Assessment of coupled Zn concentration and natural stable isotope analyses of urine as a novel probe of Zn status

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Abstract

Zinc is a common trace metal in the human body, present in about 10% of proteins. Despite numerous roles of Zn in health and disease, there is still a need for a robust biomarker of Zn status. Many parameters have been proposed, with varying levels of success, with plasma Zn often favoured. This study investigates if Zn status can be assessed from the natural stable Zn isotope composition of urine. To this end, 60 urine samples were analysed from ten healthy participants. Remarkably, samples with lower Zn concentrations are systematically enriched in heavy Zn isotopes. Most of the low-Zn urine originated from individuals who omitted dairy, meat or both from their diets. When data for blood serum from age-matched, healthy individuals are compared with the urine results, the former plot at the extension of the urine trend at higher Zn concentrations and lighter isotope compositions. The observed co-variation of Zn isotope compositions with concentrations is indicative of an isotope fractionation system where both properties are controlled by the same processes. It is interpreted as arising from filtration and/or reabsorption processes within the kidney, which are associated with absorbed dietary Zn. The data suggest that the Zn in blood serum that is bound to low molecular weight molecules has an isotope composition distinct from total serum, due to the different affinities of molecular Zn-binding residues to heavy and light Zn isotopes. This technique provides additional information into an individual's Zn status than urine or plasma Zn levels alone.

1. Introduction

After Fe, Zn is the second most abundant transition metal in the human body. As a constituent of about 10% of all proteins, including over 300 enzymes, Zn has important roles in all cells and affects growth, the endocrine and immune systems, the brain and other organs.[1–3] Globally, over a billion people are estimated to take in or be at risk of having too little Zn,[4,5] due to nutrient-limited diets with foods containing phytic acid which binds Zn and other minerals when digested, lowering their absorption.[6] This is particularly concerning for infants, who are put at higher risk of infections and impairment of physical development such as stunting if they have a Zn-poor diet.[7–9]

A sensitive, widely-accepted Zn status indicator, or 'biomarker', for the detection and monitoring of Zn deficiency is therefore important. However, such an indicator has yet to be established,[10] with most proposed parameters falling short at being sufficiently sensitive, as Zn deficiency symptoms can present without current biomarkers flagging a low Zn status. While multiple parameters have been tested,[11] the most widely used are plasma Zn concentrations, followed by urine and hair Zn concentrations.[10,11] There are, however, numerous other suggested molecular biomarkers, including levels of Zn-dependent proteins and proxies such as taste acuity which are not pathognomonic.[2,12–15]

High precision stable isotope ratio measurements for metals in human and animal tissues are increasingly utilised to probe biomedical questions.[16] Isotope fractionation, the mass-dependent change in isotope abundances of an element that partitions between two distinct system reservoirs, is commonly observed for metals in biological systems as biomolecules with different coordination environments display distinct preferences for bonding with the light or heavy isotopes of an element. In particular, the proportion of S (cysteine), O (glutamate and aspartate) and N (histidine) Zn binding sites will determine the Zn isotope compositions of different tissues, as light and heavy isotopes appear to bond preferentially to residues with S and O, N electron donor sites, respectively.[17]

Isotopic measurements of blood Fe were found to represent overall Fe status[18,19] and further analyses have been undertaken to assess how factors such as age,[20] sex,[21] diet[22] and disorders including Wilson's disease,[23] cancers,[24–26] chronic kidney disease,[27] liver cirrhosis[28] and cholestatic liver disease,[29] affect the natural Cu, Fe and Zn isotope compositions of human blood components. These investigations highlight the usefulness of stable isotope analyses for (i) understanding underlying biochemical mechanisms and (ii) the development of potential diagnostic and/or prognostic indicators of diseases. The metal isotope composition of human urine has not been explored to the same extent. Investigations are limited to using (i) Cu stable isotope analysis of dried urine spots on filter paper by laser ablation MC-ICP-MS[30] as a biomarker for Wilson's disease and (ii) the association between the Ca stable isotope composition of urine and changes in bone mineral balance[31] in a new biomarker for osteoporosis.[32] Both cases highlight the potential of isotope-sensitive techniques to improve diagnosis.

The current investigation extends urinary trace metal stable isotope analyses to the element Zn. While blood Zn concentrations are maintained during Zn limitation, renal excretion changes.[33,34] As differences in renal excretion are possibly reflected in the Zn isotope composition of urine, urinary Zn isotope compositions may provide a novel perspective for the study of Zn deficiency dynamics, and the current study explores this potential. To this end, coupled Zn concentration and stable isotope measurements were carried out on 60 urine samples from 10 healthy adult individuals with no known underlying Zn deficiency or excess. The same samples were employed in a previous investigation by Moore et al. (2018)[35] to characterise the range of urinary concentrations of four major and eight minor elements, as well as their temporal and population variability. The new data are employed together with published Zn results for blood samples from age-matched healthy individuals, to evaluate how urinary Zn isotope data can be employed as a novel probe for the Zn status of an individual.

