron normalised to the corresponding metal particle content (wt%) for the Fe, FeMo, and Mo powders exposed for 2 and 24 hours in different synthetic body fluids (corresponds to Figure 3 of Mörsdorf et al. 2015)

In the study of Jiang et al. 2012, release of **chromium** from Cr metal in gastric fluid increased between 2 hours and 168 hours, showing no saturation.

Together these results indicate that metal release is not limited by the test system but rather it depends on the nature of the material tested. In order to determine if gastric fluid will provide higher estimates of in vitro metal release (i.e. bioaccessibility) for the oral route of exposure, available

information must be reviewed to assess the effect of pH on the solubility of the metal ions for the metal substance of interest. If the solubility of the metal substance is highest at low pH then gastric fluid is appropriate for use in bioelution testing. Other fluids may be appropriate and should be evaluated for substances whose solubility is increased at neutral or high pHs (e.g. Ag, Sb, Si, Mo, W, and Hg). If oral fluids, other than gastric are used, a justification that includes metal release data at different pHs should be provided. Maximum solubility can for most test materials be predicted using simple metal speciation tools that are used in e.g. ecotoxicity testing[3]. Using these tools, those elements that might pose problems, like Ag, will be well known ahead of the bioelution testing.

In a study carried out by ECTX 2017, the effect of loading on metal release in gastric fluid was examined. The metal releases from a simple mixture containing 68.6%Fe, 16.4%Cr and 10.7% Ni were measured in gastric fluid (pH 1.5) after 2 hours at 3 different loadings: 0.2, 1, and 2 g/L (see Figure 10 below). Triplicate test samples with duplicate sampling per test vessel were included.

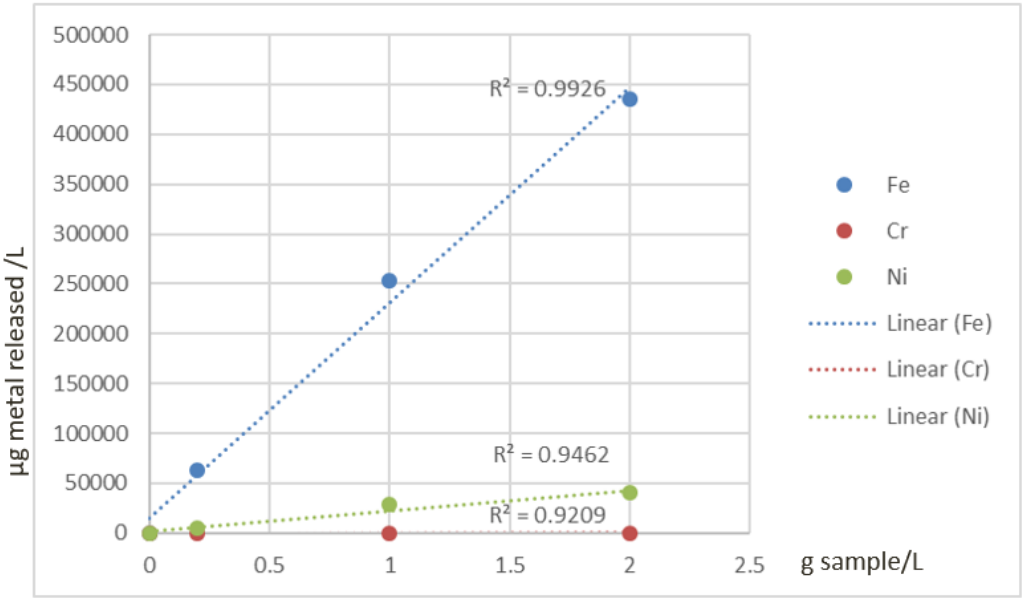


Figure 12: Effect of loading on metal release using a mixture of Fe, Cr and Ni in gastric fluid after 2 h and using 3 different loadings (ECTX 2017)

The results showed reasonably linear relationships between metal releases and loading for the 3 metals tested. They also show that different metals have different bioaccessibilities in gastric fluid (e.g. Fe and Cr differ by more than 10-fold in metal release per gram of metal). On a per gram of sample basis the releases decreased as the loading increased for Fe and Cr, while remaining the same for Ni.

[3] In aquatic ecotoxicology, determination of metal speciation is key to explain or predict effects. Direct analytical techniques to measure metal speciation are complex and not routinely used. Instead, models are used to predict metal speciation based on the physico-chemical composition of the medium (assuming equilibrium). Well known examples are Visual Minteq (https://vminteq.lwr.kth.se/) and the Windermere Humic Aqueous Model (WHAM) (https://www.ceh.ac.uk/services/windermere-humic-aqueous-model-wham).

5. Comment on other possible processes in vivo that may modify the rate of release of metal ions from the matrix and that will not be captured with the proposed in vitro method

The formulation of this question is not very clear. Is it referring to differences in composition of the synthetic versus the in vivo gastric fluid? Is it about other processes that can affect oral uptake and bioavailability (e.g. competition among metal ions for mixtures, uptake of intact particles)? The impact of fluid composition on metal release is discussed under Q2. The other processes that can affect bioavailability are discussed here.

For example, soils are mixtures of different inorganic metal compounds. The soil studies provide information on the relationship between in vivo bioavailability of individual metals (in the soil mixture) and bioaccessibility results in the soil mixture. Metal ion competition for oral absorption is already reflected in the in vivo results. While competition cannot be excluded when multiple metals are present in test samples (e.g. alloys, complex mixtures), and these processes can influence the variability around the in vivo-in vitro regression lines, the results from soil studies consistently indicate that differences in gastric bioaccessibility data for different forms of a metal can predict differences in relative oral bioavailability.

Another factor to consider is the possibility that insoluble metal-containing particles may be taken up orally (e.g. persorption) in intact form and contribute to the systemic metal loading. To assess if this exposure pathway could be contributing significantly to the bioavailability of metals, a review of the information on intact particle uptake was conducted (see Annex 3). The main conclusions from this review are that uptake of intact particles through the intestinal wall (lymphoid tissues specially) can be quite significant for nanoparticles but is expected to be low (below one percent) for micron-size particles based on studies of polystyrene and TiO_2. Importantly, the proposed gastric protocol is not applicable to nanomaterials.

The consequence of having a significant oral metal uptake that is independent of gastrointestinal metal ion release would be a higher absolute absorption and bioavailability of a metal following oral exposure than the bioaccessibility in gastric fluid. This is generally not found to be the case (Annex 2), even though there are instances where the relative bioavailability can be higher than the in vitro bioaccessibility[4].

Bioelution tests in gastric fluid measure the in vitro bioaccessibility of metals from various metal-containing substances. Relative in vitro bioaccessibilities of a given metal from target and reference substances can be used to predict the in vivo (stomach) relative bioaccessibilities.

For some metals, speciation in solution and of the surface oxide is an important factor in determining the bioavailability/toxicity of the metal-containing substance (e.g. Cr, V, Rh). Chromium oxide (Cr_2O_3) and potassium chromate (K_2CrO_4) in gastric fluid (pH 1.5) release Cr (III) and Cr (VI) ions, respectively.

[4] Example: 'soluble M' is highly dissolved in artificial gastric medium (e.g. 90% bioaccessible), while 'oxide M' is only partially dissolved (e.g. 10% bioaccessible). In vivo, 'soluble M' is 10% bioavailable while 'oxide M' is 1% bioavailable. The absolute bioavailabilities of these compounds are lower than the bioaccessibility values (10% vs 90% for 'soluble M' and 1% vs 10% for 'oxide M'). However, when relative bioavailabilities are considered and soluble compounds are used as a reference (e.g. soluble M considered as 100% bioavailable) the relative bioavailability of oxide M becomes 10%. In this example the relative bioavailabilities can be higher ('soluble M' case) than the bioaccessibilities (see Annex 1).

Cr(III) is an essential element whereas Cr(VI) pose adverse effects; there are differences in the bioavailability and toxicity of these two ions. If the in vitro and in vivo conditions are different, one could envision a scenario where from one compound (e.g. K_2CrO_4), Cr(VI) is released in vitro where it is rapidly reduced to Cr(III) due to reduction reactions governed by the chemical environment while Cr(VI) is released in vivo and not reduced to Cr(III). In this scenario, the in vitro speciation would not be predictive of the in vivo toxicity. However, this scenario is not likely as the conditions in the human body are strongly reducing; hence, Cr(VI) is rapidly reduced to Cr(III) (see e.g. Hedberg et al. 2014). Thus, when the speciation of metals (e.g. valence, complexes) released from target and reference substances in the gastrointestinal tract are similar, the relative bioaccessibility estimates are expected to reflect relative in vivo metal bioavailabilities and toxicity.

The metal bioavailability is strongly related to its chemical speciation and to the fluid chemistry including processes such as: precipitation and complexation, active transport mechanisms, feedback mechanisms, homeostasis (binding to metallothioneins / cellular ligands, chelating effects, protein binding, lipid binding) and possibility of in vivo formation of organometallic species.

These factors are expected to affect metal ions released from target and reference substances, or from alloys and pure metal ingredients, in the same way. In the case of alloys (as with soils), when releases of metals, other than the classified metal, are significant, there may be some competition for oral absorption. Thus, in vitro and in vivo bioaccessibility should be evaluated on a metal-specific basis to derive conclusions whether read-across of systemic toxicity is justified

6. Comment if and how the biotransformation by host microbiota that may occur in vivo could jeopardize the assumption that bioaccessible concentration derived with the proposed method is conservative with regard to the in vivo situation.

The Laird et al. 2007 study was the first one to report that intestinal microorganisms could increase arsenic bioaccessibility in the human gastrointestinal tract. Yet, in 2013 the same authors concluded the following: "*In vitro GI microbial activity in the distal small intestine increased arsenic release from soils; however, these effects were unlikely to be relevant since they were transient and demonstrated small effect sizes. In vivo arsenic absorption for juvenile swine was unaffected by antibiotic treatment. Therefore, it appears that microbial effects on arsenic release do not result in increased arsenic bioavailability. However, it remains to be seen whether the results for the limited set of soils described herein can be extrapolated to arsenic contaminated sites in general*." (Laird et al. 2013)

If intestinal bacteria were to increase metal bioaccessibility, it follows that in vivo bioavailability could be higher than in vitro bioaccessibility in simple gastric fluid lacking microorganisms. Yet absolute in vivo bioavailabilities of metals from soils are usually lower than bioaccessibilities (Annex 2), even if relative bioavailability values are higher in some cases (see footnote 4 and Annex 1).

7. Please explain how to deal with issues like precipitation and complexation that may occur in vitro and have an influence on the results.

Metals that are released from the test materials in vitro may further interact with components of the test medium (by e.g. precipitation at given pH, complexation with organic acids, etc.). These reactions

can influence the measured concentrations of dissolved metal in solution: e.g. precipitation of the metal ions will reduce metal in aqueous soluble fraction, while complexation may lead to higher dissolution. As the test medium is a simple solution of hydrochloric acid and there is an absence of additional complexing agents (e.g. citric acid, citrates, cyanates, hydroxyl ligands, tartrates, thiocyanates, etc.), complexation should not be a feature of, or lead to enhanced dissolution of metals and alloys in this test protocol.

With regard to precipitation, with some exceptions (e.g. mercury and silver), metal chlorides are soluble in aqueous solutions. In the case of metal release from alloys, while concentration-dependent, both lead and silver react slowly with dilute hydrochloric acid and form chloride-rich corrosion products on the metal surface as well as AgCl(s) that precipitates out from solution. This layer of poorly soluble corrosion products reduces further metal release. These processes are expected to take place both in-vitro and in-vivo. For Ag, there is a strong dependence of dissolution and precipitation on the ratio of Cl/Ag (see Figure 13).

