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Bioaccessibility of metals in alloys: Evaluation of three surrogate biofluids☆

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ABSTRACT

Bioaccessibility *in vitro* tests measure the solubility of materials in surrogate biofluids. However, the lack of uniform methods and the effects of variable test parameters on material solubility limit interpretation. One aim of this study was to measure and compare bioaccessibility of selected economically important alloys and metals in surrogate physiologically based biofluids representing oral, inhalation and dermal exposures. A second aim was to experimentally test different biofluid formulations and residence times *in vitro*. A third aim was evaluation of dissolution behavior of alloys with *in vitro* lung and dermal biofluid surrogates. This study evaluated the bioaccessibility of sixteen elements in six alloys and 3 elemental/metal powders. We found that the alloys/metals, the chemical properties of the surrogate fluid, and residence time all had major impacts on metal solubility. The large variability of bioaccessibility indicates the relevancy of assessing alloys as toxicologically distinct relative to individual metals.

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1. Introduction

Alloy dust is a byproduct of the manufacturing and use of alloy materials such as stainless steel (SS). Occupational workers and to a lesser extent the general public are exposed to alloy and metal mixtures in dust. Normally regulatory agencies attempt to define and regulate risk of mixtures relative to the sum of the individual components (U.S.EPA, 2000; Vyskocil et al., 2004) but metal alloys are a unique class of substances defined as "consisting of two or more elements so combined that they cannot be readily separated by mechanical means" (Skeaff et al., 2007; UNGHS, 2005). There are many alloys and each exhibits unique properties. Studies highlighting intrinsic differences in the solubility of metals in various alloys (Flint, 1998; Herting et al., 2008b; Skeaff et al., 2007; Stopford et al., 2003) recognize the significance of their unique qualities. These studies indicate the importance of testing metal alloys separately from metal ores or minerals when classifying hazard.

Biologically relevant exposure tests measuring the chemical dose that is available for uptake are gaining greater attention and support for public health applications (Birnbaum, 2010). One such exposure test, the bioaccessibility *in vitro* test, has been used to account for the relative bioavailability of contaminants in human health risk assessments (Brandon et al., 2006; Brock and Stopford, 2003; EN, 2009; Henderson et al., 2012; U.S.EPA, 2007). Bioaccessibility is an important facet of bioavailability, and it is frequently defined as the biologically relevant fraction of a chemical that is potentially available for uptake into a biological organism (Anderson and Hillwalker, 2008; Brandon et al., 2006; Ruby et al., 1999). The test only provides an estimate of the complex physiological and physicochemical processes that occur in human toxicokinetics, but represents the step in bioavailability that is most sensitive to the chemical behavior of materials (Brandon et al., 2006; Drexler and Brattin, 2007).

Bioaccessibility *in vitro* tests, bio-elution, offer the advantages of simplicity, speed, affordability and ethical considerations over *in vivo* bioassays. Human surrogate biofluids used in bioaccessibility tests include gastro-intestinal (saliva, stomach, intestine), dermal (sweat), lung (alveolar, interstitial, lysosomal, serum) and internal implantation (lysosomal/cytosol). Oral bioaccessibility

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tests are the most frequently investigated with the test methodology ranging from a static gastric compartment (Drexler and Brattin, 2007; EN, 2002; Stopford et al., 2003; U.S.EPA, 2007) to dynamic gastrointestinal models (Garcia et al., 2001; Juhasz et al., 2009; Rodriguez and Basta, 1999; Ruby et al., 1996; Velasco-Reynold et al., 2008). While multiple gastric methods persist, alloy dermal biofluid studies have generally used the EN 1811 reference method for allergenic responses via skin contact (Bocca et al., 2007; Flint, 1998; Julander et al., 2009; Midander et al., 2007b). However, far fewer studies have applied *in vitro* bioaccessibility tests to lung (Herting et al., 2008b; Midander et al., 2007b; Stopford et al., 2003; Thelohan and Demeringo, 1994; Twining et al., 2005; Vitarella et al., 2000) or internal implantation (Herting et al., 2008a; Stopford et al., 2003) biofluids to assess inhalation exposure to alloys. A critical barrier to this type of testing is the lack of standardization for selecting physiologically-based extraction conditions including residence time, substance mass to biofluid volume ratio, agitation, and biofluid formulation chemistries. While some test parameters have been more thoroughly investigated, such as particle size, (Hedberg et al., 2010; Midander et al., 2007a) test mass to biofluid ratio (Hamel et al., 1998; Thelohan and Demeringo, 1994), and agitation (Midander et al., 2006), other method variations, such as biofluid formulation and effect of residence time, are not as well characterized for use with alloys. These parameters are often manipulated between studies, making it difficult to compare bioaccessibility results and further preventing incorporation of a bioaccessibility test into health risk characterization.

This study evaluates metal bioaccessibility from several economically important grades of alloys in physiologically based *in vitro* biofluids representing three major exposure routes: gastric, lung and dermal. Biofluid formulations and residence times are two commonly employed test parameters that were evaluated using standard alloy reference materials. We illustrate that the *in vitro* bioaccessibility tests are applicable to assessing unique qualities of different alloy grades for health characterization purposes. We measure dissolution rates for nine alloys/metal powders in two biological surrogate biofluids. Six alloys and three elemental metal powders are compared using the major exposure route surrogate biofluids: gastric, lung and dermal.

2. Materials and methods

2.1. Materials

Four commercially available austenitic steel alloys and three metal powders were purchased from Atlantic Equipment Engineers (NJ, USA). The alloys included the American Iron and Steel Institute (AISI) stainless steel (SS) grades 316 and 304; the Ni–Cr Inconel and Ni–Cu Monel superalloys; and the metal powders included cobalt, manganese and nickel. Two alloy standard reference materials (SRMs) were purchased though National Institute of Standards and Testing (NIST, Gaithersburg, MD); SRM 101g (stainless steel, SS 304L) and SRM 14g (carbon steel). Table 1 lists the physical and chemical compositions of the alloys and metal powders.

2.2. Surrogate biofluids

Three human surrogate biofluids representing those involved in oral (gastric), inhalation (artificial lysosomal fluid [ALF]) and dermal (sweat) human exposure pathways were selected to measure alloy bioaccessibility. Different chemical formulations of the individual biofluids have been reported (Hedberg et al., 2010; Herting et al., 2007; Stopford et al., 2003), however, the effects of different biofluid formulations have not received adequate attention. To evaluate the magnitude of the effects, two commonly reported versions of ALF and two versions of gastric biofluids were applied to the SRM alloys.