2. Experimental

2.1. Sample collection

Details of the sample collection and storage procedure are given in the companion paper of Moore et al. (2018)[35] , such that only a summary is provided here. Ethical approval for both studies was provided by the Imperial College Research Ethics Committee (ICREC reference 15IC3042).

Participants were recruited via blanket email, whereby the following exclusion criteria were applied: (i) bacterial or viral infection, or any disease at the time of sample collection; (ii) history of kidney or urinary tract infections, or urethral syndrome; (iii) use of any medication or a hormonal intrauterine device; (iv) pregnancy; (v) in day 1 to 8 of the menstrual cycle at time of sample collection; (vi) under 18 years of age. Eligible volunteers were given 50 ml VWR metal-free centrifuge tubes and asked to (i) provide mid-stream samples of urine over one or two nonconsecutive days that were to include the first void of the day; (ii) provide information on age, sex and smoking status; (iii) complete a food diary for each day of sample collection.

The analytical methods were validated using suitable quality control materials. These encompassed both pure Zn solutions, in-house London Zn and IRMM-3702 Zn, as well as Seronorm Trace Elements Urine L-1 and ERM-BB184 Bovine Muscle. The bovine muscle material was taken from a previously digested aliquot.[36]

2.2. Analytical methods

A detailed description of the procedures that were employed for sample digestion, the measurement of Zn concentrations and the determination of Zn contents normalised to the specific gravity (SG) of the urine are provided in the companion study.[35] These procedures are hence only briefly summarized here and the following description focuses on the determination of the Zn isotope compositions. The sample preparation was carried out in an ISO Class 6 metal-free clean room with Class 4 laminar flow hoods at the Imperial College London MAGIC Laboratories.

Distilled 15.3 M HNO_3 and 5.8 M HCl, prepared from VWR AnalaR grade acids, were used throughout together with 18.2 MΩ cm H_2O from a Millipore system and 30%–32% Romil UpA grade H_2O_2.

Urine aliquots of 2 ml were mineralized by microwave digestion using a mixture of 15.3 M HNO_3 and H_2O_2. Aliquots of these solutions were used in the companion study[35] for the elemental analyses and employed here for the determination of Zn isotope compositions and concentrations. To this end, a ^{64}Zn-^{67}Zn double spike solution was added to sample solution aliquots with 50 to 125 ng Zn, such that the mixtures had a ratio of sample- to spike-derived Zn (S/N) of 1 ± 0.1.[36] The mixtures were equilibrated overnight at 130 °C and pure Zn fractions were then separated by anion exchange chromatography[36]. After drying and converting to the nitrate form, the Zn separates were dissolved in 0.1 M HNO_3 to obtain Zn solutions with concentrations of 50 to 100 ng ml^{-1} for isotopic analysis.

The Zn isotope measurements were carried out at the MAGIC Laboratories, using a Nu Plasma HR MC-ICP-MS operated at low mass resolution. A Cetac Aridus II desolvation system fitted with glass nebulisers was employed for sample introduction. At solution uptake rates of ~100 µl min^{-1}, the sensitivity of the instrument was typically ~130 ± 10 V/ppm using Faraday cups with 10^{11}Ω resistors. The Zn isotope compositions are reported as δ^{66}Zn values, which denote the relative difference (in ‰) between the ^{66}Zn/^{64}Zn ratio of a sample and the primary Zn isotope reference material (RM):

$$\delta^{66}\mathrm{Zn} = \left[\left(\frac{(^{66}\mathrm{Zn}/^{64}\mathrm{Zn})_{\mathrm{Sample}}}{(^{66}\mathrm{Zn}/^{64}\mathrm{Zn})_{\mathrm{RM}}}\right) - 1\right] \times 1000 \qquad \text{(Eq. 1)}$$

During the course of the study, solutions of IRMM-3702 Zn, which were doped with the ^{64}Zn-^{67}Zn double spike to obtain S/N ratios and Zn concentrations similar to the sample solutions, were employed as Zn isotope RM. Multiple runs of this standard bracketed the sample measurements to enable close monitoring of any drift in instrumental mass bias.