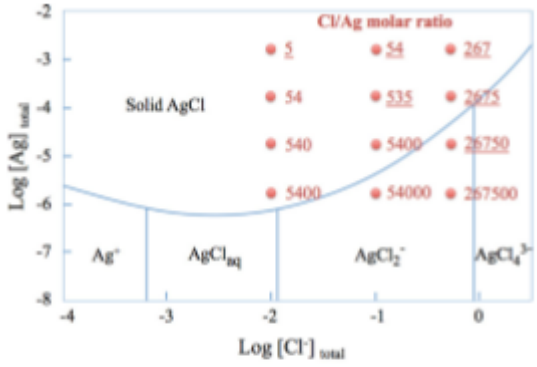


Figure 3. Predominance diagram for Ag^+ and $AgCl_x^{(x-1)-}$ species calculated using Medusa.[24] Experimental conditions with corresponding Cl/Ag molar ratios from this study are represented as red dots. The underlined ratios were the only ones that resulted in measurable releases of soluble silver species in the dissolution studies, and for the other conditions, the amount of soluble Ag species was below the detection limit of 1.8×10^{-8} mol/L.

Figure 13: Predominance diagramme for silver species calculated using Medusa (corresponds to Figure 3 of Levard et al. 2013)

In general, chemical interactions that occur in the test media are considered to reflect interactions that could also occur in vivo, and are considered relevant for in vivo bioavailability. While mercury chloride is insoluble in aqueous solutions, mercury is also non-reactive when exposed to dilute hydrochloric acid. Thus, even if $HgCl_2$ precipitates could be formed in gastric fluid there is not enough Hg ion to form them, suggesting that precipitation should not be an issue with regard to the proposed protocol. Nevertheless, for Ag, Mo, Sb, Si, W, and Hg it is also recommended that in addition to the gastric fluid, bioelution tests be conducted in saliva and/or intestinal fluid for oral route applications such as Grouping and Alloy Classification.

Depending on fluid composition and loading, precipitation could be visibly identified during the test, as it will often result in a loss of clarity of the test solution. Such observations should be included in the test results and be considered in the interpretation of the data (see also Q12).

Technically, to separate the dissolved elements from the original, undissolved test material, a 0.2µm filtering step is introduced at the sampling. The transformation-dissolution protocol defines the "dissolved metal fraction" as the fraction that passes a 0.2µm filter (OECD 2001). Dissolved metals and metal complexes and particles <0.2µm can pass the filter and will be measured. This fraction includes free ions, labile and strongly bonded metal complexes (e.g. organic metal complexes). Precipitated metal compounds will usually have particle size that precludes passing the filter. When filtration alone fails to separate dissolved metals from undissolved samples, centrifugation can be applied.

8. Provide the rationale behind the selection of the loadings (sample/fluid ratio) used

The proposed protocol includes two loadings: 2 g/L and 0.2 g/L. For substances that are highly soluble at pH 1.5 and release a lot of metal, the lower loading will avoid limiting the dissolution reaction (i.e. avoid saturation). For substances with very low releases, the higher loading will maximize the probability that the values are > LOQ (e.g. some alloys or very water insoluble metals or metal compounds) (see also response to Q4).

A loading of 20 g/L is specified as the initial loading in ASTM 5517. If metal release is higher than 250 ppm, ASTM 5517 recommends to lower the loading to 1 g/L. Similar loadings were used by Stopford et al. 2003. Some soil studies have used loadings of 10 g/L (OSWER 2007); some alloy and metal studies have used loadings of 0.1 g/L (e.g. Herting et al. 2014; Mörsdorf et al. 2015).

We believe that the proposed loadings of 2 g/L and 0.2 g/L (10-fold lower) represent a good compromise between having sufficient amounts of sample to accurately detect metal release and preserving a linear relationship between sample concentration and metal release, as already suggested from the examples discussed in Q4.

9. Provide the rationale behind the relative measurement of bioaccessibility between pure metal (used as reference material) and alloy that is used to calculate the "Bioaccessible Concentration". Considering that the pure metal is always used as reference material to calculate the relative bioaccessibility in an alloy, independently of the amount of metal present in the alloy, the assumption that there is a linear relationship between the fraction of metal ion released from a material in the absence of a matrix effect and the total content of that metal in the material should be demonstrated.

This question pertains to application 2-Alloy classification of the gastric bioelution protocol. The basic premise is that the systemic toxicity effects of metal-containing materials are related to the bioavailability of the metal ion after systemic absorption and that a relationship between in vitro bioaccessibility in gastric fluid and relative in vivo bioavailability can be defined for those metals that are classified for systemic effects when in elemental form (see answer to Q15). In the case of alloys, the metallic bonds can affect the metal release (bioaccessibility) and the results often deviate from what would be expected if the alloy behaved as a simple mixture (e.g. an alloy containing 10% of a metal would be expected to release 10% of the measured release from the pure ingredient). For alloys where matrix effects affect metal releases, the bulk concentration of a metal will not be predictive of in vitro and in vivo bioaccessibility in gastric fluid or in vivo bioavailability of that metal. Bioelution tests provide a tool to refine the bulk concentration of metals in alloys in a manner that more closely matches the expected in vivo bioaccessibility. When data on released amounts of metals per mass of

sample from alloys and reference metal ingredients are considered, the bioaccessible concentration of the metal in the mixture can be calculated using the following equation.

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

When applying bioelution test data from alloys to refine a metal's bulk concentration, the bioaccessible concentration is compared to the bulk concentration in order to assess the presence of a matrix effect.

Example: data from Hillwalker and Anderson 2014 paper is used below to illustrate the ways in which data from gastric bioelution tests are reported for metals and alloys and can be used to calculate the bioaccessible concentration of a metal (e.g. Mn) in an alloy (e.g. carbon steel) using these data. The bulk concentration of Mn in carbon steel is 0.456%. The Supplemental Table S2 of the Hillwalker and Anderson paper presents metal release data (metal bioaccessibility) from carbon steel in terms of µg metal/mL fluid (e.g. average 0.84 µg Mn/mL).

Supplementary Information
Table S2. Metal Concentration in Biofluid Extract (µg metal in extract / mL fluid)

Alloy / °grade	Biofluid	Cobalt	Chromium	Copper	Iron	Manganese	Nickel	Zinc
Carbon steel NIST 14g	gastric	<0.0054	<0.0038	<0.0055	186	1.0	<0.015	<0.016
					158	0.79		
					157	0.79		
					170	0.89		
					147	0.75		

Table 4: *Metal concentration in biofluid extract (Hillwalker and Anderson 2014)*

Data on Mn bioaccessibility from carbon steel in terms of µg metal/g sample, corresponding to a loading of 2 g sample/L fluid, is reported in the Supplemental Table S3 of the paper (e.g. average 420 µg Mn/g carbon steel, calculated as 0.84 µg Mn/mL / 2 g/1000 mL= 420 µg Mn/g).

Supplementary Information
Table S3 Metal Concentration in Material (µg metal in extract / g alloy material); n = 2 to 5

Alloy / °grade	Biofluid	Cobalt Avg±SD	Chromium Avg±SD	Copper Avg±SD	Iron Avg±SD	Manganese Avg±SD	Nickel Avg±SD	Zinc Avg±SD
Carbon steel NIST 14g	gastric	<2.7	60 ± 7	<2.8	8.2 ± 0.7	420 ± 52	<7.5	<8.0

Table 5: Metal concentration in material (Hillwalker and Anderson 2014)

Table 6 presents data on metal bioaccessibility for the pure Mn in terms of µg Mn/mL fluid at a loading of 2 g/L. An average release of 1467 µg Mn/mL corresponds to an average release of 733,333 µg/g of Mn sample (calculated as 1467 µg Mn/mL / 2 g/1000 mL= 733,333 µg Mn/g).

Mn metal	gastric	1300
		1570
		1530

Table 6: Mn metal bioaccessibility µg Mn/mL fluid at a loading of 2 g/L (Hillwalker and Anderson 2014)

This information can be used to calculate the bioaccessible concentration of Mn in the carbon steel alloy as follows:

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

$$BC\% = \frac{420}{733{,}333}\ x\ 100 = 0.057\%$$

The bioaccessible concentration indicates that carbon steel alloy behaves as a mixture containing 0.057% of Mn metal in it. Since the content of Mn in the alloy is 0.456%, there is a matrix effect in the alloy that decreases Mn release (~8-fold) compared to what could be expected from a simple mixture. This example illustrates how the bioaccessible concentration of a metal can differ from its bulk concentration due to alloy matrix effects.

Other examples where the bioaccessible concentration of metals in alloys differ from their bulk concentrations exist. Mörsdorf et al. 2015 showed that the 2 h gastric fluid bioaccessible concentration of Fe and Mo in a ferromolybdenum alloy (7% Fe and 26% Mo) were lower than their respective bulk concentrations (30.2% Fe and 59.8% Mo) by 2-4-fold. Hedberg et al. 2016 showed that when tested for 168 hours in lysosomal fluid, Fe in Inconel had a bioaccessible concentration of <0.5% (calculated based on 2 different Fe metal reference samples) while its bulk concentration was 14.6% (Figure 14).

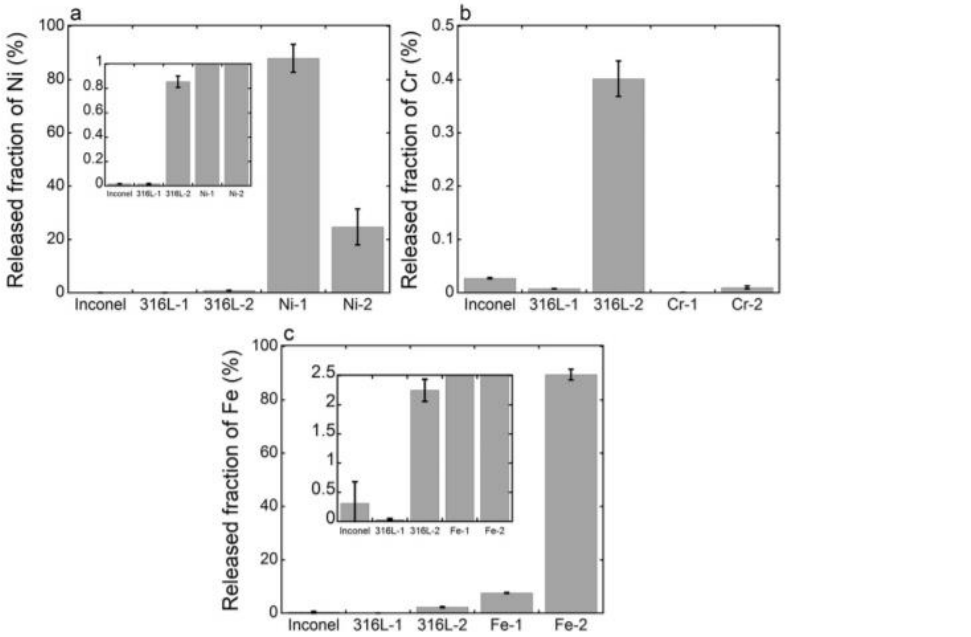


Fig. 4. Released fractions of Ni (a), Cr (b), and Fe (c), for the Inconel powder compared with reference powders of stainless steel 316L, nickel, chromium, and iron metal, and normalized to the nickel, chromium, and iron content in the powders. All release data is shown for ALF (pH 4.5) after 24 h (a) or 168 h (b and c) exposure. The reference powders were exposed at similar conditions. Data shown is from (Hedberg et al., 2011) for Ni release from the 316L powders (24 h in ALF), from (Mazinanian et al., 2013) for Ni release from the nickel powders, and from (Hedberg et al., 2010) for Cr and Fe release from the 316L, iron, and chromium powders. The error bars show the standard deviation of triplicate samples.

Figure 14: Released fractions of Ni, Cr, Fe for the Inconel powder compared to stainless steel, Ni, Cr and Fe metal (corresponds to Figure 4 of Hedberg et al. 2016)

Stockmann-Juvala et al. 2013 reported nickel releases from pure Ni metal and SS316L after 168 hours in lysosomal lung fluid of 0.87 and 0.00088 µg/g sample, respectively. From these data, the bioaccessible concentration of Ni in S316L powder (0.1%) can be calculated; the BC of Ni in SS316L was 100-fold lower than its bulk content (10.3%).