Gastric biofluids from the static gastric compartment model are simple surrogates with low pH levels (pH 1.2–1.5) representing a worst-case fasting exposure scenario for a conservative bioaccessibility assessment (Brock and Stopford, 2003; EN, 2009; Juhasz et al., 2009; U.S.EPA, 2007). Two different gastric solution compositions were selected; a 0.07 N HCl solution further developed by Stopford et al. for determining metals in art material (ASTM, 2007; Stopford et al., 2003) and an approximately 1 N HCl solution buffered with 2.5 M glycine (herein described as gastric–GLY) used by the US EPA to assess gastric bioaccessibility of lead in soil (Drexler and Brattin, 2007). This oral bioaccessibility model was selected because the static approach has undergone extensive inter-laboratory round robin testing (ASTM, 2007; Drexler and Brattin, 2007; EN, 2002; U.S.EPA, 2009) and validation with *in vivo* studies with soil matrices (Rodriguez and Basta, 1999; U.S.EPA, 2007).

ALFs are composed of complex salts and organic acids with low pH (pH 4.5) simulating phagocytosis of particulates by lung alveolar cells and interstitial macrophages (Stopford et al., 2003; Thelohan and Demeringo, 1994) and inflammatory response connected with surgical implants in the body (Herting et al., 2008a). Two ALF compositional differences reported in the literature include either the use of glycine (Thelohan and Demeringo, 1994) or an equivalent mass of glycerol (Stopford et al., 2003), herein described as glycine–ALF and glycerol–ALF, respectively. Surrogate sweat (pH 6.4–6.6) that used was prepared according to the EN 1811 standardized test, which is commonly used for allergenic response from nickel, chromium, cobalt and other metals in alloys (Bocca et al., 2007; EN, 2009; Julander et al., 2009; Midander et al., 2007b).

The complete compositions of the five biofluids used are listed in Supplementary information (SI) Table S1. All solutions were prepared using 18 MΩ cm water and analytical grade reagents and chemicals. ALF and sweat were used within a week and 3 h of preparation, respectively. The gastric fluids were considered stable throughout the study duration.

2.3. Experimental conditions

Test parameters evaluated included multiple formulations of biofluids and three residence times. Two formulations each for gastric and lung biofluids were tested. Gastric was tested with and without glycine ($C_2H_5NO_2$) and ALF was tested with glycine or glycerol ($C_3H_8O_3$). Complete compositions of all test biofluids are described in SI Table SI. Three residence times, 2, 24 and 72 h, were tested for lung and dermal biofluids and two residence times, 2 and 72 h were tested for gastric solutions, Table 2 and Fig. 1. The gastric and glycerol–ALF formulations were used for the residence time studies, Table 2 and Table S1. All test parameters were performed with two SRMs, carbon steel (NIST 14g) and stainless steel (NIST 101g), which represent vastly different alloys.

We then evaluated the bioaccessibility of 4 alloys and 3 elemental metal powders in the following biofluids: gastric, lung and dermal (Table 3). Here we focused on one formulation and one residence time for each biofluid. Gastric employed HCl for 2 h, lung utilized ALF with glycerin for 72 h, and dermal sweat was tested as described above for 72 h.

The preparation consisted of 0.1 g (±10%) of test alloy/metal powder with 50 mL of surrogate biofluid representing a 1:500 g/mL extraction ratio. This exposure ratio

Table 1
Chemical composition (wt%) and particle size of test materials.

Test material (grade[a])	Co	Cr	Cu	Fe[b]	Mn	Ni	Mo	P	Particle size[c]
Carbon steel 1078 (NIST 14g)	0.0030	0.0810	0.0470	–	0.4560	0.0300	0.0110	0.0060	0.5–1.18 mm
Stainless steel 304L (NIST 101g)	0.0900	18.46	0.0290	–	0.0850	10.0	0.0040	0.0070	75–710 μm
Stainless steel 304	0.09	18.02	0.0290	68.30	0.15	11.14	–	0.012	44–149 μm
Stainless steel 316	–	16.74	–	69.58	0.08	11.69	2.15	0.03	44–149 μm
Inconel (Ni–Cr)	–	15.78	0.500	9.000	0.07	74.19	–	–	<44 μm
Monel (Ni–Cu)	–	–	28.9	0.080	2	67.11	–	–	<44 μm
Co metal	99.8	–	–	–	–	–	–	–	<36 μm
Mn metal	–	–	–	–	99.8	–	–	–	44–297 μm
Ni metal	–	–	–	–	–	99.8	–	–	44–149 μm

[a] American Iron and Steel Institute.
[b] Approximate iron balance.
[c] Sieve analysis.

Table 2
Comparison of test parameters, residence times and different biofluid formulations on metal bioaccessibility.

SRM (n = 5 or 3)	Biofluid	Method modification	*A.* $\%_{metal}$					*B.* $\%_{alloy}$	
			Cobalt avg ± SD	Copper avg ± SD	Chromium avg ± SD	Manganese avg ± SD	Nickel avg ± SD	Iron avg ± SD	Zinc avg ± SD
Carbon steel NIST 14g	gastric	2 h time/gastric[a], %	<0.00027	<0.00028	<0.00019	9.3 ± 1	<0.00075	15 ± 5	<0.00080
		2 h GLY–gastric[b], % formulation p-value	<0.00027	<0.00028	<0.00019	5.5 ± 0.1 0.004	<0.00075	7.8 ± 0.2 <0.001	<0.00080
		72 h time/gastric[a], % residence time p-value	46 ± 7	<0.00028	<0.00019	78 ± 6 <0.001	25 ± 8	27 ± 1 0.004	0.0062 ± 0.0005
	ALF	2 h time/ALF[a], %	<0.00027	<0.00028	6.3 ± 0.4	1.1 ± 0.5	<0.00075	2.8 ± 0.4	0.023%
		2 h GLY–ALF[c], % formulation p-value	<0.00027	<0.00028	5.2 ± 0.6 0.016	0.1 ± 0.01 0.009	<0.00075	1.7 ± 0.6 0.018	0.023%
		72 h time/ALF[a], % residence time p-value	<0.00027	74 ± 14	~100 ± 6 0.016	96 ± 6 0.036	~100 ± 6	67 ± 3 0.018	0.024 ± 0.0006
	Sweat	2 h time[c], %	<0.00027	<0.00028	<0.00019	0.56 ± 0.1	<0.00075	0.022 ± 0.006	<0.00080
		72 h time[c], % time p-value	<0.00027	<0.00028	<0.00019	1.8 ± 0.1 <0.001	<0.00075	0.025 ± 0.004 0.868	<0.00080
Stainless steel NIST 101g	Gastric	2 h time/gastric[a], %	<0.00027	<0.00028	0.35 ± 0.1	<0.000070	0.39 ± 0.1	0.26 ± 0.08	<0.00082
		2 h GLY–gastric[b], % formulation p-value	<0.00027	<0.00028	0.44 ± 0.1 0.322	<0.000070	0.46 ± 0.1 0.474	0.31 ± 0.08 0.428	<0.00082
		72 h time/gastric[a], % residence time p-value	24 ± 3	<0.00028	23 ± 3 <0.001	16 ± 3	24 ± 3 0.004	12 ± 1.0 <0.001	0.018 ± 0.002
	ALF	2 h time/ALF[a], %	<0.00027	<0.00028	<0.00019	<0.000070	<0.00075	<0.00065	<0.00080
		2 h GLY–ALF[c], % formulation p-value	<0.00027	<0.00028	<0.00019	<0.000070	<0.00075	<0.00065	<0.00080
		72 h time/ALF[a], % residence time p-value	<0.00027	<0.00028	0.12 ± 0.01	0.96 ± 0.4	<0.00075	<0.00065	<0.00080