Following acquisition of the 'raw' isotope data, all further data reduction, including instrumental mass bias correction via the double spike technique,[37,38] corrections for spectral interferences from

Ni^{+} and Ba^{2+}, and calculation of the $\delta^{66}Zn$ values relative to IRMM-3702 Zn, were carried out offline.[38,39] These $\delta^{66}Zn$ results were further corrected for an isotopic offset of +0.30‰ between IRMM-3702 Zn and the JMC Lyon Zn isotope RM, so that the final $\delta^{66}Zn$ data are reported relative to the latter standard.[40] The Zn concentrations of the samples were determined using standard isotope dilution equations from data obtained during the double spike isotope measurements.

3. Results

3.1. Quality control

A full procedural Zn blank was processed along with each batch of samples and this varied between 1 and 3 ng during the course of the study. The blank contributed 0.3 to 1.2% to the total Zn budget of samples and altered $\delta^{66}Zn$ values by less than 0.02‰, assuming a maximum isotopic offset of 1‰. Accordingly, the Zn concentrations of samples were corrected for the corresponding procedural blank, but no corrections were applied to the $\delta^{66}Zn$ results. Zinc yields were typically >90% and any separation chemistry-induced isotope fractionation is corrected for by the prior addition of the Zn double spike and subsequent data deconvolution. The 2SD uncertainties that are quoted for the $\delta^{66}Zn$ results of samples are based on the between-run 2SD repeatability that was obtained for multiple analyses of IRMM-3702 Zn solutions.

Data quality was further assessed by repeated analyses of the secondary pure Zn isotope quality control material, which was analysed with and without processing through the anion exchange chemistry, as well as the two biological RMs. The Zn isotope data for these materials are in accord with previously published results (Table S1). The Zn concentrations determined for ERM-BB184 Bovine Muscle are also identical to the reference values whilst the results for Trace Elements Urine L-1 are about 10% lower than the reference data but identical, within error, to results previously acquired in the same laboratory using the current procedure.[36]

3.2. Participants and samples

Urine samples were obtained from ten volunteers, six women and four men. The participants, with ages of between 23 and 33 years, lived and worked in southeast England. Of these ten, six gave urine samples on two nonconsecutive days and four gave samples on only one day. The samples are representative of three broad diet groups: omnivorous (all foods), no-meat, which includes vegetarian, pescatarian and vegan diets, and no-dairy, which encompasses vegan and dairy-free but otherwise omnivorous diets.

The companion study of Moore et al. (2018) reported both 'raw' and SG normalised concentration data for major (Na, K, Mg, Ca) and trace (Co, Cu, Zn, As, Rb, Sr, Mo, Pb) elements of 65 urine samples from the 10 participants. Coupled Zn concentration and isotope data are available for 60 of these samples (Table S2).

3.3. Zn concentrations and isotope compositions

The samples have highly variable Zn concentrations, with SG normalised data that range from 44 to 1498 ng ml^{-1} and a mean result of 341 ± 261 ng ml^{-1} (± 1SD). Notably, the mean, geometric mean, median and 95th percentile data are in good agreement with recent reference values from studies conducted in the UK, Germany, Belgium and Canada.[35] Further focusing on the SG-normalised Zn concentrations, the overall variability of the individual results have a coefficient of variation (CV) that is larger at 82% than the variability defined by the mean results for the 10 individuals (CV = 56%). This reflects that the urinary Zn concentrations of the participants also show significant differences over time with mean CV values of 43% and 50% for urine collected within a day and two non-consecutive days, respectively (Table 1).

The δ^{66}Zn values of the urine samples fall between 0.11‰ and 1.42‰, with mean and median results of 0.60‰ ± 0.26‰ (± 1SD) and 0.60‰, respectively (Table 1). No published Zn data are available from previous studies for comparison. The δ^{66}Zn values of urine samples (n = 3 to 9) from each participant vary by an average of 0.13‰ (1 SD) when the data from Participant #3 are omitted,

which are almost as variable (with results from 0.46‰ to 1.42‰) as the complete dataset. This reflects urinary Zn isotope variability for the participants over time, which is ±0.11‰ to ±0.12‰ for urine collected within a day and two non-consecutive days (Table 1). In comparison, the standard deviation of the mean δ^{66}Zn values for each of the ten participants is larger at ±0.24‰ (1 SD) (Table 1, Fig 1), indicating variations between individuals are greater than temporal variations.

A z-score was employed to assess how representative the first void urine sample is for the daily mean urinary Zn concentration and isotope composition of an individual. The z-score was calculated as $z = (x - \bar{x})/SD$, where x is the first void result, whilst $\bar{x}$ and SD are the mean result and standard deviation for the same day. For both the Zn concentrations and isotope compositions, the 10 participants returned mean z-scores of 1.0, corroborating the previous observation that temporal variability is smaller than differences between individuals. This confirms that spot urine samples provide a reasonable approximation of daily mean urinary Zn isotope compositions and concentrations for an individual.