To demonstrate how the presence of a matrix effect in alloys can affect metal releases compared to those from simple mixtures, the following experiment was carried out by T Brouwers (ECTX 2017) for Eurometaux at ECVAM's request. The release of nickel from a sample of SS316L alloy powder containing 10.7% of Ni (68.6%Fe, 16.4%Cr) was compared to the release of nickel from a simple mixture containing 10.7% Ni, 68.6%Fe, and 16.4%Cr powders, both samples tested at the same sample loading of 2 g/L. Gastric fluid corresponded to an HCl solution at pH 1.5 and the duration of the test was 2 hours. The release from the pure Ni metal powder was also reported. The results shown in Table 7 below demonstrate approximately a 300-fold difference [(20.1 mg Ni/g) / (0.065 mg Ni/g)] between the Ni releases from a simple mixture or from the SS316L alloy (ECTX, 2016), even if the concentration of Ni in both samples was the same. Using the release of pure Ni metal as a reference, the bioaccessible concentrations (BC) of Ni in the simple mixture (12%) and SS316 L (0.04%) were calculated. The BC of Ni in the simple mixture (12%) coincides with its bulk concentration (11%) indicating that no matrix effect was present. By contrast, the BC of Ni in SS316L (0.04%) is almost 300-fold lower than its bulk concentration and 300-fold lower than the BC of the simple mixture. This illustrates how the alloy matrix effect can affect metal bioaccessibility.

Sample	***Particle diameter***	***Mass loading (g/L)***	***Nickel loading (g Ni/L)***	***Nickel release (mg Ni/L)***	***Nickel release (mg Ni/g sample)***	***Bioaccessible Concentration of Ni in mixture (%)***
Simple mixture: *68.6% Fe, 16.4% Cr, 10.7% Ni*[a]	D_{90}= 2 µm	2	0.21	40.1	20.1	12.3
SS316L: *68.6% Fe, 16.4% Cr, 10.7% Ni*[b]	D_{80}= 5 µm	2	0.214	0.13	0.065	0.04**
Ni metal, *100% Ni*[a]	D_{90}= 2 µm	0.2	0.2	32.5	163*	

*[a.] ECTX X02a.130; [b.] ECTX X02a. 144; * previous results for this sample were 209 (rounded from 208.6) mg Ni/g sample (ECTX X02a. 144) and 182.6 mg Ni/g sample (ECTX X02a. 085). ** 0.03-0.04% when using * samples as a reference.*

Table 7: Differences in metal release in gastric fluid from a simple mixture and an alloy, both containing the same bulk concentration of nickel (ECTX 2017).

The second part of the question pertains to the assumption that there is a linear relationship between the fraction of metal ion released from a material in the absence of a matrix effect (simple mixture) and the total content of that metal in the material. This was addressed by examining the relationship between metal release and metal added to the gastric fluid (these results have been already presented under Q4) and by considering the comparability of results between alloy and pure ingredient at equal sample as well as metal loading. To address this last point further, a study (ECTX 2018a) was carried out to measure Ni release (after 2 hours in gastric fluid) from the following three samples: Ni metal powder (0.21 g/L sample loading), Ni metal powder (0.21 g/L loading) + SiO_2 (1.79 g/L loading) for a total sample loading of 2 g/L, and SS316L with a content of 10.7% Ni (2 g/L sample loading). These

samples were chosen to examine the influence of sample mass, either as inert material (SiO_2) or as alloy material on the release of Ni. Single test samples with duplicate samplings per test vessel were included for nickel and SS316L powder, with triplicate test samples included for the mixture of nickel metal and SiO_2. The results from this study are shown in Table 8 below.

Sample powders	***Total Mass loading (g/L)***	***Nickel loading (g Ni/L)***	***Nickel release (mg Ni/L)***	***Nickel release (mg Ni/g sample)***	***% Ni released of sample***
Ni metal	0.214	0.214	44.6	209 (rounded from 208.6)	20.9
Ni metal + SiO_2	2.0	0.214	53.0	26.5	2.7
SS316L	2.0	0.214	0.13	0.065	0.007

Table 8: Gastric bioelution results for Ni containing samples tested at equal mass or equal nickel loading (nickel measurements in blank and pure SiO2 samples were below the reporting limit of 0.5 µg/L) (ECTX 2018a, X02a-144).

Table 8 results confirm that the lower percent nickel release from SS316L powder compared to pure nickel metal powder of similar particle characteristics is not due to a mass effect (i.e., a higher mass loading used for alloy than for pure metal) but it is due to the matrix alloy effect. Furthermore, they indicate that the addition of 10-fold higher mass of substance does not affect the release of Ni from Ni metal powder to any significant extent. The same Ni^o and SS316L samples were used in the studies reported in Tables 7 and 8.

This study complements the previous results (see Q4) indicating that metal releases (Cr, Ni, Fe) are reasonably linear at 3 different loadings: 0.2, 1, and 2 g/L for the 3 metals tested (in gastric fluid pH 1.5 after 2 hours). They also show that different metals in elemental form have different bioaccessibilities in gastric fluid (e.g. Fe and Cr differ by more than 10-fold in metal release per gram of metal). On a per gram of sample basis the releases decreased as the loading increased for Fe and Cr, while remaining the same for Ni. ***This would suggest that when calculating the BC of metals in alloys, the results from the pure ingredients at the higher loading may need to be considered to get the highest (most conservative) calculated %BC values.***

10. Justify the appropriateness of the analytical methods proposed.

Bioelution tests aim at measuring the release of constituent elements from metal containing substances and mixtures. For many decades the proposed analytical methods (e.g. ICP-MS, ICP-OES, AAS) have been used for (geo)chemical analyses and are well standardised (e.g. NEN-EN-ISO 17294-2) and worldwide-accepted. Each technique has its advantages and disadvantages mostly related to detection limits, cost per analysis, and suitability for various matrices. A more detailed comparison of these techniques can be found in the Perkin-Elmer brochure attached as Annex 4.

For the gastric bioelution test protocol, we recommend that either an inductively-coupled plasma spectrometer (ICP-OES, optical emission, or ICP-MS, mass spectrometer) or an electro thermal excitation atomic absorption spectrometer (graphite furnace or flame -atomic absorption spectrophotometer, AAS) technique be used with validated instrumentation (e.g. according to ISO

17025 or equivalent). If a different technique is used, this will require justification and a demonstration that the technique can accurately measure the element of interest in benchmark materials.

It is important that the analyses 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 the limits of quantification and the limits of detection for the elements of interest shall be reported.
While the physical characterization of the sample is required for a proper interpretation of the gastric bioelution test data, the SOP does not cover the analysis of e.g. material composition, specific surface area, particle size distribution in solution, surface or morphology analysis.

11. Clarify the nature of the test items that will be used with the test method.

On the basis of the experimental evidence, application 1 (Grouping) of the test method is suitable to metals, inorganic metal compounds, or metal(metalloid)-containing complex materials (specifically, alloys, metal-related ores and ore concentrates). For each material selected, sufficient information needs to be provided so that the results from the bioelution tests can be properly interpreted (CAS number, composition, basic phys-chem, etc). For application 2 (Alloy classification), alloys and their metal(metalloid) ingredients are the test items. A separate guidance note for the sample preparation is provided in addition to the SOP for the gastric fluid test itself (Annex 5).

For the gastric fluid, test items tested as powders are recommended to be <100 µm in diameter in order to limit the variability as much as possible and to be relevant to the fraction of the inhalation exposure that is swallowed and becomes bioavailable through the oral route. Some of the alloys and metallic substances placed on the market are often present in massive form. Massive forms of metals (M^o) and alloys can be tested as received or epoxy-embedded; some bioelution tests conducted after 2012 have utilized epoxy-embedded massive forms of alloys. For massive forms of these substances, techniques such as BET cannot be used to assess the surface area. In this case, it is possible to embed the massive samples in epoxy to expose a known geometric surface area to the gastric solution.

In some instances it may be desirable to reduce massive alloys to a smaller size for testing. 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 recommended when sufficient material is available, 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 is important to test the metal ion release from the material both as initially received and after being crushed/ground/milled to establish whether the crushing/grinding/milling operation has had an impact on the release properties of the test material.

12. Provide a list of metals and/or alloys that may not be covered by the proposed test method (exceptions/applicability domain):

When relevant, the answers to these questions are considered separately for each of the two applications (Grouping and Alloy Classification).

a) Are they metals and metal ions for which systemic toxic effects may occur independently of solubility?

Nanoparticles of metals or metal compounds may be absorbed as such from the gastrointestinal tract and can be distributed to different organs (Frohlich and Roblegg 2012). It is not known whether the toxicity of nanoparticles is due to direct nanoparticle effects or to the delivery of metal ions to these organs. Moreover, the proposed gastric protocol is not applicable to nanomaterials as its usefulness for these materials remains to be checked (i.e. the protocol would require more advanced techniques to ensure sufficient particle separation). For massive and micron-size powders of metals, inorganic metal compounds, or metal-containing complex materials (specifically, alloys, metal-related ores and ore concentrates), the *bioavailable metal ion is considered to be responsible for the systemic toxicity.*

b) Provide examples of systemic (oral) toxicity driven by the metal compound rather than the metal ion

One example is mercury where the toxicity of inorganic mercury is different from that of methyl mercury (organometallic). Methyl mercury can be rapidly absorbed via ingestion, skin or inhalation, and be systemically circulated. Behaviour and target organs are different from inorganic mercury (e.g. Broussard et al. 2002). Nickel carbonyl is another example. The acute inhalation toxicity of this organometallic compound is much higher than that of any of the inorganic nickel compounds (Sunderman et al. 1961).

c) Are there metals with different solubility in vitro and in vivo? Some metal compounds were mentioned by PARERE members during the consultation.

It seems that the PARERE members referred to cadmium compounds, mentioning in particular the following review "Toxicologic Assessment of the Army's Zinc Cadmium Sulfide Dispersion Tests: Toxicity and Related Data on Selected Cadmium Compounds https://www.ncbi.nlm.nih.gov/books/NBK233504/. The review mentions that *'a substance can be insoluble in water or acids in vitro but soluble in vivo; cadmium oxide (CdO) is an example."* However, there are no further primary references supporting this statement and the text does not allow us to identify the "acids" referred to in the review. Further references to solubility cited in the review -including those in Table 4-1 (shown as Table 9 below)- present water solubility results only (CdO appearing as slightly soluble).

TABLE 4-1 Water Solubilities of Selected Cd Compounds

Name	Formula	Water Solubility at 20°C
Cadmium metal	Cd	Insoluble
Cadmium chloride	$CdCl_2$	1,680,000 mg/L
Cadmium oxide	CdO	5 mg/L, but soluble in lung
Cadmium sulfide	CdS	1.3 mg/L
Cadmium carbonate	$CdCO_3$	Insoluble
Cadmium red	70% Cd, 16% Se, 13% S	Insoluble
Cadmium yellow	77% Cd, 22% S, 0.3 % Zn	Insoluble
Cadmium selenide	CdSe	Insoluble
Cadmium sulfate	$CdSO_4$	608,000 mg/L
Zinc Cadmium Sulfide	ZnCdS	Insoluble

Source: Adapted from Weast 1985.

Table 9: water solubilities of selected Cd compounds (National Research Council (US) Subcommittee on Zinc Cadmium Sulfide. Washington (DC): National Academies Press (US); 1997.)

The solubility of CdO in gastric juice was further explored in the context of the EU Cadmium metal/Cadmium oxide risk assessment (EU RAR 2007- Cadmium Oxide). The report mentions the following:

> Iijima (1972) and Wada et al. (1972) (cited in Tsuchiya, 1978) performed an experiment on the solubility of various cadmium compounds in artificial gastric and intestinal juices. The solubility of CdO was estimated to be 94% in the artificial gastric juice and 0.15% in artificial intestinal juice. Therefore, it should be considered that, although the water solubility of Cd-salts used in most experimental studies is probably substantially higher than that of CdO, differences in oral bioavailability may be less marked because of the almost complete solubilisation of CdO in gastric juice.