A) $\%_{metal}$ [((μg metal in solution/μg metal in alloy) * 100], *B*) $\%_{alloy}$ [(μg metal in solution/μg alloy) * 100]. < = below detection limit. SS = stainless steel.
[a] Biofluid solutions prepared according to gastric and ALF (Stopford et al., 2003).
[b] Biofluid solutions prepared according to glycine–gastric (Drexler and Brattin, 2007).
[c] Biofluid solutions prepared according to glycine–ALF (Thelohan and Demeringo, 1994); all metals from SRM 304L NIST 101g were below detected limits in the sweat biofluid.

is associated with minimal solution saturation effects, increasing the potential for dissolution reproducibility even for soluble compounds (Hamel et al., 1998). Each mixture was placed on an orbital shaker bath set at 100 oscillations min^{-1} (3 cm × 3 cm stroke path) at a temperature of 37 ± 2 °C. Agitation was applied to the sweat biofluid for consistency amongst the methods through EN 1181 requires extraction without agitation. Exposure to light was minimized. The pH of the solution was monitored throughout the exposure period and adjusted to ±0.5 pH units of the expected level. At the end of the exposure, an aliquot was filtered through a PVDF 0.45 μm filter, representing an estimate of the dissolved fraction of the extract. Extractions were prepared in duplicate or triplicate for the commercially available alloys and in triplicate or quintuplicate for the SRMs. All filtrates were stored at <4 °C until analysis. Quality assurance protocols used included employing calibrated scales and transfer pipettes (verification of calibration prior to daily use) as well as class A volumetric glassware.

2.4. Metal analysis and quality control

Sixteen elements were quantified by inductively coupled plasma atomic emission spectroscopy (ICP-AES); aluminum, arsenic, barium, beryllium, cadmium, chromium, cobalt, copper, iron, lead, manganese, molybdenum, nickel, phosphorus, strontium and zinc. Detection limits ranged from 0.0014 to 0.016 μg/mL in the biofluid extract, from 0.70 to 8.0 μg/g in the metal and from 0.000070 to 0.00080 relative to $\%_{metal}$ bioaccessibility; Supplemental information, Tables S2–S4. Quality control samples accounted for 30% of each batch and included reagent blanks, fortifications and instrument standard check samples. Recoveries of fortification samples and check standards were from 85 to 110% and 93 to 109%, respectively. The dissolution reproducibility was assessed by determining the bioaccessibility of all biofluids with three to five SRMs in a series of batches over the course of multiple days. The test procedures exhibited good reproducibility as indicated by low coefficients of variance across all batches for all biofluids, ranging from 0.087 to 0.36%.

2.5. Statistical analysis

Comparison of bioaccessibility between individual method modifications for each metal was carried out by a t-test. When data failed the equal variance test, a Mann–Whitney rank sum test was applied. A p-value of <0.05 was considered statistically significant. All statistical calculations were performed using Sigmaplot v. 11 (Systat Software Inc., San Jose, CA, USA).

3. Results and discussion

Seven of the sixteen metals were frequently detected in the biofluid extracts of the nine alloys/metal powders tested: cobalt, chromium, copper, iron, manganese, nickel and zinc, SI Table S2. To compare metal solubility across biofluids and alloys, bioaccessibility values were either normalized to the certified metal mass in the alloy ($\%_{metal}$) in Tables 2 and 3 or relative to the mass of the alloy ($\%_{alloy}$) which can be found in SI (Table S2) and Table 2.

Most alloys are developed specifically to discourage corrosion or to encourage other unique properties. Pure metals or even soils are not sufficiently similar to alloys to be considered as a good proxy for evaluating the bioaccessibility of metals. Although soil SRMs have been used in bioaccessibility tests for comparisons (Hamel et al., 1998), they would not be good surrogates to assess the effects of test parameters for bio-elution of alloys. We selected two distinctly dissimilar SRM alloys to assess different biofluid formulations and residence times.

3.1. Evaluation of in vitro biofluid testing modifications

3.1.1. Surrogate biofluid formulations

Two formulations of gastric biofluids were tested for a 2 h residence time, gastric with and without glycine (NH_2CH_2COOH), Table 2. Ni, Cr and Fe were all slightly higher in the gastric glycine formulation, although there was no statistical difference between the two gastric formulations in stainless steel 304L (NIST 101g). However, Mn and Fe were lower in the gastric glycine formulation both by about a factor of 2 and statistically significant in carbon steel.

Two formulations of lung biofluids were tested: artificial lysosomal fluid (ALF) with glycine (NH_2CH_2COOH) or glycerol ($C_3H_8O_3$). In carbon steel the Cr and Fe were slightly lower in the glycine ALF formulation, while the Mn was about a factor of 10 lower in the glycine ALF. While the bioaccessibility data is limited due to many results being below detection limits (BDL), Ni and Cr do not appear to be especially sensitive to the formulations for the alloys tested. In contrast, the Mn and Fe results suggest that some metals in some alloys could be quite sensitive to these modest formulation