3.4. Correlations

In a diagram of Zn isotope compositions versus Zn concentration, the urine data display a broad but clear trend of increasing δ^{66}Zn with decreasing Zn content (Fig. 2), which is apparent regardless of whether raw measured or SG normalised Zn concentrations are plotted (Fig. S1). Also of note is that female urine tends to have lower Zn concentrations and heavier isotope compositions than that of males (Fig. S1). A similar grouping is seen when the diet groups of the participants are considered, whereby the urine from participants with no-dairy and no-meat diets generally have lower Zn contents and heavier Zn isotope compositions. The observation that variations in the urinary Zn data show apparent links to both sex and diet is not unexpected, as the study participants harbour a correlation between diet groups and sex, whereby most omnivores are male whilst most no-meat and no-dairy participants are female (Table S3).

The observed systematics are further confirmed by statistical analyses of the results. In detail, there is a significant difference in urinary Zn concentrations between sexes, whereby the higher urinary Zn contents of females are documented by *p* values of 0.01 and 0.001 for the raw and SG-normalised Zn data, respectively (Table 2). Similarly, the heavier Zn isotope composition of urine from female participants is significant with a *p* value of 5×10^{-5} (Table 2).

Considering the results grouped according to diet, samples from participants with omnivorous diets generally have higher mean Zn concentrations than those from participants on no-meat and particularly no-dairy diets (Table 2). The no-dairy Zn concentrations are statistically different from urine of omnivorous participants with a *p* value of 0.01 (Table 2). A clearer difference between diets can be seen in the δ^{66}Zn results. In detail, the majority of urine with heavier Zn isotope compositions are from individuals following no-meat and no-dairy diets (Fig. 3 and 4A, Table 2). The urine samples from omnivorous participants thereby have a lower mean δ^{66}Zn value (0.49‰) compared to those from participants on no-meat (0.79‰) and no-dairy (0.69‰) diets, whereby these differences are significant with *p* values of 0.001 and 0.01, respectively (Table 2).

4. Discussion

In the following discussion, the inferences of the data are interrogated, incorporating previously published natural Zn isotope data from blood and current understanding of human Zn metabolism.

4.1. Comparison of urinary Zn data with results for blood and blood components

As urine is produced as a filtrate from blood, it is useful to compare the Zn results obtained for urine with previously published data for whole blood, red blood cells (RBCs) and blood serum. Although blood plasma and serum are different due to plasma containing clotting factors, both are discussed together in the following, since they share a common purpose in studies of Zn homeostasis. A number of previous investigations have determined healthy ranges for the Zn

concentrations of whole blood and its components. In detail, such work shows that adult whole blood, RBCs and serum/plasma typically have higher Zn concentrations than urine, ranging from ~5000 to 7000 ng ml^{-1}, ~7500 to 16000 ng ml^{-1} and ~700 to 1200 ng ml^{-1}, respectively.[21,41–44]

Whilst Zn isotope results are scarcer than Zn concentrations for blood, sufficient data are available to establish some clear systematics. Of particular interest are results that enable a robust comparison with the present urine data. Employed to this end are coupled Zn isotope and concentration data for whole blood, RBCs and serum from a recent study of 47 healthy volunteers with ages of 19 to 35 years from France and additional $\delta^{66}Zn$ results from 12 healthy Belgians adults with ages of 21 to 30.[21,22] The Zn concentrations of whole blood, RBCs and serum are significantly less variable than the Zn contents of urine, with CV values of about 15% to 20% (Table 3). Whilst temporal changes cannot be assessed, it was previously suggested that Zn in serum and plasma of people with healthy Zn homeostasis does not vary as a direct impact of daily diet fluctuation, though diurnal variations have been reported.[41,45,46]

Focussing on the Zn isotope compositions, it is notable that the overall range of urinary $\delta^{66}Zn$ is 2 to 2.5× larger than that reported for age-matched 'healthy' whole blood, RBCs and serum (Table 3). It is furthermore apparent that whole blood and RBCs have similar $\delta^{66}Zn$ values with nearly identical means of about 0.45‰, which are slightly but significantly higher than the blood serum mean of 0.17‰ (Table 3, Figure 3). In contrast, urine has a heavier mean Zn isotope composition of $\delta^{66}Zn$ = 0.60‰ (Table 1). The difference in $\delta^{66}Zn$ between RBCs and serum thereby most likely reflects the distinct Zn bonding environments of the two reservoirs.