It is therefore likely that the discrepancy cited in the review refers to water solubility vs. solubility in gastric fluid (assumed to correspond to in vivo); this discrepancy was indeed demonstrated and it does not imply that Cd substances would be have different solubility in vivo than in vitro, provided that the relevant fluids are used.

d) Which types of matrix are covered/excluded with the proposed test method?

For application 1 (Grouping), the following metal-containing materials and matrices are included: inorganic metal(metalloid) compounds, metals in elemental state, alloys, UVCBs (including ores, concentrates and pigments) in powder or massive form. Examples of substances tested under this application are listed in Annex 6.

For application 2 (Alloy Classification), only metal containing alloys in powder or massive form and their metal(metalloid) ingredients are included.

Nanomaterials are excluded in both applications.

13. Since the test method is proposed for the oral route of exposure, provide the most likely route of exposure for the test items that should be evaluated. Please provide examples to illustrate the answer

Exposure to metal-containing materials can take place via oral, dermal and inhalation routes of exposure. Inhalation and dermal exposures predominate in the workplace (e.g. workers exposed to Zn oxide powder during production). Oral and dermal exposure can be more common for consumers and the general public exposed to articles (e.g. person swallowing a metal coin, person drinking water carried by metal pipes). The bioelution protocol in gastric fluid and the two applications of test result are focused on systemic effects only. Thus, dermal exposure is of little relevance as systemic absorption of inorganic metals through the skin is very low, <1-2% for soluble metal compounds and ≤0.1-0.2% for insoluble compounds and metals (HERAG, 2008, see also Q14).

It is noted that after inhalation of metal-containing particles at the workplace, a significant fraction (particles of > 4-10 µm size) does not enter the deeper lung but is re-distributed out of the respiratory tract towards the gastro-intestinal tract by mucociliary action and swallowing. The proposed protocol for gastric test is relevant to the oral route of exposure and to that fraction of the inhalation exposure that is also swallowed and absorbed through the gastrointestinal tract (e.g. Goix et al. 2016).

In the case of application 2 (Alloy Classification), inhalation or dermal exposure of workers can occur during manufacturing or processing (e.g. welding, grinding) of alloys. In hot processes such as welding of alloys, however, the particles generated are not alloy particles but rather spinels and complex oxides. As mentioned above, a fraction of the alloy particles that is ingested is swallowed and metals are absorbed through the gastrointestinal tract. Consumers are usually exposed to alloys in massive forms (no particles are generated). Exposure is mostly dermal. In some instances, the accidental ingestion of alloys can take place (e.g. coin or toy part swallowed). Alloys can also be in contact with food and water (pipes, fittings) and metal ions may be transferred to the food-water that is ingested orally.

14. Please comment on the oral route of exposure in relation to dermal and inhalation routes. Can oral be considered worst case scenario in relation to the other routes? Please provide examples to illustrate the answer.

Inhalation and dermal exposures predominate in the workplace (see also Q13), while oral and dermal exposure can be more common for consumers and the general public. Importantly, the absorption of metals and the systemic bioavailability of metals can be very different for each of these routes of exposure. For example, dermal absorption of inorganic metals is generally very low (≤1-2%) for water soluble compounds and even lower for the more insoluble ones (≤0.1-0.2%) (HERAG 2008 contains specific references for various metals). This is summarised in Table 10 below. Similarly, dermal absorption of As from soil was measured at ≤0.5% (Lowney et al. 2007). Thus, while dermal exposure is quite relevant for local skin effects, it does not contribute significantly to the systemic bioavailability of most metals.

Table 21: Dermal absorption data for metals and inorganic metal compounds

Metal/compound	Test system	Results	References
Data as extracted and concluded upon in the various existing EU RA reports:			
Zinc oxide / Zinc sulphate	*in vitro*, porcine skin	2 % from liquid media 0.2 % from dust exposure (EU RAR assessment, Rapporteur: The Netherlands)	Grötsch (1999)
Cadmium metal, Cadmium oxide	(analogy)	< 1 % (EU RAR assessment, Rapporteur: Belgium)	EU RAR (2004)
Nickel metal, Nickel sulphate, Nickel chloride, Nickel nitrate, Nickel acetate	*in vivo*, human skin, tape stripping	0.2 % (EU RAR assessment, Rapporteur: Denmark)	Hostýnek et al. (2001a) Hostýnek et al. (2001b)
Nickel sulphate, Nickel chloride, Nickel nitrate, Nickel acetate	*in vitro*, human skin	2 % (EU RAR assessment, Rapporteur Denmark) 1 % when material bound to stratum corneum is discounted	Tanojo et al. (2001)
Diantimony trioxide	*in vitro*, human skin	0 - 0.1 %	Roper & Stupart (2006)
Copper compounds (not specified)	*in vitro* (unspecified)	0.3% soluble/insoluble Cu compounds (VRA Copper)	Roper (2003) Cage (2003)
Lead oxide	*in vitro*, human skin	0 - 0.1 % (VRA Lead)	Toner & Roper (2004)
Additional (non-exhaustive compilation) data made available from metal industries participating in HERAG:			
Zinc oxide	*in vitro*, porcine skin	< 0.1%	Gamer et al. (2006)
Aluminium chlorohydrate (^{26}Al-labelled)	*in vivo*, two human volunteers	0.012 % uptake (industry data)	Priest (2004), citing from Flarend et at. (2001)
Cobalt metal	*in vitro* (Franz diffusion cell, human skin)	Absorption not given as a percentage of the applied dose but as a steady-state flow of (0.0123 ± 0.0054) µg cm^{-2} h^{-1} with a lag time of (1.55 ± 0.71) h. Significant absorption only took place, when the metal was oxidised to Co^{2+} by stirring in artificial sweat for 30 minutes.	Filon et al. (2004)
Titanium dioxide	*in vitro*, porcine skin	< 0.1%	Gamer et al. (2006)

Table 10. Dermal absorption studies with a variety of metal compounds (corresponds to Table 21 of HERAG 2008)

Regarding inhalation exposure, a fraction of the inhaled material, the one with larger aerodynamic diameter and greater metal mass, will be deposited in the trachea-bronchial and extrathoracic regions of the respiratory tract (e.g. Anjivel and Asgharian 1995; Asgharian and Price 2009; Oller and Oberdorster 2016). With knowledge of the particles size distribution and density of the metal-containing aerosol, dosimetric models can be used to calculate the fraction of the inhaled dose that will be deposited in the non-alveolar regions of the respiratory tract. Particles deposited in these regions are systemically absorbed through the gastrointestinal tract after they are swallowed. Thus, the oral route is relevant for the fraction of the inhaled dose that is not deposited in the alveolar region of the respiratory tract (Goix et al. 2016). The fluids that are considered representative of the respiratory tract are the interstitial fluid (Gamble, neutral pH) and the lysosomal fluid (pH 4-5 and high citric acid). With regard to the particles that are deposited in the alveolar region, these particles will come into contact with interstitial fluid, and only the ones that are taken up by cells (e.g. macrophages) will come in contact with lysosomal fluid. Metal release in lysosomal fluid can, for some metals, be higher than in gastric fluid due to the presence of complexing agents (see Q1 answer). However, some studies suggest that the in vitro lysosomal fluid may overestimate the intracellular dissolution of metal particles; cultured cells observed 24 h post exposure to nickel particles still show a full loading of particles, while lysosomal fluid results predicted complete particle dissolution (Latvala et al. 2016). Altogether, bioelution tests in gastric fluid can be used to assess the systemic bioavailability of metals from oral and inhalation routes. Gastric fluid bioelution tests are not relevant to assess local respiratory tract effects after inhalation. These tests are as such not applicable to nanomaterials.

15. In the light of using existing in vitro/in vivo correlations, the following should be provided:

- ***All available relevant in vitro and in vivo data.***
- ***The relevance of the available data with regard to humans. A comprehensive comparison between the protocols used in the available literature and the one proposed in the TPF (e.g. the Unified Bioaccessibility Method (UBM) developed by BARGE). List all differences and similarities and how they may impact the results generated.***
- ***The uncertainty associated with the available correlations***

Annex 1 (Alloy LLC report) corresponds to a literature review looking at the relationships between in vitro bioaccessibility and relative in vivo bioavailabilities of metals in soil samples. The basic premise is that the systemic toxicity effects of metal-containing materials are related to the bioavailability of the metal ion after systemic absorption. Thus, establishing that a relationship can be defined between in vitro bioaccessibility in gastric fluid and relative in vivo bioavailability for a particular metal based on metal-containing soils or metal compounds is relevant to other metal-containing substances (e.g. alloys, M^o) that are also absorbed systemically. This principle applies to both applications of the bioelution test method. The Annex 1 review is limited to those elements that in metallic form carry classifications for systemic effects.

Alloy LLC (Annex 1) conducted a review of the available information on the relationship between in vivo relative bioavailability (RBA) and in vitro bioaccessibility (IVBA) of As, Pb, Be, Se, Sb, Cd, Co, Ni, and Hg from soils. There is a broad base of research to understand and predict the bioavailability of metals from soil, because this information forms part of the risk assessment process that dictates the need for remedial action at contaminated sites. Findings from the studies of soil (mixtures of metal compounds), including evaluation of in vitro extraction testing to predict bioavailability inform how bioelution test data can predict (or provide a conservative estimate of) the bioavailability of metals from these and other metal containing substances and mixtures. Of the 39 studies reviewed by Alloy LLC, 22 included information on both relative bioavailability (RBA) as measured in animals (mouse, rat, and swine) and IVBA (in vitro bioaccessibility) measured in laboratory extraction tests. RBA corresponds to the bioavailability of metals from soils relative to that from a fully water soluble metal compound. RBA does not indicate absolute bioavailability of metals from soils; the absolute bioavailability of metals from soil (ABA) is always lower than the RBA (see Annex 2).

Across all studies and all metals evaluated, the data provide strong evidence that soil- and site-specific factors exert control on the bioavailability of metals from soil relative to metal bioavailability from soluble forms. Strong predictive relationships between RBA and IVBA emerged for lead and arsenic, with fewer available data for Sb, Cd, Co, Ni, and Hg, and no data for Be and Se. An example of the relationship between RBA and relative IVBA in stomach fluid, or stomach and intestinal fluids is shown below for Sb (Figure 15 corresponds to Figure 5a of Annex 1).