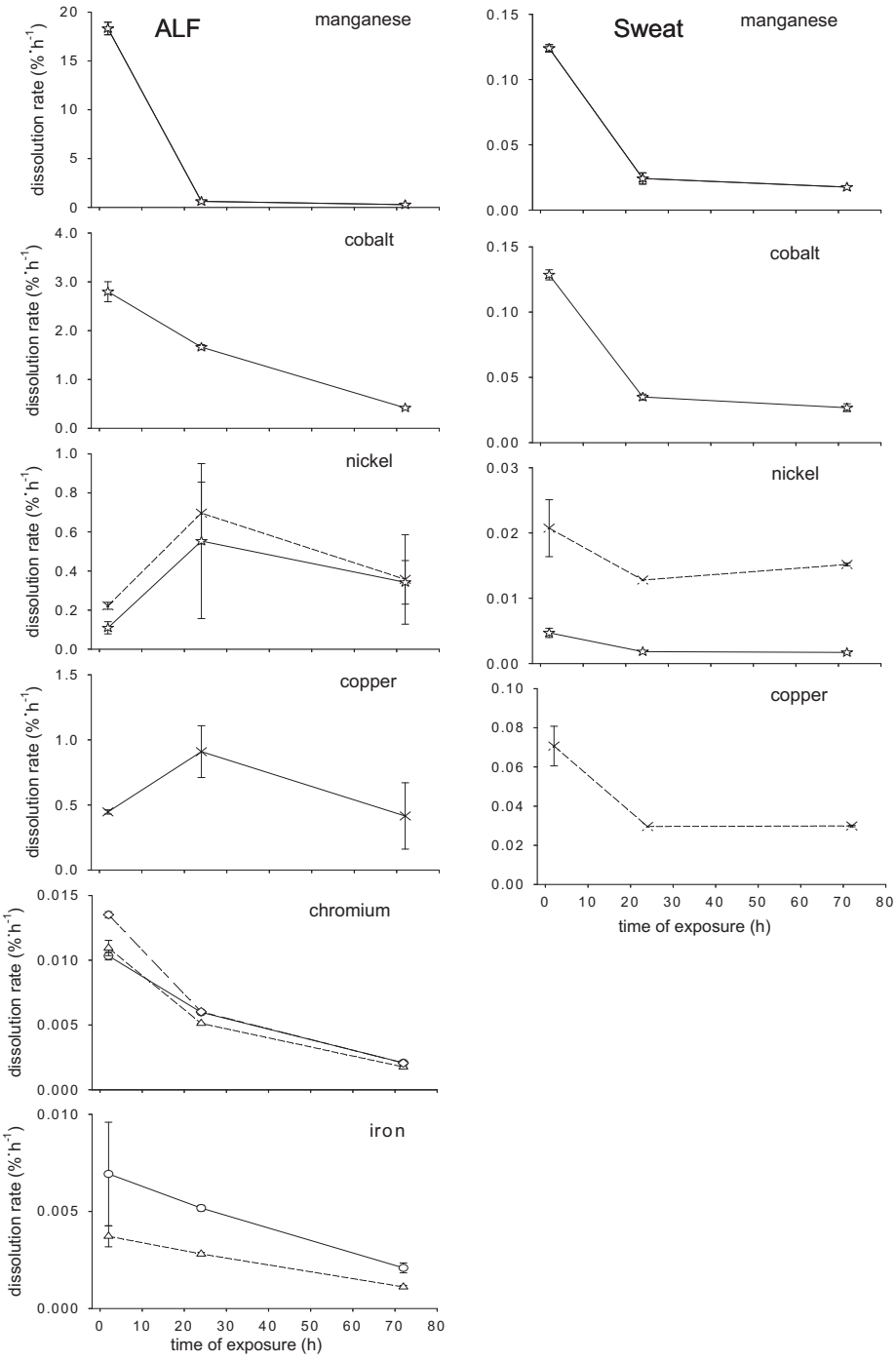


Fig. 1. Metal bioaccessibility dissolution rates per residence time [($\%_{metal}$ [((μg metal in solution/μg in substance]·100)·h^{-1}] for SS316 (○), SS304 (◇), Inconel (△) and Monel (×) and the metal powders (fx1) exposed to ALF (Stopford et al., 2003) and sweat (EN, 2009) surrogate biofluids ($n = 2$ or 3).

differences. Formulation differences are negligible for some metals and alloy reactivity may be comparable between studies using these two gastric biofluids. However, some metals appear to be sensitive to slight variations of biofluid composition.

3.1.2. Residence times

Three extraction times (2, 24, and 72 h) were tested in sweat and ALF (Fig. 1). Two extraction times (2 and 72 h) were tested in the gastric biofluid (Table 2). Large differences between extraction times were observed for gastric biofluid for both alloys. In the carbon steel (NIST14g) we found that Co, Ni and Zn were BDL in the 2 h extracts but were significantly extracted by the 72 h test set. Similarly Co, Mn and Zn were BDL at 2 h in the stainless steel yet significantly extracted at 72 h. The extraction of Mn and Fe increased about 8- and 2-fold respectively from 2 to 72 h. Interestingly, both Cr and Ni increased about 60-fold from 2 to 72 h in the gastric biofluid and Fe increased about 50-fold. Like gastric, we found that several metals in ALF extracts were BDL at 2 h but detected at 72 h, specifically Cu, Ni and Zn in carbon steel and Co, Mn and Zn in stainless steel. We observed a 20-fold increase in Cr, a

Table 3
Metal bioaccessibility[a] as a percentage of the metal in the alloys or elemental metal powders with three biofluids.

Alloy/[b]grade	Biofluid[c]	Cobalt avg ± SD	Chromium avg ± SD	Copper avg ± SD	Iron avg ± SD	Manganese avg ± SD	Nickel avg ± SD
Stainless steel (304)	gastric	<0.00027	0.018 ± 0.002	<0.00028	0.087 ± 0.005	<0.000070	0.10 ± 0.007
	ALF	<0.00027	0.13 ± 0.001	<0.00028	0.080 ± 0.006	0.44 ± 0.009	1.8 ± 0.07
	sweat	<0.00027	<0.00019	<0.00028	<0.00065	<0.000070	<0.00075
Stainless Steel (316)	gastric	na	0.063 ± 0.004	na	0.18 ± 0.01	<0.000070	0.26 ± 0.02
	ALF	na	0.15 ± 0.0005	na	0.15 ± 0.02	4.7 ± 0.6	0.24 ± 0.009
	sweat	na	<0.00019	na	<0.00034	<0.000070	<0.00075
Inconel (Ni–Cr)	gastric	na	0.0523 ± 0.004	<0.00028	0.082 ± 0.004	<0.000070	0.067 ± 0.004
	ALF	na	0.149 ± 0.002	8.2 ± 4	<0.00065	0.73 ± 0.1	0.10 ± 0.06
	sweat	na	<0.00019	<0.00028	<0.00065	<0.000070	<0.00075
Monel (Ni–Cu)	gastric	na	na	3.9 ± 1.0	3.4 ± 0.8	<0.000070	2.8 ± 0.8
	ALF	na	na	30 ± 18	<0.00065	0.13 ± 0.1	26 ± 16
	sweat	na	na	2.2 ± 0.03	<0.00065	<0.000070	1.1 ± 0.02
Co metal	gastric	16 ± 2	na	na	na	na	na
	ALF	30 ± 2	na	na	na	na	na
	sweat	1.9 ± 0.2	na	na	na	na	na
Mn metal	gastric	na	na	na	na	73 ± 7	na
	ALF	na	na	na	na	20 ± 0.2	na
	sweat	na	na	na	na	1.3 ($n = 1$)	na
Ni metal	gastric	na	na	na	na	na	0.95 ± 0.2
	ALF	na	na	na	na	na	25 ± 8
	sweat	na	na	na	na	na	0.12 ± 0.01

na = certified value not available; < = below method detection limit.
[a] ($\%_{metal}$, [μg metal in extract/μg metal in alloy]·100).
[b] American Iron and Steel Institute.
[c] Elimination time: 2 h (gastric), 72 h (ALF, sweat).