4.2. Origin of the $\delta^{66}Zn$ versus Zn concentration trend

In the following, the origin of the trend seen in Fig. 2, whereby samples with lower Zn concentrations are increasingly enriched in heavy Zn isotopes is discussed, along with its relationship to blood serum data. Notably, the trend is clearly present regardless of whether the raw or the SG-normalised urinary Zn concentrations are considered (Fig. S1). Furthermore, it is

noteworthy that the large variation in δ^{66}Zn values is mostly due to differences between individuals, rather than temporal differences (Table 1, Fig. 1) and such a significant variation is not present in whole blood, RBCs or serum (Fig. 3, Table 3).

Although urine from female participants typically features higher δ^{66}Zn values and lower Zn contents than samples from males, this requires further evaluation with additional measurements from a larger cohort. However, if the trend of Fig. 2 is indeed related to sex, the sex-related differences in urinary Zn systematics would have to originate from blood serum. While male whole blood has been found to contain slightly more Zn than female,[42,47] and there are differences in Zn requirements between the sexes, previous studies revealed no sex-related differences in the Zn concentrations and isotope compositions of blood serum or plasma (Fig. 4B, Table 3).[21,22,41]

An alternative to consider is that the urinary δ^{66}Zn – Zn concentration trend is a direct consequence of the dietary choices of the participants. This interpretation follows from the observation that urine with the highest Zn concentrations and lowest δ^{66}Zn values is primarily from omnivores, whilst urine from individuals on no-meat and no-dairy diets have less Zn and heavier Zn isotope compositions (Fig. 2, Table 2, S3). Support for this conclusion is provided by previous studies, which revealed that the Zn concentrations of blood serum and urine are impacted by the dietary uptake of Zn.[48] In particular, it was shown that the phytic acid contained in many vegetables, grains and cereals inhibits uptake of Zn in the intestines and consumption of such foods has been linked with lower blood serum and urine Zn levels,[49,50] including in the companion study of Moore et al. (2018).[35]

Zinc isotope data indicate, however, that the trend seen in Fig. 2 is not directly linked to differences in dietary Zn uptake. If this interpretation were correct, one would expect that urinary Zn isotope variations are also apparent in the results for blood serum. Whilst serum Zn isotope data for different diets are currently not available, the δ^{66}Zn values of whole blood from those on vegetarian diets do appear to be higher compared to those of omnivores, even though the dataset is small (Table 3).[22] The difference in whole blood Zn isotope compositions between the diet groups

is, however, much smaller compared to that seen for urine. Whilst this observation may, in part, reflect the dominant effect of RBCs on the Zn isotope composition of whole blood, the Zn isotope data available for blood serum of young, healthy adults with unknown diets is insufficiently variable to explain the large spread of urinary δ^{66}Zn. This conclusion is supported by Zn concentrations, which are also much less variable in blood serum compared to urine samples, with CVs of 19% and 82% respectively (Tables 2 and 3).

As such, the available data rule out that the urinary δ^{66}Zn – Zn concentration trend is *directly* linked to dietary Zn uptake. In fact, the much lower variability of both Zn isotope compositions and concentrations in serum compared to urine also argue against an origin of the trend in other relevant mechanisms of Zn processing in the human body, such as transfer of Zn from the intestines to blood serum or utilisation of Zn in the liver and RBCs. Considering this, the Zn correlation of Fig. 2 is, in all likelihood, a consequence of processes that occur during the handling of Zn within the kidneys and its excretion with urine.

4.3. Zn isotope fractionation in the kidneys

Within the kidneys there are two viable processes which are most likely to be responsible for the Zn trend of Fig. 2: glomerular filtration of the blood or reabsorption of Zn from the initial glomerular filtrate. These mechanisms are discussed in the following section. Whilst active secretion is a third viable mechanism and is known to impact metals such as K,[51] there is currently no evidence that active secretion of Zn follows reabsorption.

4.3.1. Zn isotope fractionation as a result of glomerular filtration

It is conceivable that the trend of Fig. 2 is generated during the initial blood filtration process whereby the glomerular filtrate is produced. This mechanism is feasible if there is a significant isotopic difference between the Zn bound to low molecular weight molecules (LMWMs) and high molecular weight molecules (HMWMs) in blood. In addition, it is reasonable to assume that the

urinary δ^{66}Zn values represent only Zn bound to LMWMs in the blood, because the glomerular filtration barrier will not allow HMWMs to pass. If the HMWM fraction is, furthermore, enriched in isotopically light Zn and the mass balance of Zn in the two fractions changes, the filtration process can generate correlated variations of increasing δ^{66}Zn with decreasing urinary Zn content.