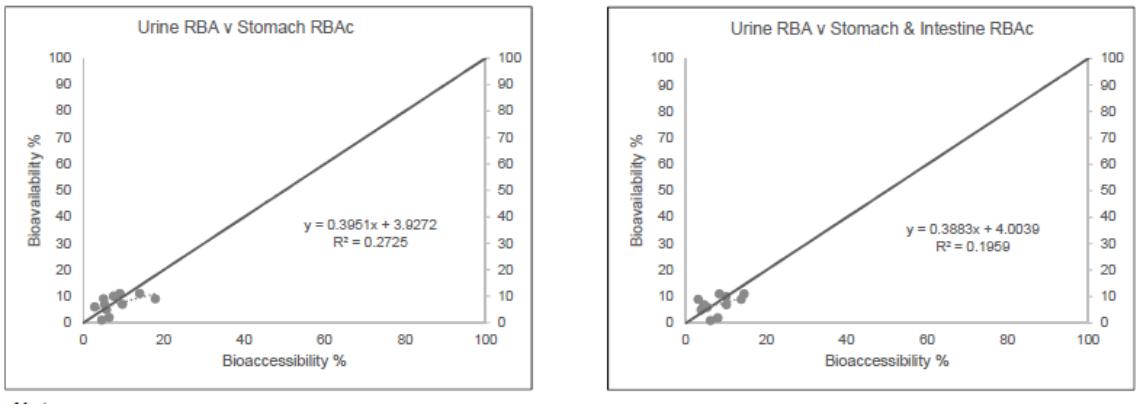
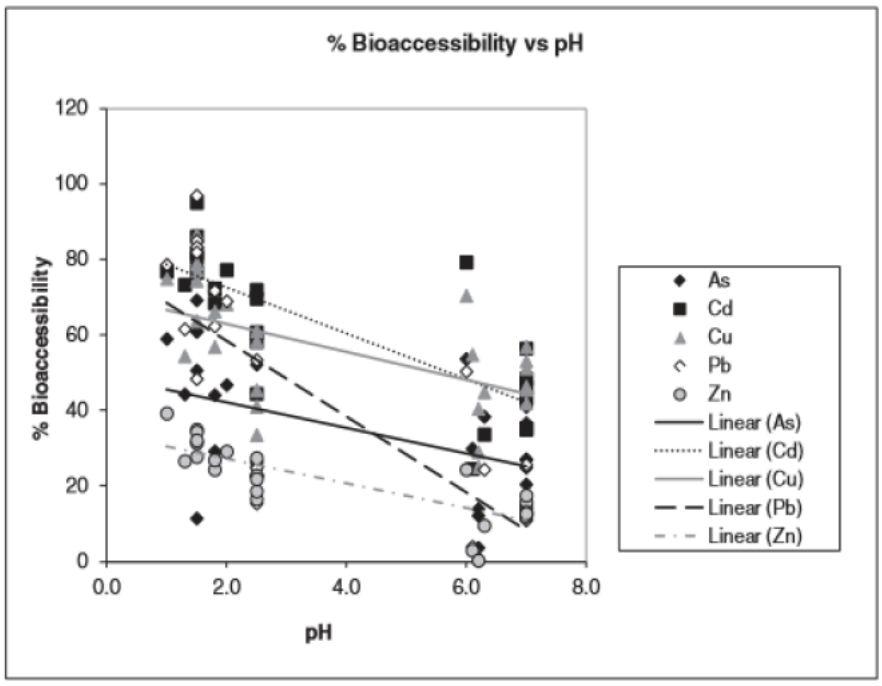


Figure 15: Plots of IVBA vs. RBA for antimony extracted from Denys et al. 2012

For Cd, Co, Pb and Ni, higher IVBA are achieved with lower pH conditions and this effect of pH outweighs other components of the extraction fluid that are intended to create a more "physiological" environment. For arsenic, solubility from soil appears to have a lower dependence on pH, although for some soils, low pH is associated with higher IVBA. Arsenic results from the low pH extraction methods are more closely correlated with RBA when studied using paired soils. Figure 16 (Figure 6 from Annex 1) demonstrates the pH dependence of metal release for As, Cd, Cu, Pb and Zn.



The study published by Koch et al. investigates the IVBA of one soil using multiple IVBA methods. The methods include several pH conditions, and reported data for 5 metals. This Figure from Koch et al. depicts the general negative relationship between pH and bioaccessibility as measured by a variety of IVBA methods, when applied to NIST SRM 2710. Koch et al. report these as: "negative correlations, meaning that percent bioaccessibility decreases with increasing pH, were confirmed for all elements," and that the "strongest correlations (highest R values) were statistically significant and occurred for lead, zinc, and cadmium, and were not as pronounced for arsenic, copper and zinc, in particular arsenic."

Figure 16: Percent bioaccessibility vs. pH for some elements of interest (figure excerpted from Koch et al. 2013).

Figure 17 below corresponds to metal releases (Pb, Zn, Cd, Cu, Ni, As) from NIST standard reference material soils at different oral fluid pH (corresponds to Figure 4 of Annex 1). The red arrows indicate the pH (1.5) of the proposed gastric fluid in the bioelution test.

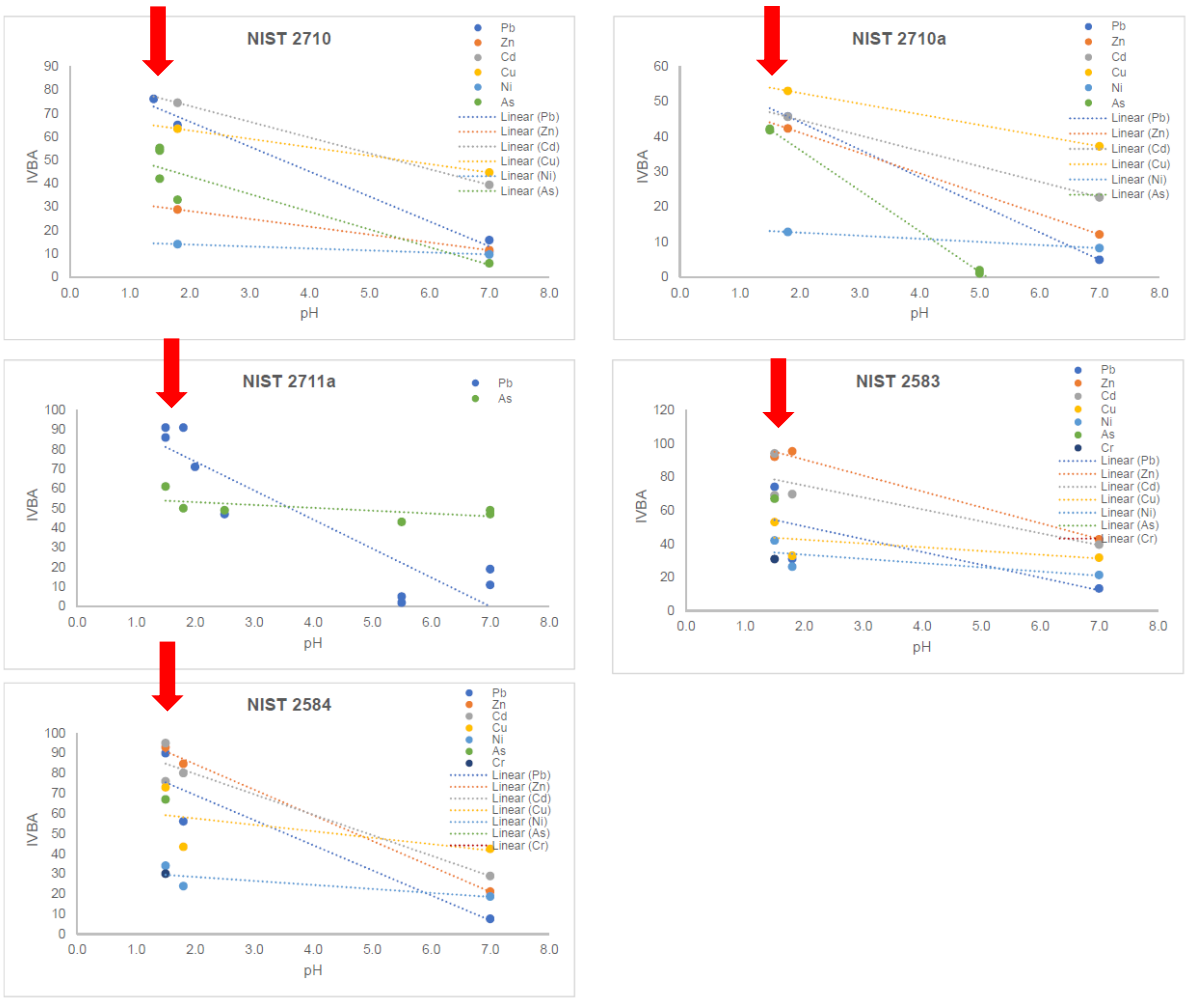


Figure 17: Effect of pH on IVBA for NIST standard reference material soils

As discussed above, for most metals, the pH of the proposed gastric fluid composition (pH =1.5) is expected to result in the higher metal releases, with some exceptions. In contrast to the findings described above, the IVBA of mercury is higher at higher pH (Welfringer and Zagury 2009).
In many cases the IVBA values are higher than the RBA ones and in other cases they are equal or lower. In all cases studied, a relationship between IVBA and RBA was established (see Annex 1 for a detailed report).

In addition to soil studies, in vitro bioelution and in vivo toxicokinetic or toxicity studies were conducted with compounds of cobalt and nickel. Studies of bioelution in gastric fluid (2h) and bioavailability (area under the curve) in rats after oral ingestion of two cobalt compounds demonstrated similar differences (100 to 150-fold) in IVBA and RBA (see Attachment 1 to Annex 1 for more details). Bioelution studies in gastric (2h) and intestinal (24h) fluids (in parallel) were conducted with 12 different nickel compounds (Henderson et al. 2012). The same samples were tested in rat acute oral ingestion studies. A good dose-response between oral LD50 (i.e. acute toxicity) and IVBA of nickel in gastric fluid was found. Additional consideration of the bioaccessibility results in intestinal fluid did not significantly improve the regression compared to using results from gastric fluid alone.

Similarly, IVBA of various nickel compounds in gastric fluid correlated with in vivo bioavailability reported in rat absorption studies (Ishimatsu et al. 1995) (see Attachment 2 to Annex 1 for more details).

To compare the performance of the proposed gastric fluid composition relative to other possible fluids, Figure 18 below shows a comparison of IVBA values in various fluids and their respective RBAs for As and Pb (corresponds to Figure 7 of Annex 1). The values circled in red are those corresponding to our proposed gastric fluid composition. They show that this fluid will yield high (conservative) estimates of metal bioaccessibility.

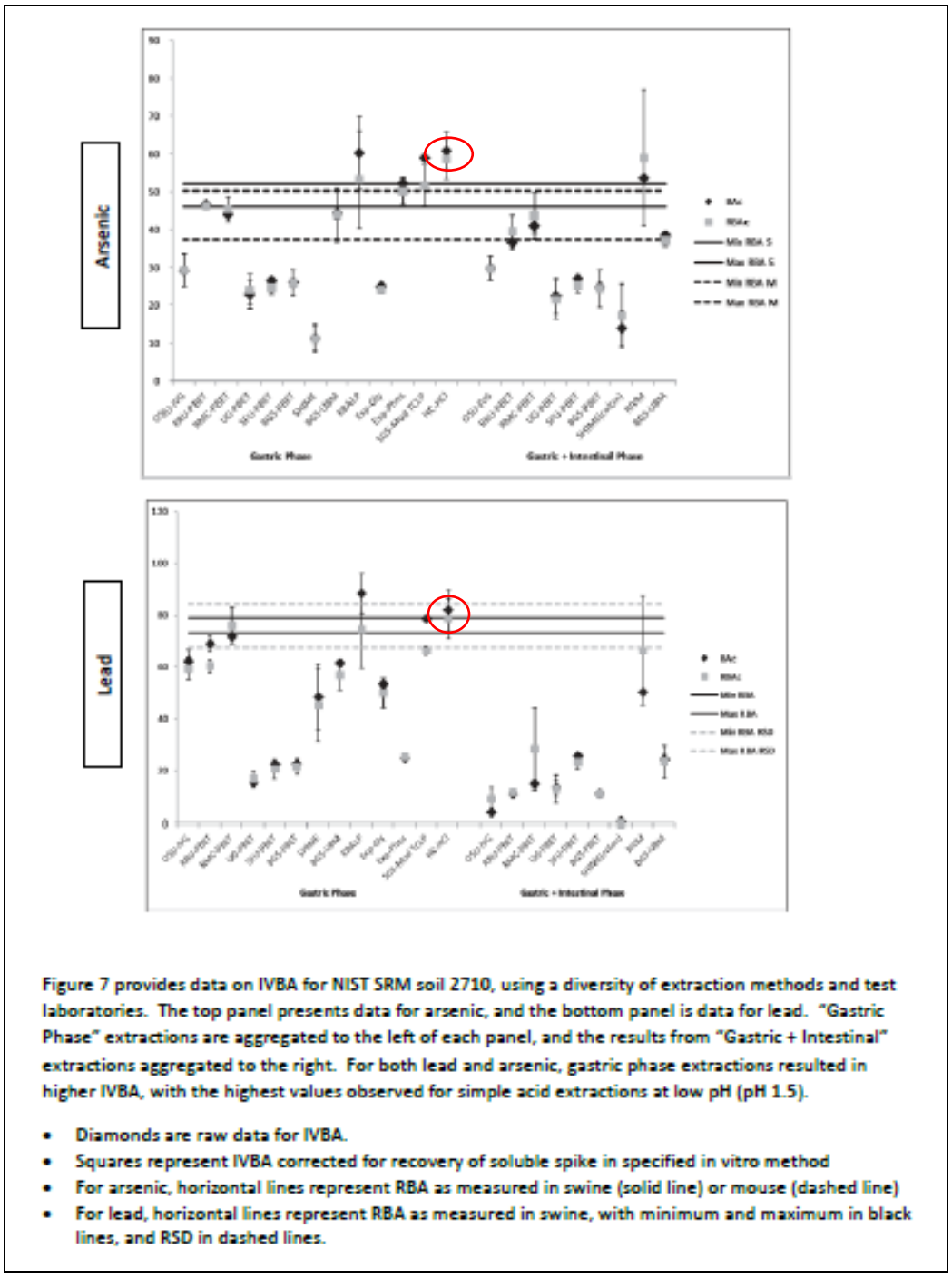


Figure 7 provides data on IVBA for NIST SRM soil 2710, using a diversity of extraction methods and test laboratories. The top panel presents data for arsenic, and the bottom panel is data for lead. "Gastric Phase" extractions are aggregated to the left of each panel, and the results from "Gastric + Intestinal" extractions aggregated to the right. For both lead and arsenic, gastric phase extractions resulted in higher IVBA, with the highest values observed for simple acid extractions at low pH (pH 1.5).