90-fold increase in Mn and a 25-fold increase in Fe between 2 and 72 h with ALF in the carbon steel. Unlike gastric and ALF, few metals were extracted at either residence time from the sweat biofluid. As expected the dermal bioaccessibility of many metals was BDL in sweat biofluid at both residence times, including Co, Cr, Cu, Ni and Zn. Mn, however, had a 3-fold increase from 2 to 72 h residence time in sweat.

Different residence times have been used in bioaccessibility studies. Data from bioaccessible gut fluid tests with longer residence times have been used to calculate conservative risk rankings after incorporation in threshold ingestions (Twining et al., 2005). However, use of a gastric residence time longer than 3 h is not physiologically appropriate (Twining et al., 2005). Unlike gastric, lung and sweat multi-day residence times generally are physiologically supported (Chen and Lippmann, 2009). Bioaccessibility tests investigating surrogate lung biofluids have been conducted from 2 to 72 h (Midander et al., 2007b; Stopford et al., 2003; Vitarella et al., 2000) while even longer times are used in some standardized sweat bioaccessibility methods, such as a seven day exposure period (EN, 2009).

The upper limits of bioaccessibility ($\%_{metal}$) may have been reached for several metals at 72 h. In the gastric biofluid Co, Mn and Ni were ≥25% for carbon steel and ≥23% for Co, Cr and Ni in stainless steel (NIST 101g), Table 2. More dramatic than gastric extraction, the upper limits of bioaccessibility for the ALF biofluid may have been reached at ≥74% for Cr, Cu, Mn and Ni for the carbon steel, in contrast to the ALF 72 h residence time extractions for the stainless steel (NIST 101g). The metal bioaccessibility was <1% for all metals tested in stainless steel (NIST 101g) after 72 h in ALF biofluid. These results are consistent with the known physical–chemical properties of the two alloy grades used.

We intentionally chose two distinctly different types of steels to reveal the potential difference the changes in test parameters might have on different alloys. The carbon steel (NIST14g) has poor corrosion resistance while the stainless steel (NIST 101g) is equivalent to a 304 alloy grade which has strong corrosion resistance. This corrosion resistant characteristic is primarily associated with >10% by weight of Cr and Ni. Many alloys dissolve incongruently, meaning the composition of the solid and the dissolved solute do not stoichiometrically match (Herting et al., 2008b; Midander et al., 2007a). Solubilization of metals from alloys is accompanied by alteration of the alloy and possibly formation of a secondary solid phase on the alloy surface consisting of oxides and/or hydroxides. This alteration may take the form of a passive surface film of Cr (III) oxide as a secondary solid phase formed on the surface. With continuous aqueous exposure the secondary oxide solid phase acts as a surface barrier against additional metal dissolution (Hedberg et al., 2010; Herting et al., 2008b; Midander et al., 2007a). Our results are consistent with incongruent solubilization and oxide surface film formation for the high Cr test sample, stainless steel (NIST 101g). The effect of changing residence times had distinctly different effects on the two different alloys. If only corrosion resistant steels are evaluated with various biofluid formulations and test parameters, then only modest differences might be observed. For example, the ALF 2 h and 72 h are only slightly changed for stainless steel, but are vastly different for carbon steel. Residence times had a profound effect on biofluids and test materials evaluated, but these effects depended strongly on the alloy.

3.1.3. Comparison of in vitro surrogate gastric, lung and dermal biofluids

All biofluids used for this study are acidic, ranging from near neutral to highly acidic: sweat (pH 6.5), ALF (pH 4.5), and gastric (pH 1.2). The lowest bioaccessibility was observed for sweat, Tables 2 and 3. Comparing ALF with gastric at 2 h extraction time, we found that Cr was higher in ALF while Mn was lower in ALF in carbon steel alloy. In contrast, at 2 h the stainless steel results suggest potentially higher Cr and Ni in gastric than ALF although due to slight differences in detection limits this is not conclusive. However, comparing the 72 h gastric with ALF we find that ALF had substantially higher bioaccessibility for Cr, Cu, Mn and Ni in carbon steel. In contrast the gastric extracts were higher than ALF extracts from stainless steel for Co, Cr, Mn and Ni. Depending on the alloy the relative bioaccessibility of metals is not simply a function of acidity. While typically more acidic biofluids are associated with greater release of metals from alloys/metals (Midander et al., 2007b; Stopford et al., 2003) and soils (Oomen et al., 2002) we observed high bioaccessibility in ALF for some metals in some

alloys. The ALF is composed of several complexing salts and agents, such as organic acids, that have been shown to weaken covalent bonds in the metal oxide and hydroxide surface layers, resulting in the release of metal–ligand complexes (Hedberg et al., 2010). This is likely associated with increased chemical dissolution of alloys in the ALF solution as compared to the gastric and sweat solutions.

3.2. Evaluation of dissolution behavior of alloys with in vitro lung and dermal surrogates

Dissolution kinetics was investigated in ALF and sweat biofluids for the commercially available alloys to evaluate reactivity (Fig. 1). The rates of dissolution for Mn, Co, Cr and Fe were similar to those reported by other studies of metal alloys exposed to lung and sweat biofluids (Herting et al., 2008a, b; Midander et al., 2007a, 2007b). Maximum early release was followed by fast dissolution quickly approaching steady-state by 24 h. Fast dissolution approaching steady-state in 24 h has been reported in other studies of metal alloys exposed to lung and sweat biofluids (Herting et al., 2008a, b; Midander et al., 2007a, 2007b). Metal release over time may be reduced by the formation of an oxide passive surface layer in corrosive resistant materials as discussed above. Potentially other surface chemistries such as calcium and/or phosphate film(s) on the surfaces of particles exposed to lung biofluids (Midander et al., 2007b) may also influence bioaccessibility reduction. The formation of passive surface layer formation on metal composites has been shown to be time dependent (Chen et al., 1999).