The proposed scenario is not unrealistic given current understanding of Zn speciation in blood. A number of previous studies have shown that the majority of serum Zn in healthy people is reversibly bound to a mixture of O- and N- ligands in albumin (~60% to 70%) whereas about 30% are strongly bound to the thioester residues of α-2-Macroglobulin (α2M).[52,53] Due to their large molecular size, only a small fraction (of ~1% or less) of albumin and essentially no α2M pass into the ultrafiltrate in a healthy kidney.[54] The remaining serum Zn, which is often referred to as "free" or "labile" Zn,[55] is most likely bound largely to amino acids and other LMWMs.[56,57]

As Zn bound to S-containing cysteine residues in proteins is typically isotopically light, the strong bonding of Zn to α2M may well generate the inferred difference in Zn isotope composition between HMWM and LMWM, whereby the main Zn-binding residues of the latter pool are not clearly defined. This is in accord with the suggestion that Zn bonding to α2M may be responsible for the difference in Zn isotope composition between serum and RBCs (Fig. 3).[58]

4.3.2. Zn isotope fractionation during reabsorption from the glomerular filtrate

Given the mass balance of urinary Zn excretion compared to the standing Zn pool of blood, which is filtered 25 times per day, it follows that a large proportion of the Zn that passes into the glomerular filtrate is subsequently reabsorbed into the bloodstream. While recent studies have estimated that around 80% of filtered Zn may be reabsorbed into blood,[59] the mechanism is poorly understood.[60] Information available on the reabsorption of LMW proteins, for which the cumulative Zn binding capacity is unknown, indicate much lower estimates of around 10%.[54] Although the extent is uncertain, the reabsorption of Zn in the kidneys from the glomerular filtrate will likely be up-regulated when Zn availability is low, further reducing the amount of Zn in urine.[61] If reabsorption

is to be responsible for higher δ^{66}Zn values of urine by isotopic fractionation, this requires a mechanism associated with preferential reabsorption of light Zn isotopes from the glomerular filtrate. However, this conclusion is not in accord with current understanding of Zn transporters, including those found in kidneys.[62] This reflects that the Zn-binding residues in the transport sites of ZIP and ZnT transporters have N- and O-donor atoms in their coordination environments.[63–65] As these residues preferentially bond with heavy Zn isotopes, the δ^{66}Zn values of urine are expected to be lower with up-regulated reabsorption.

4.4. Modelling of Zn isotope fractionation in the kidneys

Isotope fractionation curves can be constructed that predict the relationship of urinary $^{66}Zn/^{64}Zn$ isotope ratios with Zn concentrations as a result of Zn processing in the kidneys by a mechanism characterised by an isotope fractionation factor α. For closed system Rayleigh fractionation, the relationship is given by

$$R = R_0 \times f^{\alpha-1} \qquad \text{(Eq. 2)}$$

where *f* is the fraction of Zn that passes to the urine after processing (and which can vary from 1 to 0), R is the $^{66}Zn/^{64}Zn$ isotope ratio of the urine, whilst R_0 represents the initial or bulk $^{66}Zn/^{64}Zn$ ratio of the system. For open system steady state isotope fractionation, the δ^{66}Zn – Zn concentration relationship can be determined by:

$$R = {R_0}\Big/{\big(f + \alpha(1-f)\big)} \qquad \text{(Eq. 3)}$$

If glomerular filtration is the dominant process responsible for the δ^{66}Zn of urine, depending on whether the isotopic difference between the Zn bound to the HMWM and LMWM is reversible on a fast or slow time scale or essentially irreversible, the isotopic fractionation between the pools may define a closed system Rayleigh or an open system steady state isotope fractionation trend, or an intermediate relationship.

Shown in Fig. 5 are calculated closed and open system isotope fractionation lines that are able to encompass the variable Zn concentrations and isotope compositions of the urine samples, whereby the defining parameters for the models are summarized in Table 4. It is, however, arguable whether the given fractionation equations are rigorously applicable to the scenarios that are considered here. Problematic in particular are that (i) the Zn concentrations of urine are not solely determined by the processes responsible for generating the observed Zn isotope fractionation but also by the variable dilution in urine; and (ii) the initial or bulk Zn concentration of the system $[Zn]_0$ is not clearly defined and likely differs within and between individuals. Given this, it is unsurprising that the urinary Zn data do not fall on a single, well-defined fractionation trend in Fig. 5. As such, the fractionation models of Fig. 5 and Table 4 should not be taken as providing robust estimates for system parameters that are of interest, such as the isotope fractionation factor.

However, the fractionation models do suggest that the observed broad but clear relationship between $\delta^{66}Zn$ and Zn concentrations is most likely produced by a process (or processes) that affect(s) both properties in a coupled manner and which takes place within the kidneys. Also notable is that the initial Zn isotope composition of the system is relatively well constrained as this is likely to be essentially identical to the $\delta^{66}Zn$ value of blood serum and such values are hence employed in the modelling of Fig. 5 (Table 4).