- Diamonds are raw data for IVBA.
- Squares represent IVBA corrected for recovery of soluble spike in specified in vitro method
- For arsenic, horizontal lines represent RBA as measured in swine (solid line) or mouse (dashed line)
- For lead, horizontal lines represent RBA as measured in swine, with minimum and maximum in black lines, and RSD in dashed lines.

Figure 18: Comparison of IVBA to RBA for Pb and As (excerpted from Koch et al. 2013)

To understand the uncertainty associated with the observed relationships between IVBA and RBA, it is important to estimate the variability associated with both of these values. This is shown in Figure 19 below for As (corresponds to Figure 2B of Annex 1). In this example it can be seen that even taking into account the variability in the estimates of IVBA and RBA, the relationship between these parameters is robust.

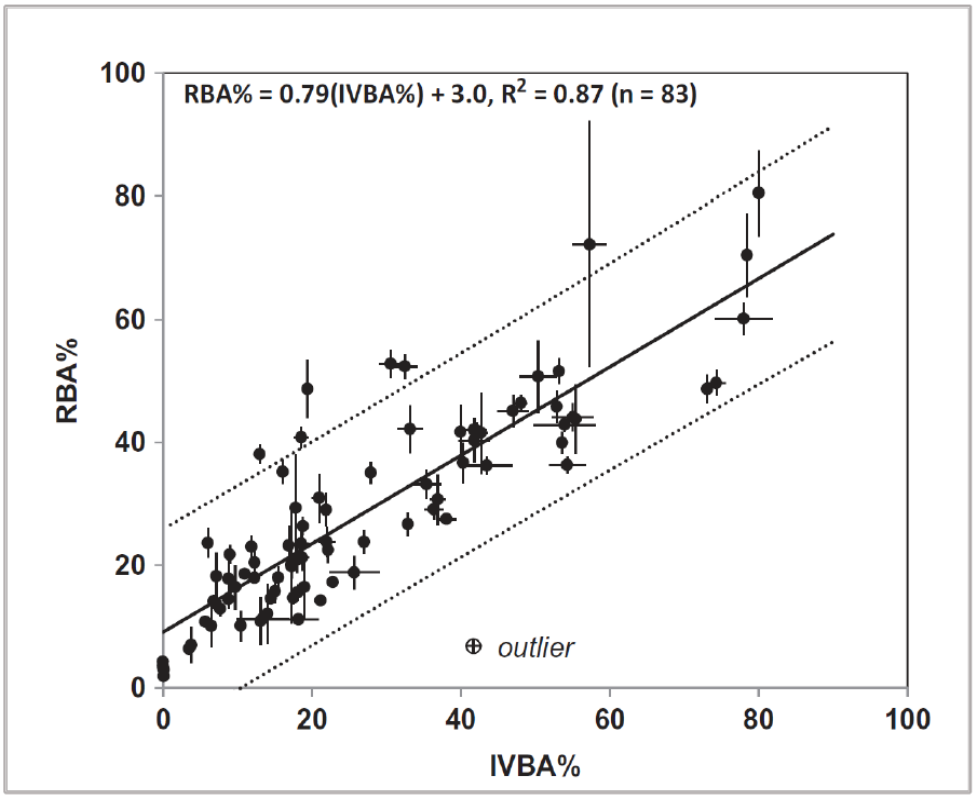


Figure 2b demonstrates the process of developing an in vitro to in vivo correlation (IVIVC). In this instance, data for in vitro bioaccessibility data (IVBA) and relative oral bioavailability (RBA) of arsenic from soil are each plotted with their associated variability. The IVIVC is reflected in the regression equation provided at the top of the figure. With this equation, IVBA data can be used to estimate RBA. Full derivation is provided in Diamond et al., 2016.

Notes:
IVBA – in vitro bioaccessibility
RBA – Relative oral bioavailability (assessed in animal studies)
Solid Line represents the weighted regression model
Dotted lines are 95% prediction limits for the weighted model.

Figure 19: In vitro in vivo correlation (IVIVC)

16. One issue raised during the PARERE consultation regards the fact that for alloys potential changes of the matrix may happen during the life cycle and in particular during handling, which may affect the release of ions. Could you comment on this in relation to the proposed in vitro method?

No material is totally inert and changes may occur over time (e.g. through wear and tear) depending on the service conditions. For example, shampoo can become more concentrated by evaporation over time and plastics degrade by exposure to sunlight and sulphur dioxide in the atmosphere. In this

respect, metals and alloys are no different. The question is whether any of the changes in the level of metal release that can occur during life cycle is relevant to human health. It is also important to remember that, it is because of their durability and their unique properties, that metals and alloys form a major part in the infrastructure and products that sustain modern life.

The following information relates to application 2 (Alloy classification):
The metals and other substances in alloys are bound within the metal matrix and all chemical/electrochemical reactions take place at the metal or alloy surface. In order for metallic ions (and indeed non-metallic ions) to be freed from the metal matrix, energy must be provided to break the metallic (chemical) bonds. Those energies are set by nature and do not vary with time. However, the handling, processing and service conditions may vary with time and, as such, these changes could lead to an increase or, indeed, a decrease in the rate of metal release. It is also possible that no change in metal release may occur throughout the lifetime of a metal, alloy or a metallic product.

The mechanical and thermal history (i.e. processing and service conditions) of metals and alloys can have a specific influence on both the crystal structure of the metal matrix and the nature of the metal/alloy surface (e.g. the presence or absence of a protective oxide or a corrosion product than may also inhibit or facilitate metal release). For example, the lead sulphate that forms on electrode plates of lead-acid batteries inhibits their dissolution.

Another example is provided by carbon steel, which may be used in the annealed (softened) or heated treated (hardened and tempered) conditions. In the softened condition, carbon steel exhibit a metal matrix consisting of ferrite (body centered cubic α-iron) and cementite. While the metal matrix of hardened and tempered carbon steel will consist of tempered martensite (a body centered tetragonal structure). In both the softened and hardened-tempered conditions, carbon steel is subject to general (uniform) surface corrosion. Yet, despite the differences in the crystal structure of the matrix, the corrosion rates of these steels (measured in mm per year) are almost identical.

It is possible, however, for the incorrect processing of metals and alloys to lead to increased levels of corrosion and, therefore, metal release. For example, incorrectly heat treated or poorly welded stainless steel can be “sensitised” due to chromium depletion at the grain boundaries and a form of localised corrosion can occur. An example of this “sensitisation” can be seen in the blades of domestic knives manufactured from martensitic stainless steel, where small pits occur close to the joint between the knife blade and the handle. While this phenomenon is unsightly, the level of increased metal release is extremely low. This is because the grain boundaries are typically 0.2-2 nanometers wide and the corrosion media must penetrate the narrow grain boundaries and transport the released ions to the exterior of the knife blade. Thus, the volume of material consumed in the dissolution of the “sensitized” grain boundaries is very small and unlikely to significantly influence the outcome of bioelution tests.

It should also be remembered that the properties of metals and alloys are well-established. Therefore, product designers take precautions to protect metals and alloys that are susceptible to corrosion (and metal release). For example, surfaces can be anodised or coated with metals, organic coatings and paint to prevent or reduce the potential for corrosion and metal release. Passive metals and alloys

(e.g. stainless steel and titanium) do not routinely require additional protective coatings, as the surface oxide on these materials is sufficiently protective.
In the context of the bioelution protocol related to oral exposure to metals and alloys, it is important to remember that corrosion and metal release are time-dependent phenomena. Corrosion and metal release processes must first be initiated and then progress with time. The transit time for food through GI tract is estimated to be 12-18 hours for healthy individuals, but transit times greater than 30 hours have been reported depending on the material. An average half time for gastric emptying of 17.7 min has been reported with a complete emptying of the stomach of 91 min in human volunteers (Tomlin et al. 1993; Wang et al. 2001). In this respect, the duration of the proposed bioelution test method is set at 2 hours. The duration of exposure in the proposed bioelution protocol provides a limited window for metal release and is reflective of the biological processes in the stomach.

In conclusion,

(i) Massives should be tested in a form that is representative of normal handling and use
(ii) While the handling and processing of metallic materials and metallic articles may have an adverse impact on the metal matrix, it is the impact at the surface that will have the greatest influence on the rate of metal release.
(iii) In general, changes in the metal matrix may increase or decrease the rate of metal release; yet, alloys are not normally selected for applications where increases in metal release are known or reasonably foreseen
(iv) Changes in the matrix can lead to reduced and stable metal release over the lifetime of consumer products (e.g. release of metals from new and old stainless steel pans, Flint and Packrisamy 1997).
(v) Corrosion and metal release processes are time dependent.

In cases where processing of the alloy can affect its crystalline structure, it is industry's duty to assess whether the classification of the alloy shall be adapted.

The size of the particles used in the bioelution test will affect the bioaccessibility. Guidance on the selection of the test material can be found for example in CLP: Chapter 8, Article 8(6): *Tests that are carried out for the purposes of this Regulation shall be carried out on the substance or on the mixture in the form(s) or physical state(s) in which the substance or mixture is placed on the market and in which it can reasonably be expected to be used*. OECD 29 (TDp) states: powders are tested at the smallest representative particle size as placed on the market, while massives are tested at a particle size representative of normal handling and use.

Importantly, for application 2, similar physical forms (powders or massives) should be tested for the alloy and its classified ingredient. The most important is to ensure that bioelution conditions provide a realistic maximum estimate of the released metal that may be available for oral absorption. Importantly, a Guidance Note for sample selection and preparation has been prepared (Annex 5). For the classification of an alloy based on bioelution test results (% bioaccessible concentration), the physical form of the sample to be tested should be consistent with the form that is placed on the market as well as with the form that can be reasonably expected to be generated during its use.
Industry understands regulatory concerns that when an alloy's classification is not warranted based on the bioaccessible concentration being < Generic Concentration Limit (GCL) or Specific

Concentration Limit (SCL), the presence of a carcinogen in the alloy still needs to be communicated to downstream users. This would be achieved by industry agreeing to list the ingredients of the alloy with their respective classifications, in addition to including the classification profile of the alloy itself.

17. Provide guidance on a) the selection of reference materials and b) list the quality/acceptance criteria (sections missing in the submitted protocol).

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

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

Proficiency materials: are well characterized and defined materials (e.g. regarding elemental composition and particle size) that may or may not include the metal of interest but need to match the physical form of the test materials (e.g. samples of powder metals or metal compounds, embedded and non-embedded massive forms of alloys). These materials are tested initially by the lab when establishing the SOP and are run periodically afterwards (at least once a year). Results corresponding to ± 20% of reported values are deemed acceptable. Proficiency materials are independent of the application of the test data. A physical repository and Shewart charts with test results will be made available.