In the ALF extracts Cu and Ni from the metal powders, and Ni from Monel suggest a possible bi-phase dissolution behavior comprised of an initial increase from 2 to 24 h with a slow decline. This could also suggest alloy/metal incongruent solubilization and new oxide, phosphide and/or calcium solid surface formation. We observe different solubilization with time consistent and suggestive of changes in the surface chemistries. Certainly an assessment of multiple time points to attain relative steady-state behavior of different alloy grades is relevant for characterizing exposure over time and appears prudent for a complete human health exposure characterization.

The dissolution results are consistent with the known alloy properties. For example, high corrosion resistant alloys had minimal release of Cr and Fe and were BDL for all other metals tested. In contrast, bioaccessibility rates of Mn, Co, Ni and Cu for the Monel alloy, poor corrosive resistant alloys, and the metal powders were significantly higher. The differences between alloys and dissolution rates are profound. Several orders of magnitude differences in ALF (0.004–16% h^{-1}) and the sweat biofluid (0.02–0.14% h^{-1}) were observed.

3.3. Comparisons of bioaccessibility of metals from nine alloys/metal powders in surrogate biofluids

Tables 2 and 3 summarize the metal bioaccessibility results ($\%_{metal}$) for gastric (2 h), and ALF and sweat (72 h), surrogate biofluids. The corrosion resistant samples, stainless steels 304, 316, Inconel and NIST 101g, had low bioaccessibility of metals for all three biofluids. Many metals were BDL for the corrosion resistant alloys. For instance, Cr, Cu, Fe, Mn, Ni were $<0.3\%_{metal}$ in gastric biofluid. In contrast, in the poor or moderately corrosion resistant alloys and elemental metal powders we found several metals to be significantly more bioaccessible. In the Monel sample Ni, Fe and Cu had 2.77, 3.43 and 3.87$\%_{metal}$ respectively in gastric biofluid. Unlike gastric biofluid extracts, ALF extractions of the corrosion resistant stainless steels (304, 316, Inconel and NIS101g) had low to moderate bioaccessibility. While some ALF extracts were low for corrosive resistant steels, some were notably higher. For example, Cu in Inconel was about 8%, Mn in SS316 was over 4% and finally Ni in SS304 was 1.79% bioaccessible. The ALF extracts of the non-corrosive alloys were quite high for some metals, as previously discussed above for carbon steel. In addition, the Monel Cu and Ni were 30 and 25.7$\%_{metal}$ respectively for ALF extracts. As expected, the elemental metal powders (Co-metal, Mn-metal and Ni-Metal) were found to have very high bioaccessibilities relative to the alloys for all biofluids, Table 3. The $\%_{metal}$ bioaccessibility for any given alloy for a given biofluid ranged widely, Tables 2 and 3. As but one example, Ni in gastric was about 0.067$\%_{metal}$ for the Inconel alloy and 2.77$\%_{metal}$ for the Monel alloy, over a 40-fold difference. Other than the elemental metal powders the sweat biofluids resulted in little bioaccessibility, typically BDL. The exceptions were in the Monel alloy where both Cu and Ni were found to be about 2 and 1$\%_{metal}$, respectively.

The metal alloy bioaccessibility levels overall were lowest in the corrosion resistant SS 304, 316, NIST 101g, and then Inconel. The poor corrosion resistant steels Monel and carbon steel were found to have overall the highest bioavailabilities. The corrosion resistant stainless steel alloys contained >11% chromium composition by mass which provides a protective chromium-rich oxide layer and limits metal release. The carbon steel and Monel alloy have little to no Cr and were characterized with higher bioaccessibility for many metals and biofluids. While the higher Ni content in the Monel compared to the carbon steel provided some protection from solubilization of metals in the biofluids, this effect was not as strong as seen with the Cr-containing alloys. The Monel and carbon steel alloys and the elemental metal powders can be considered more reactive than the other stainless steels tested.

With six alloys and three elemental metal powders tested on average for 72 h residence times, the sweat extraction resulted in the lowest bioaccessibility and the ALF the highest bioaccessibility. Although the gastric was the most acidic of the biofluids tested, it did not result in the highest bioaccessibility because the physiologically-based residence time of 2 h was used. Within the physiologically-based approach the dominant factor in determining bioaccessibility was the reactivity of the individual alloys.

The potential impact of the bioaccessibility test on hazard classification of alloys is important as increasingly greater incidences of cancer and non-cancer health risks are associated with workplace (Gorell et al., 1999) and urban (Willis et al., 2010) exposure from ambient metal generated in industrial metal facilities. The bioaccessibility of metals from the elemental metal powders was much higher than from the metal in the alloys. For example the Co, Ni and Mn elemental metal powders were at least 10-fold higher than the corresponding metal bioaccessibility from any of the alloys, Table 3. These results illustrate and support that alloy grades have unique chemical reactivity that is not adequately explained by their individual metal compositions. For example, the solubility may be over-estimated, as is the case for Mn and Co in the more reactive alloys, or under-estimated, as for Ni in the less reactive alloy grades.

4. Conclusion

These results support testing alloy grades as unique from their metal components to avoid over or under-estimating their health risk to humans. All alloy grades, independent of biofluid, revealed time-dependent release. We demonstrated the value of using distinctly different standardized alloy materials ranging in reactivity and/or corrosion resistance to determine the sensitivity of test parameters within bioaccessibility methods. What may seem like minor changes in biofluid formulations may have significant effects on bioaccessibility. This was demonstrated with Mn in the gastric with and without glycine and the ALF with glycine or

glycerol. The dissolution studies illustrated the profound effects that alloy characteristics and media have on metal dissolution. These studies measured and compared metal solubility in a series of alloys and elemental metal powders in various surrogate biofluids. They yield data that are useful in improving estimates of exposure to these materials.

Financial interest declaration

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2013.10.006.

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Supplementary Information

Table S1. Composition (g/L, unless otherwise indicated) and pH of surrogate biofluids; gastric (Stopford et al., 2003) , GLY-gastric (Drexler and Brattin, 2007); artificial lysosomal fluid (ALF) (Stopford et al., 2003), Glycerol LY-ALF (Thelohan and Demeringo, 1994); sweat (EN, 2009)

Reagents	gastric	GLY-gastric	ALF	GLY-ALF	sweat
HCl	0.07N	0.07N	—	—	—
NaCl	—	—	3.21	3.21	5
NaOH	—	—	6	6	—
Citric acid	—	—	20.8	20.8	—
$CaCl \cdot 2H_2O$	—	—	0.128	0.128	—
$NaHPO_4$ dibasic	—	—	0.071	0.071	—
Na_2SO_4	—	—	0.039	0.039	—
$MgCl_2$ hexahydrate	—	—	0.106	0.106	—
Glycerol also called Glycerine ($C_3H_8O_3$)	—	—	0.059	—	—
Glycine (NH_2CH_2COOH)	—	30.03	—	0.059	
$C_6H_5Na_3O_7 \cdot 2H_2O$ (Na_3 citrate $\cdot 2H_2O$)	—	—	0.077	0.077	—
$C_4H_4O_6Na_2 \cdot 2H_2O$ (Na_2 tartrate $\cdot 2H_2O$)	—	—	0.09	0.09	—
$C_3H_5NaO_3$ (Na lactate, 60% in water)	—	—	0.065 mL	0.065 mL	—
$C_3H_5O_3Na$ (Na pyruvate)	—	—	0.086	0.086	—
Formaldehyde	—	—	1 mL	1 mL	—
Urea	—	—	—	—	1
Lactic acid (90% soln)	—	—	—	—	0.926 mL
pH	1.2	1.2	4.5	4.5	6.5

[a]Also called glycerol ($C_3H_8O_3$). The pH of the biofluid solutions were adjusted to within ± 0.5 pH units of the designated pH using hydrochloric or sodium hydroxide.