4.5. Assessment of urinary Zn isotope compositions as a potential indicator for Zn status

In summary, the analytical results indicate that urinary Zn concentrations and, in particular, $\delta^{66}Zn$ values may vary in response to the size of the serum Zn pool bound to LMWMs relative to the total amount of serum Zn. This is an important finding, which supports the hypothesis that the serum Zn pool bound to LMWMs is dynamic,[66] whereby its proportion appears to vary relative to total serum Zn as a consequence of homeostatic regulation to accommodate changes in Zn absorption and body requirements.[67,68] In particular, it can be hypothesised that the LMWM Zn pool is reduced when overall Zn availability is limited. Furthermore, it was suggested that omnivores have higher

406 homocysteine levels in plasma compared to those on meat-free diets[69] and, as a consequence,
407 individuals on different diets may have different proportions of Zn-binding residues in the LMWM
408 Zn pool. Whilst such diet-dependent changes in Zn speciation are unlikely to be primarily
409 responsible for the large changes that can be inferred for the proportion and δ^{66}Zn value of the
410 LMWM serum Zn pool, such a mechanism may well strengthen the observed urinary isotope
411 fractionation trend (Fig. 5). To corroborate the findings of this study, it will be useful for future
412 studies to investigate the inferred difference in Zn isotope composition between the LMWM pool of
413 blood and bulk blood serum, using a suitable separation technique.

414 A number of previous studies have suggested urinary Zn levels or plasma Zn concentrations as
415 suitable indicators of Zn status. This conclusion has been questioned repeatedly, however, in
416 particular because plasma Zn levels are under tight homeostatic control and as urinary Zn contents
417 are also controlled by variable dilution, which may be difficult to correct accurately in some
418 cases.[70,71] In contrast, unlike Zn concentration, the isotopic offset between the δ^{66}Zn values of urine
419 and serum is solely linked to the mass balance of total Zn in serum and the Zn pool that is removed
420 from the body by renal excretion. As such, Zn isotope data used in conjunction with normalised
421 urinary Zn concentrations are likely to be a more robust indictor of Zn status than urinary Zn contents
422 alone. In support of this are the statistical differences between the Zn isotope compositions of
423 individuals that follow different diets, which are more readily resolvable than those seen in the Zn
424 concentrations (Table 2).

425 The isotope fractionation models suggest that changes in Zn status may be best characterised by
426 the δ^{66}Zn offset between blood serum and urine, as this should be small when the LMWM Zn pool
427 is large. The finding that low urinary Zn concentrations coupled with high δ^{66}Zn values may be a
428 reliable indicator of when the overall Zn status of the body is under pressure to conserve Zn deserves
429 further investigation. A study with larger sample numbers is desirable to robustly test this
430 hypothesis, and this should ideally encompass Zn isotope composition and concentration analyses
431 of urine and serum samples from the same person. In detail, cohorts that should be investigated are

individuals with normal and low Zn status, as based on dietary Zn uptake, and those with Zn deficiency symptoms and/or disorders directly associated with Zn-dyshomeostasis, such as acrodermatitis enteropathica[72]. Another important variable to consider is age and analyses are desirable for individuals with age-related changes in glomerular filtration rate, post-menopausal women who may have altered Zn homeostasis due to declining oestrogen and α2M levels,[73] as well as infants and young children, as these are most at risk from Zn deficiency. Additional considerations are how the δ^{66}Zn values of urine will be impacted by compromised glomerular filtration barriers and kidney disease or other conditions that affect the renal handling of trace metals, such as diabetes.[74–76]

5. Conclusions

Based on Zn isotope analysis of 60 urine samples from apparently healthy people, urine emerges as a promising medium to investigate Zn status. The difference between the isotope composition of serum and urine most likely reflects that Zn bound to LMWMs may have higher δ^{66}Zn values than the Zn associated with HMWMs in serum. The Zn isotope fractionation seen in urine is likely expressed during glomerular filtration, as this allows essentially only LMWMs to pass. Although it cannot be ruled out, no evidence has been found to indicate any role of Zn reabsorption or active secretion within the kidneys in inducing further isotope fractionation, although the former is likely to affect the Zn concentration of the urine.

Although the Zn isotope compositions of blood and blood serum are significantly less variable compared to urine, paired serum and urine Zn isotope analyses will be valuable for understanding the partitioning of Zn between HMWMs and LMWMs at different Zn status. Such coupled urine and serum analyses may also better constrain the overall Zn status of individuals, as it encapsulates both LMWM and HMWM Zn pools, which would further reduce uncertainty caused by temporal variations.