Such proficiency materials should be:

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

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

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

Note: The proposed data acceptance criteria would be based on the following:

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

When the CV values of the test materials exceed the recommended values (leading to data rejection) but the benchmark values are met, the proficiency material should be run. If the values for the proficiency materials are met but not for the 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 releases 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 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.

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.

Annexes

PARERE Annex 1. 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

PARERE Annex 2. Oral absorption of metals from selected water soluble metal compounds

PARERE Annex 3. Review on particle uptake by Samuel Buxton (NiPERA) (August 2017) revised

PARERE Annex 4. Perkin-Elmer guidance

~~PARERE Annex 5. Guidance on sample handling and preparation of test materials in the gastric bioelution test~~ (content moved into the SOP)

PARERE Annex 6. List of metal-containing materials tested in bioelution

PARERE Annex 7. Key references

List of tables and figures

Tables:

- Table 1: releases of antimony compounds in various artificial fluids (Hedberg et al. 2010b)
- Table 2: Percent nickel release of available nickel from various nickel-containing substances after 24 hours in gastric or ALF (lysosomal fluid) (KMHC 2010).
- Table 3: Composition and parameters utilized in common in vitro bioaccessibility assays (Annex 1)
- Table 4: Metal concentration in biofluid extract (Hillwalker and Anderson 2014)
- Table 5: Metal concentration in material (Hillwalker and Anderson 2014)
- Table 6: Mn metal bioaccessibility µg Mn/mL fluid at a loading of 2 g/L (Hillwalker and Anderson 2014)
- Table 7: Differences in metal release in gastric fluid from a simple mixture and an alloy, both containing the same bulk concentration of nickel (ECTX 2017).
- Table 8: Gastric bioelution results for Ni containing samples tested at equal mass or equal nickel loading (nickel measurements in blank and pure SiO2 samples were below the reporting limit of 0.5 µg/L) (ECTX 2018a, X02a-144).
- Table 9: water solubilities of selected Cd compounds (National Research Council (US) Subcommittee on Zinc Cadmium Sulfide. Washington (DC): National Academies Press (US); 1997.)
- Table 10. Dermal absorption studies with a variety of metal compounds (corresponds to Table 21 of HERAG 2008)

Figures

- Figure 1: Effect of pH on Ti ion elution from specimens of CP Ti immersed in simulated body fluids solutions for 24, 48 or 72 h (corresponds to Figure 1 of Suito et al. 2013).
- Figure 2: Influence of biological media and pH on the release rate of iron from stainless steel particles (corresponds to Figure 4 of Mörsdorf et al. 2015).
- Figure 3: Released fractions [µg Si/µg] of Si from SiHG immersed in PBS of pH 7.2 and pH-adjusted PBS solutions (A) and corresponding released fractions of Si after 168 h of exposure in synthetic biological fluids of similar pH (GST, ALF and ASW) (B) (corresponds to Figure 4 of Herting et al. 2014
- Figure 4: Released amounts of molybdenum (Mo) normalized to the powder particle content of molybdenum (wt%) for the Mo, FeMo, MoO2 and MoO3 powders exposed for 2 and 24 hours in different synthetic body fluids (GST-artificial gastric solution; ALF-artificial lysosomal fluid; ASW- artificial sweat; PBS-Phosphate Buffered Saline; GMB-Gamble's solution) (corresponds to Figure 2 of Mörsdorf et al. 2015).
- Figure 5: Percent bioaccessibility vs. pH (corresponds to Figure 3 of Koch et al. 2013).
- Figure 6: Time-dependent metal release in gastric fluid for pure metals and compounds (powder forms).
- Figure 7: Time-dependent metal release in gastric fluid for SS316L (powder form) (ECTX 2018b).
- Figure 8: Time-dependent Cu release in gastric fluid from Z45 and pure copper metal (massive forms) (ECTX 2018c).
- Figure 9: Sb releases from various Sb-containing substances in gastric fluid (corresponds to Figure 8 of Hedberg et al. 2010b, unpublished). Antimony transformed [mass%], equivalent to percentage of the amount particles dissolved since normalized to the antimony content of the test item. Data presented as average of values of triplicate samples in different media. Blank values for each individual media and exposure period have been subtracted. Abbreviations: GMB-Gamble's solution, PBS-phosphate buffered saline, ASW- artificial sweat, ALF-artificial lysosomal fluid, GST-gastric fluid.
- Figure 10: (identical to Figure 4): Released amounts of molybdenum (Mo) normalized to the powder particle content of molybdenum (wt%) for the Mo, FeMo, MoO2 and MoO3 powders exposed for 2 and 24 hours in different synthetic body fluids (GST-artificial gastric solution; ALF-artificial lysosomal fluid; ASW- artificial sweat; PBS-Phosphate Buffered Saline; GMB-Gamble's solution) (corresponds to Figure 2 of Mörsdorf et al. 2015)

- Figure 11: Release of molybdenum or iron normalised to the corresponding metal particle content (wt%) for the Fe, FeMo, and Mo powders exposed for 2 and 24 hours in different synthetic body fluids (corresponds to Figure 3 of Mörsdorf et al. 2015)
- Figure 12: Effect of loading on metal release using a mixture of Fe, Cr and Ni in gastric fluid after 2 h and using 3 different loadings (ECTX 2017)
- Figure 13: Predominance diagramme for silver species calculated using Medusa (corresponds to Figure 3 of Levard et al. 2013)
- Figure 14: Released fractions of Ni, Cr, Fe for the Inconel powder compared to stainless steel, Ni, Cr and Fe metal (corresponds to Figure 4 of Hedberg et al. 2016)
- Figure 15: Plots of IVBA vs. RBA for antimony extracted from Denys et al. 2012
- Figure 16: Percent bioaccessibility vs. pH for some elements of interest (figure excerpted from Koch et al. 2013).
- Figure 17: Effect of pH on IVBA for NIST standard reference material soils
- Figure 18: Comparison of IVBA to RBA for Pb and As (excerpted from Koch et al. 2013)
- Figure 19: In vitro in vivo correlation (IVIVC)

References

This is the complete list of references used in the Responses to PARERE document. All these papers are referred in the PARERE text as supporting evidence. Copies of key studies are provided in Annex 7 (zip file).

- Anjilvel S, Asgharian B. 1995. A multiple-path model of particle deposition in the rat lung. Fundamental and Applied Toxicology, 28: 41–50.
- Asgharian B, Price O. 2009. Multiple Path Particle Deposition Model, (MPPD Version 2.1), Software (with graphical user interface) is available for free download from Bahman Asgharian, Ph.D. at Applied Research Associates. www.ara.com.
- ASTM (American Society for Testing and Materials). 2007. Standard test method for determining extractability of metals from art materials. In: ASTM, Annual Book of ASTM Standards, vol. 06. 02, D5517–03. ASTM, Philadelphia, US.
- Axson JL, Stark DI, Bondy AL, Capracotta SS, Maynard AD, Philbert MA, Bergin IL, Ault AP. 2015. Rapid Kinetics of Size and pH-Dependent Dissolution and Aggregation of Silver Nanoparticles in Simulated Gastric Fluid. J Phys Chem C Nanomater Interfaces, 119(35):20632-20641.
- Barão VA, Mathew MT, Assunção WG, Yuan JC, Wimmer MA, Sukotjo C. 2011. Stability of cp-Ti and Ti-6Al-4V alloy for dental implants as a function of saliva pH - an electrochemical study. Clin Oral Implants Res, 23(9):1055-62.
- Brandon EF, Oomen AG, Rompelberg CJM, Versantvoort CHM, Van Engelen GM, Sips AJAM. 2006. Consumer product in vitro digestion model: bioaccessibility of contaminants and its application in risk assessment. Regulatory Toxicology and Pharmacology, 44: 161- 171.
- Brattin W, Drexler J, Lowney Y, Griffin S, Diamond G, Woodbury L. 2013. An in vitro method for estimation of arsenic relative bioavailability in soil. J Toxicol Environ Health, 76:458–478.
- Broussard L, Winecker RE, - Ropero-Miller J, Hammett-Stabler CA. 2002. The Toxicology of Mercury Laboratory Medicine, 33(8):614-625.
- Cai Z, Nakajima H, Woldu M, Berglund A, Bergman M, Okabe T . 1999. In vitro corrosion resistance of titanium made using different fabrication methods. Biomaterials, 20(2):183-90.
- Chrysikopoulos and Kruger, 1986. report SGP-TR-99 Chelated Indium Activable Tracers for Geothermal Reservoirs.
- Denys S, Caboche J, Tack K, Rychen G, Wragg J, Cave M, Jondreville C, Feidt C. 2012. In vivo validation of the unified BARGE method to assess the bioaccessibility of arsenic, antimony, cadmium, and lead in soils. Environ Sci Technol, 46:6252–6260.
- Drexler JW, Brattin WJ. 2007. An in vitro procedure for estimation of lead relative bioavailability: with validation. Hum Ecol Risk Assess, 13: 383–401.
- Eckert E, Bamdad F, Chen LY. 2014. Metal solubility enhancing peptides derived from barley protein. Food Chemistry, 159:498–506.
- ECTX. 2016: **X02a-084**: 2 hours bioelution study on stainless steel 316L powder at a 2 g/L loading in simulated gastric fluid.
- ECTX. 2017. **X02a-130**: A 2 hours bioelution 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.
- ECTX. 2018a. **X02a-144**: 2 hours bioelution study on stainless steel 316L powder at a 2 g/L loading in simulated gastric fluid.

- ECTX. 2018b. **X02a-145**: 4 hours bioelution kinetics study on stainless steel 316L powder at a 2 g/L loading in simulated gastric fluid.
- ECTX. 2018c. **X02a-146**: 4 hours bioelution kinetics study on Z45 (massive) at a 2 g/L loading (surface equivalent) in a simulated gastric fluid.
- EU RAR (Risk Assessment Report). 2007. Cadmium oxide-part II human health. http://publications.jrc.ec.europa.eu/repository/bitstream/JRC37161/cadmiumoxidehhreport302.pdf
- Flint GN and Packirisamy S. 1997 Purity of Food Cooked in Stainless Steel Utensils; NiDI Report, (Reprinted from Food Additives and Contaminants, Volume 14, number, 1997).
- Frohlich E, Roblegg E. 2012. Models for oral uptake of nanoparticles in consumer products. Toxicology, 291(1-3):10-17.
- Galia E, Nicolaides E, Hörter D, Löbenberg R, Reppas C, Dressman JB. 1998. Evaluation of various dissolution media for predicting in vivo performance of class I and II drugs. Pharm Res, 15:698-705.
- Goix S, Uzu G, Oliva P, Barraza F, Calas A, Castet S, Point D, Masbou J, Duprey JL, Huayta C, Chincheros J, Gardon J. 2016. Metal concentration and bioaccessibility in different particle sizes of dust and aerosols to refine metal exposure assessment. J Hazard Mater, 5: 317:552-562.
- Hedberg YS, Hedberg JF, Isaksson S, Mei N, Blomberg E, Wold, Odnevall Wallinder I. 2017. Nanoparticles of WC-Co, WC, Co and Cu of relevance for traffic wear particles - Particle stability and reactivity in synthetic surface water and influence of humic matter. Environ Pollut, 224: 275-288.
- Hedberg YS, Herting G, Latvala S, Elihn K, Karlsson HL, Odnevall Wallinder I. 2016. Surface passivity largely governs the bioaccessibility of nickel-based powder particles at human exposure conditions. Regul Toxicol Pharmacol, 81:162-170.
- Hedberg YS, Odnevall Wallinder I. 2015. Metal release from stainless steel in biological environments – a review. Biointerphases – special issue on Ions and Solvation at Biointerphases, 11(1).
- Hedberg Y, Midander K. 2014. Size matters: Mechanism of metal release from 316L stainless steel particles is governed by size-dependent properties of the surface oxide. Mater Lett, 122: 223-226.
- Hedberg Y, Hedberg J, Odnevall Wallinder I. 2012. Particle characteristics and metal release from natural rutile (TiO2) and zircon particles in synthetic body fluids. Journal of Biomaterials and Nanobiotechnology, 3: 37-49.
- Hedberg Y, Gustafsson J, Karlsson HL, Möller L, Odnevall Wallinder I. 2010a. Bioaccessibility, bioavailability and toxicity of commercially relevant iron- and chromium-based particles: in vitro studies with an inhalation perspective, Particle and Fibre Toxicology, 7:23.
- Hedberg Y, Jiang T, Odnevall-Wallinder I. 2010b. Bioaccessibility of antimony released from four different antimony compounds in synthetic biological media. Commissioned by International Antimony Association (i2a).
- Henderson RG, Cappellini D, Seilkop SK, Bates HK, Oller AR. 2012. Oral bioaccessibility testing and read-across hazard assessment of nickel compounds. Regul Toxicol Pharmacol, 63 (1): 20–28.
- Henderson R, Verougstraete V, Anderson K, Arbildua JJ, Brock TO, Brouwers T, Cappellini D, Delbeke K, Herting G, Hixon G, Wallinder IO, Rodriguez PH, Assche FV, Wilrich P, Oller AR. 2014. Interlaboratory validation of bioaccessibility testing for metals. Regulatory Toxicology Pharmacology, 70(1):170-181.