Supplementary Information

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

Alloy / [a]grade	Biofluid	Cobalt	Chromium	Copper	Iron	Manganese	Nickel	Zinc
Carbon steel NIST 14g	gastric	<0.0054	<0.0038	<0.0055	186 158 157 170 147	1.0 0.79 0.79 0.89 0.75	<0.015	<0.016
	ALF	<0.0054	2.1 2.0 1.9	0.56 0.71 0.82	1390 1360 1280	9.2 8.9 8.2	0.85 0.84 0.79	<0.016
	sweat	<0.0054	<0.0038	<0.0055	0.93 0.33 0.41	0.15 0.17 0.17	<0.015	<0.016
SS 304L NIST101g	gastric	<0.0054	1.9 1.3 1.1 1.5 0.80	<0.0055	7.6 5.3 4.4 5.8 3.2	<0.0014	1.1 0.78 0.67 0.86 0.46	<0.016
	ALF	<0.0054	<0.0038	<0.0055	<0.013	0.013 0.013 0.023	<0.015	<0.016
	sweat	<0.0054	<0.0038	<0.0055	<0.013	<0.0014	<0.015	<0.016
SS 304	gastric	<0.0054	0.058 0.071 0.063	<0.0055	1.1 1.3 1.2	<0.0014	0.19 0.22 0.20	<0.016
	ALF	<0.0054	0.46 0.45	<0.0055	1.2 1.1	0.013 0.013	4.1 3.9	<0.016
	sweat	<0.0054	<0.0038	<0.0055	<0.013	<0.0014	<0.015	<0.016
SS 316	gastric	<0.0054	0.20 0.22 0.21	<0.0055	2.3 2.6 2.5	<0.0014	0.43 0.50 0.46	<0.016
	ALF	<0.0054	0.50 0.50	<0.0055	2.2 1.9	0.081 0.068	0.54 0.57	<0.016
	sweat	<0.0054	<0.0038	<0.0055	<0.013	<0.0014	<0.015	<0.016
Monel	gastric	<0.0054	0.024	20	0.070	<0.0014	32	0.24

(Ni-Cu)			0.024 0.021	29 19	0.046 0.049		53 31	0.40 0.23
	ALF	5.6 5.4	0.52 0.92	250 98	<0.013	0.014 0.091	502 190	4.4 2.0
	sweat	<0.0054	<0.0038	12 13	<0.013	<0.0014	15 15	0.15 0.16
Inconel (Ni-Cr)	gastric	<0.0054	0.16 0.18 0.16	<0.045	0.15 0.16 0.14	<0.0014	1.0 1.1 1.0	<0.016
	ALF	<0.0054	0.47 0.46	1.1 0.56	<0.013	0.0092 0.011	2.1 0.95	<0.016
	sweat	<0.0054	<0.0038	<0.0055	<0.013	<0.0014	<0.015	<0.016
Co metal	gastric	270 340 330	<0.0038	<0.0055	60 63 66	<0.0014	<0.015	<0.016
	ALF	630 570	<0.0038	<0.0055	<0.013	<0.0014	5.3 5.1	<0.016
	sweat	36 42	<0.0038	<0.0055	<0.013	<0.0014	<0.015	<0.016
Mn metal	gastric	<0.0054	<0.0038	<0.0055	135 135 126	1300 1570 1530	<0.015	<0.016
	ALF	<0.0054	<0.0038	<0.0055	<0.013	400 401	<0.015	<0.016
	sweat	<0.0054	<0.0038	<0.0055	<0.013	25 (n=1)	<0.015	<0.016
Ni metal	gastric	<0.0054	<0.0038	<0.0055	61 61 60	<0.0014	23 14 20	0.16 0.081 0.13
	ALF	<0.0054	<0.0038	<0.0055	<0.013	<0.0014	380 606	16 24
	sweat	<0.0054	<0.0038	<0.0055	<0.013	<0.0014	2.3 2.6	<0.016

[a]American Iron and Steel Institute; residence time: 2h (gastric), 72 hours (ALF, sweat); < = below method detection limit

Supplementary Information

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

Alloy / [a]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
	ALF	<2.7	100 ± 40	350 ± 60	6.7e5 ± 3e4	4400 ± 300	410 ± 18	<8.0
	sweat	<2.7	<1.9	<2.8	280 ± 200	81 ± 5	<7.5	<8.0
SS 304L NIST101g	gastric	<2.7	650 ± 200	<2.8	2600 ± 800	<0.70	390 ± 100	<8.0
	ALF	<2.7	<1.9	<2.8	<6.5	8.1 ± 3	<7.5	<8.0
	sweat	<2.7	<1.9	<2.8	<6.5	<0.70	<7.5	<8.0
SS 304	gastric	<2.7	32 ± 3	<2.8	590 ± 40	<0.70	101 ± 7	<8.0
	ALF	<2.7	230 ± 2	<2.8	560 ± 40	6.6 ± 0.1	<7.5	<8.0
	sweat	<2.7	<1.9	<2.8	<6.5	<0.70	<7.5	<8.0
SS 316	gastric	<2.7	105 ± 7	<2.8	1200 ± 86	<0.70	230 ± 20	<8.0
	ALF	<2.7	250 ± 0.9	<2.8	1000 ± 100	38 ± 5	280 ± 10	<8.0
	sweat	<2.7	<1.9	<2.8	<6.5	<0.70	<7.5	<8.0
Monel (Ni-Cu)	gastric	<2.7	11 ± 0.8	1.1e4 ± 3e3	27 ± 7	<0.70	1.9e4 ± 6e3	150 ± 50
	ALF	2770 ± 71	360 ± 100	8.6e4 ± 5e4	<6.5	26 ± 30	1.7e5 ± 1e5	1600 ± 800
	sweat	<2.7	<1.9	6200 ± 80	<6.5	<0.70	7300 ± 100	79 ± 4
Inconel (Ni-Cr)	gastric	<2.7	83 ± 6	<2.8	74 ± 3	<0.70	505 ± 30	<8.0
	ALF	<2.7	230 ± 3	410 ± 200	<6.5	5.1 ± 0.8	760 ± 400	<8.0
	sweat	<2.7	<1.9	<2.8	<6.5	<0.70	<7.5	<8.0
Co metal	gastric	1.6e5 ± 4e3	<1.9	<2.8	3.2e4 ± 800	<0.70	<7.5	<8.0
	ALF	4210 ± 460	<1.9	<2.8	<6.5	<0.70	<7.5	<8.0
	sweat	1.9e4 ± 2e3	<1.9	<2.8	<6.5	<0.70	<7.5	<8.0
Mn metal	gastric	<2.7	<1.9	<2.8	6.6e4 ± 3e3	7.3e5 ± 1e4	<7.5	<8.0
	ALF	<2.7	<1.9	<2.8	<6.5	2.0e5 ± 2e3	<7.5	<8.0
	sweat	<2.7	<1.9	<2.8	<6.5	13000 (n=1)	<7.5	<8.0
Ni metal	gastric	<2.7	<1.9	<2.8	3e4 ± 300	<0.70	9500 ± 2000	62 ± 20
	ALF	<2.7	<1.9	<2.8	<6.5	<0.70	2.5e5 ± 8e4	9900 ± 3000
	sweat	<2.7	<1.9	<2.8	<6.5	<0.70	1200 ± 100	<8.0