- HERAG. 2008: https://www.icmm.com/herag
- Herting G, Jiang T, Sjöstedt C, Odnevall Wallinder I. 2014. Release of Si from silicon metalloids, a FeSi alloy and a silicate mineral in simulated biological media. PLoS ONE 9(9).
- Hillwalker WE, Anderson KA. 2014. Bioaccessibility of metals in alloys: Evaluation of three surrogate biofluids. Environmental Pollution, 185:52–58.
- Ishimatsu S, Kawamoto T, Matsuno K, Kodama Y. 1995. Distribution of various nickel compounds in rat organs after oral administration. Biol Trace Elements Res, 49(1):43-52.
- IITRI 2010. IIT Research Institute, Life Sciences group, Chicago IL, at https://www.itia.info/assets/files/ACGIH/21_IITRI_Bioaccessibility_Studies_5_Tungsten_Compounds.pdf.
- Jantratid E, De Maio V, Ronda E, Mattavelli V, Vertzoni M, Dressman JB. 2009. Application of biorelevant dissolution tests to the prediction of in vivo performance of diclofenac sodium from an oral modified-release pellet dosage form. Eur J Pharm Sci, 37: 434−441.
- Jiang T, Odnevall Wallinder I, Herting G. 2012. Chemical stability of chromium carbide and chromium nitride powders compared with chromium metal in synthetic biological solutions. ISRN Corrosion.
- Juhasz AL, Herde P, Herde C, Boland J, Smith E. 2014. Validation of the Predictive Capabilities of the Sbrc- G in Vitro Assay for Estimating Arsenic Relative Bioavailability in Contaminated Soils. Environmental Science & Technology, 48: 12962- 12969.
- Juhasz AL, Weber J, Smith E, Naidu R, Marschner B, Rees M, Rofe A, Kuchel T, Sansom L. 2009. Evaluation of SBRC- Gastric and SBRC- Intestinal Methods for the Prediction of In Vivo Relative Lead Bioavailability in Contaminated Soils. Environmental Science & Technology, 43:4503– 4509.
- Kalantzi L, Goumas K, Kalioras V, Abrahamsson B, Dressman JB; Reppas C. 2006. Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. Pharm Res, 23: 165−176.
- KMHC (Kirby Memorial Health Center). 2010. Compiled analysis reports for 15 nickel substances: Solubility in simulated fluids. Analyses were conducted during 2008-2010. Testing laboratory: Kirby Memorial Health Center. Owner company: Nickel REACH Consortia Members.
- Koch I, Reimer KJ, Bakker MI, Basta NT, Cave MR, Denys S, Dodd M, Hale BA, Irwin R, Lowney YW, Moore MM, Paquin V, Rasmussen PE, Repaso-Subang T, Stephenson GL, Siciliano SD, Wragg J, Zagury GJ. 2013. Variability of bioaccessibility results using seventeen different methods on a standard reference material, NIST 2710. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering, 48(6):641–655.
- Laird BD, James KJ, Van de Wiele TR, Dodd M, Casteel SW, Wickstrom M, Siciliano SD. 2013 An investigation of the effect of gastrointestinal microbial activity on oral arsenic bioavailability Journal of Environmental Science and Health, Part A Vol. 48(6).
- Laird BD, Van de Wiele TR, Corriveau MC, Jamieson HE, Parsons MB, Verstraete W, Siciliano SD. 2007. Gastrointestinal microbes increase arsenic bioaccessibility of ingested mine tailings using the simulator of the human intestinal microbial ecosystem. Environ Sci Technol, 41(15):5542-7.
- Latvala S, Hedberg J, Di Bucchianico S, Möller L, Odnevall Wallinder I, Elihn K, Karlsson HL. 2016. Nickel Release, ROS Generation and Toxicity of Ni and NiO Micro- and Nanoparticles. PLoS One. 19, 11(7).

- Levard C, Mitra S, Yang T, Jew AD, Badireddy AR, Lowry GV, Brown GE Jr. 2013. Effect of chloride on the dissolution rate of silver nanoparticles and toxicity to E. coli. Environ Sci Technol, 47(11):5738-45.
- Lowney YW, Wester RC, Schoof RA, Cushing CA, Edwards M, Ruby MV. 2007. Dermal absorption of arsenic from soils as measured in the rhesus monkey. Toxicol Sci, 100(2):381-92.
- Maltby J R, Lewis P, Martin A. 1991. Gastric fluid volume and pH in elective patients following unrestricted oral fluid until three hours before surgery. Canadian Journal of Anaesthesia, 38 (4): 425.
- Molina RM, Schaider LA, Donaghey TC, Shine JP, Brain JD. 2013. Mineralogy affects geoavailability, bioaccessibility and bioavailability of zinc. Environmental Pollution, 182: 217-224.
- Midander K, de Frutos A, Hedberg Y, Darrie G, Odnevall Wallinder I. 2010. Bioaccessibility studies of ferro-chromium alloy particles for a simulated inhalation scenario. A comparative study with the pure metals and stainless steel. Integrated Environmental Assessment and Management, 6(3): 441-455.
- Mörsdorf A, Odnevall Wallinder I, Hedberg Y. 2015. Bioaccessibility of micron-sized powder particles of molybdenum metal, iron metal, molybdenum oxides and ferromolybdenum - Importance of surface oxides. Regulatory Toxicology and Pharmacology, 72(3): 447-457.
- Mudie D M, Amidon G L, Amidon GE 2010. Physiological parameters for oral delivery and in vitro testing. Mol Pharmaceutics, 7: 1388−1405.
- National Research Council (US) Subcommittee on Zinc Cadmium Sulfide. Toxicologic Assessment of the Army's Zinc Cadmium Sulfide Dispersion Tests 1997. Washington (DC): National Academies Press (US).
- OECD. 2001. OECD Series on Testing and Assessment Number 29. Guidance Document on Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media. ENV/JM/MONO(2001)9.
- Oliver DP, McLaughlin MJ, Naidu R, Smith LH, Maynard EJ, Calder IC. 1999. Measuring Pb bioavailability from household dusts using an in vitro model. Envir Sci Technol, 33 (24): 4434–4439.
- Oller AR, Oberdörster G. 2016. Incorporation of dosimetry in the derivation of reference concentrations for ambient or workplace air: a conceptual approach. J Aerosol Science, vol 99: 40-45.
- OSWER (Office of Solid Waste and Emergency Response). 2007. Estimation of relative bioavailability of lead in soil and soil- like materials using in vivo and in vitro methods. OSWER 9285: 7- 77.
- Rodriguez RR, Basta NT, Casteel SW, Pace LW.1999. An In Vitro Gastrointestinal Method To Estimate bioavailable Arsenic in Contaminated Soils and Solid Media. Environ Sci Technol, 33: 642-649.
- Ruby M V, Schoof R, Brattin W, Goldade M, Post G, Harnois M, Mosby DE, Casteel SW, Berti W, Carpenter M, Edwards D, Cragin D. Chappell W. 1999. Advances in Evaluating the Oral Bioavailability of Inorganics in Soil for Use in Human Health Risk Assessment. Env Sci Technol, 21: 3697–3705.
- Safruk AM, Berger RG, Jackson BJ, Pinsent C, Hair AT, Sigal EA. 2015. The bioaccessibility of soil-based mercury as determined by physiological based extraction tests and human biomonitoring in children. Science of the Total Environment, 518–519:545–553.

- Schroder JL, Basta NT, Casteel SW, Evans T J, Payton ME, Si J. 2004. Validation of the In Vitro Gastrointestinal (IVG) Method to Estimate Relative Bioavailable Lead in Contaminated Soils. J Environ Qual, 33: 513–521.
- Stefaniak AB, Virji MA, Harvey CJ, Sbarra DC, Day GA, Hoover MD. 2010. Influence of artificial gastric juice composition on bioaccessibility of cobalt- and tungsten-containing powders. Int J Hyg Environ Health, 213: 107–115.
- Stockmann-Juvala H, Hedberg Y, Dhinsa NK, Griffiths DR, Brooks PN, Zitting A, Odnevall Wallinder I, Santonen T. 2013. Inhalation toxicity of 316L stainless steel powder in relation to bioaccessibility. Hum Exp Toxicol, 32: 1137.
- Stopford W, Turner J, Cappellini, D, Brock T, 2003. Bioaccessibility testing of cobalt compounds. J Environ Monit, 5: 675–680.
- Suito H, Iwawaki Y, Goto T, Tomotake Y, Ichikawa T. 2013. Oral Factors Affecting Titanium Elution and Corrosion: An In Vitro Study Using Simulated Body Fluid. PLoS One, 8(6): e66052.
- Sunderman FW, Range CL, Sunderman W Jr, Donnelly AJ, Lucysevn GW. 1961. Nickel Poisoning: XII. Metabolic and pathologic changes in acute pneumonitis from nickel carbonyl. Am J Clin Pathology, 36 (6): 477-491.
- Tomlin J, Brown N, Ellis A, Carlsson A, Bogentoft C, Read NW. 1993. The effect of liquid fibre on gastric emptying in the rat and humans and the distribution of small intestinal contents in the rat. Gut, 34(9):1177-81.
- Vasiluk L, Dutton MD, Hale B. 2011. In vitro estimates of bioaccessible nickel in field contaminated soils, and comparison with in vivo measurement of bioavailability and identification of mineralogy. Science of the Total Environment, 409: 2700- 2706.
- Walsh PL, Bothe JR, Bhardwaj S, Hu M, Nofsinger R, Xia B, Persak S, Pennington J, Bak A. 2015. A canine biorelevant dissolution method for predicting in vivo performance of orally administered sustained release matrix tablets. Drug Dev Ind Pharm, 4:1- 9.
- Wang L, Pérez-Magueda, LA, Matijevic, E. 1998. Rapid preparation of uniform colloidal indium hydroxide by the controlled double-jet precipitation, Colloid & Polymer Science 276 (1998), S. 847-850.
- Wang SC, Lu KY, Chen SM, Young TK. 2001 Gastric emptying and intestinal transit of liquid and solid markers in rats with chronic uremia. Chin J Physiol, 44(2):81-7.
- Welfringer B, Zagury G. 2009. Evaluation of two in vitro protocols for determination of mercury bioaccessibility: Influence of mercury fractionation and soil properties. Journal of Environmental Quality, 38:2237–2244.
- Zhang S, Du C, Wang Z, Han X, Zhang K, Liu L. 2013 Reduced cytotoxicity of silver ions to mammalian cells at high concentration due to the formation of silver chloride. Toxicol In Vitro Volume 27 (2): 739-744.