[a]American Iron and Steel Institute; residence time: 2h (gastric), 72 hours (ALF, sweat); < = below method detection limit; µg metal in extract / g alloy material is calculated by multiplying the concentration in the biofluid extract (µg/mL, see table S2) by 50 mL (final extract volume) and dividing by 0.1 g (mass of alloy and metal powder in the extract solution).

Supplementary Information

Table S4. Metal Bioaccessibility as Percentage of Alloy ($\%_{alloy}$, [µg metal in extract / µg alloy] · 100) in biofluids; n = 2 to 5

Alloy / [a]grade	Bio-fluid	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	<0.00027	0.0060 ± 0.0007	<0.00028	15 ± 5	0.042 ± 0.005	<0.00075	<0.00080
	ALF	<0.00027	0.10 ± 0.005	0.035 ± 0.007	67 ± 3	0.44 ± 0.03	0.041 ± 0.002	<0.00080
	sweat	<0.00027	<0.00019	<0.00028	0.028 ± 0.02	0.0081 ± 0.0005	<0.00075	<0.00080
SS 304L NIST101g	gastric	<0.00027	0.0653 ± 0.02	<0.00028	0.26 ± 0.08	<0.000070	0.039 ± 0.01	<0.00080
	ALF	<0.00027	<0.00019	<0.00028	<0.00065	0.00081 ± 0.0003	<0.00075	<0.00080
	sweat	<0.00027	<0.00019	<0.00028	<0.00065	<0.000070	<0.00075	<0.00080
SS 304	gastric	<0.00027	0.0032 ± 0.0003	<0.00028	0.059± 0.004	<0.000071	0.010 ± 0.0007	<0.00080
	ALF	<0.00027	0.023 ± 0.0002	<0.00028	0.056 ± 0.004	0.00066 ± 0.0001	<0.00075	<0.00080
	sweat	<0.00027	<0.00019	<0.00028	<0.00065	<0.000070	<0.00075	<0.00080
SS 316	gastric	<0.00027	0.011 ± 0.0007	<0.00028	0.12 ± 0.009	<0.000070	0.023 ± 0.002	<0.00080
	ALF	<0.00027	0.025 ± 0.001	<0.00028	0.10 ± 0.01	0.0037 ± 0.001	0.028 ± 0.001	<0.00080
	sweat	<0.00027	<0.00019	<0.00028	<0.00065	<0.000070	<0.00075	<0.00080
Monel (Ni-Cu)	gastric	<0.00027	0.0011 ± 0.0001	1.1 ± 0.3	0.0027 ± 0.001	<0.000070	1.9 ± 0.6	0.015 ± 0.005
	ALF	0.28 ± 0.007	0.036 ± 0.01	8.7 ± 5	<0.00065	0.0026 ± 0.003	17 ± 10	0.16 ± 0.08
	sweat	<0.00027	<0.00019	0.62 ± 0.008	<0.00065	<0.000070	0.73 ± 0.01	0.0079 ± 0.0004
Inconel (Ni-Cr)	gastric	<0.00027	0.0083 ± 0.001	<0.00028	0.0074 ± 0.0003	<0.000070	0.051 ± 0.003	<0.00080
	ALF	<0.00027	0.024 ± 0.001	0.041 ± 0.02	<0.00065	0.00051 ± 0.0001	0.076 ± 0.04	<0.00080
	sweat	<0.00027	<0.00019	<0.00028	<0.00065	<0.000070	<0.00075	<0.00080
Co metal	gastric	16 ± 2	<0.00019	<0.00028	3.2 ± 0.1	<0.000070	<0.00075	<0.00080
	ALF	30 ± 2	<0.00019	<0.00028	<0.00065	<0.000070	<0.00075	<0.00080
	sweat	1.9 ± 0.2	<0.00019	<0.00028	<0.00065	<0.000070	<0.00075	<0.00080
Mn metal	gastric	<0.00027	<0.00019	<0.00028	6.6 ± 0.2	73 ± 7	<0.00075	<0.00080
	ALF	<0.00027	<0.00019	<0.00028	<0.00065	20 ± 0.2	<0.00075	<0.00080
	sweat	<0.00027	<0.00019	<0.00028	<0.00065	1.3 (n=1)	<0.00075	<0.00080
Ni metal	gastric	<0.00027	<0.00019	<0.00028	3.0 ± 0.03	<0.000070	0.95 ± 0.2	0.0062 ± 0.002
	ALF	<0.00027	<0.00019	<0.00028	<0.00065	<0.000070	25 ± 8	0.99 ± 0.3
	sweat	<0.00027	<0.00019	<0.00028	<0.00065	<0.000070	0.12 ± 0.01	<0.00080

[a]American Iron and Steel Institute; residence time: 2h (gastric), 72 hours (ALF, sweat); < = below method detection limit; $\%_{alloy}$ calculated by multiplying µg metal in extract / g alloy material (see table S3) by a g to µg conversion factor (1e-6) and converting to percentage

Detection limits ranged from 0.0014 to 0.016 ug/mL in the biofluid extract, from 0.70 to 8.0 ug/g in the metal and from 0.000070 to 0.00080 relative to % metal bioaccessibility; Supplemental Information, Tables S2 to S4.