A further benefit of this investigative technique is that specialised storage or preservation methods that keep the internal components of a sample structurally intact are not required for isotope analyses that involve complete digestion of tissues by mineral acids. The continued development of technologies that allow greater sample throughput[77–79] will, furthermore, enable more exhaustive biomedical investigations and increase the potential for routine use of metal stable isotope analysis for clinical and medical purposes.

Conflicts of interest

There are no conflicts to declare.

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Table 1: Zn concentrations and isotope compositions of the urine samples

	Zn_{Raw}	$Zn_{SG\text{-}norm}$	$\delta^{66}Zn$
	ng ml^{-1}	ng ml^{-1}	‰
Overall variation			
Range	16 - 2060	44 - 1498	0.11 - 1.42
Mean	282	321	0.60
Median	160	275	0.60
SD	375	264	0.26
CV (%)	133	82	
Variability between participants using individual mean results			
Mean	265	322	0.62
SD	231	180	0.24
CV (%)	87	56	
Variability for individuals over 1 or 2 non-consecutive days*			
Daily SD	193	136	0.11
Daily CV (%)	66	43	
2 day SD	177	178	0.12
2 day CV (%)	52	50	
Comparison of first-void concentrations to daily means			
z score	1.1	1.0	1.0

The mean, median, SD and CV values were calculated based on 60 individual samples (not the individual participant means), *Data from 6 individuals who provided samples on two non-consecutive days.

Table 2: Evaluation of effect of sex and diet on Zn concentrations and isotope compositions of healthy human urine

	Zn_{Raw}	$Zn_{SG\text{-}norm}$	$\delta^{66}Zn$
	ng ml^{-1}	ng ml^{-1}	‰
Female (n = 36)			
Range	16 - 569	44 - 657	0.34 - 1.42
Mean	182	228	0.70
Median	129	232	0.66
SD	152	149	0.26
CV (%)	84	65	
Male (n = 24)			
Range	64 - 2060	160 - 1498	0.11 - 0.79
Mean	535	459	0.44
Median	201	325	0.44
SD	433	335	0.17
CV (%)	81	73	
p (vs. female)	0.01	0.001	0.00005
Omnivorous (n = 34)			
Range	26 - 2060	44 - 1498	0.11 - 1.13
Mean	394	401	0.49
Median	264	309	0.44
SD	462	307	0.21
CV (%)	117	77	
No-meat (n = 18)			
Range	16 - 384	66 - 566	0.42 - 1.42
Mean	134	247	0.79
Median	368	513	0.98
SD	107	158	0.27
CV (%)	80	64	
p (*vs. omv*)	0.05	0.17	0.001
No-dairy (n = 11)			
Range	31 - 350	52 - 241	0.42 -1.11
Mean	112	124	0.69
Median	68	102	0.64
SD	96	66	0.19
CV (%)	86	53	
p (*vs. omv*)	0.05	0.01	0.01

Table 3: Zn concentrations and isotope compositions of blood samples

	Zn ng ml-1	**δ^{66}Zn** ‰	**Zn** ng ml^{-1}	**δ^{66}Zn** ‰	**Zn** ng ml-1	**δ^{66}Zn** ‰
	Whole Blood[21,22]		**Red Blood Cells[21]**		**Blood Serum[21]**	
Overall						
n	59		47		46	
Range	3608 - 7778	0.21 - 0.63	5539 - 15213	0.17 - 0.74*	1004 - 2352	-0.03 - 0.31
Mean	5855	0.43	10263	0.46	1369	0.17
Median	5730	0.43	10048	0.45	1341	0.15
SD	873	0.10	2039	0.16	266	0.07
CV (%)	15		20		19	
Female						
n	31		25		25	
Range	4689 - 7778	0.25 - 0.61	5539 - 15213	0.29 - 0.61	1004 - 2352	0.09 - 0.31
Mean	5997	0.43	10917	0.46	1304	0.17
Median	5826	0.43	10815	0.46	1392	0.15
SD	850	0.08	2329	0.09	165	0.09
CV (%)	14		21		13	
Male						
n	28		22		21	
Range	3608 - 7181	0.21 - 0.63	6961 - 12572	0.17 - 0.74*	1034 - 1530	-0.03 - 0.30
Mean	5694	0.42	9519	0.46	1447	0.16
Median	5673	0.43	9428	0.44	1293	0.16
SD	891	0.10	1347	0.22	340	0.05
CV (%)	16		14		23	
p (vs. female)	0.2	0.7	0.02	0.4	0.07	0.4
Omnivorous**						
n		6				
Range		0.34 - 0.56				
Mean		0.44				
Median		0.44				
SD		0.07				
No-Meat**						
n		6				
Range		0.51 - 0.61				
Mean		0.56				
Median		0.56				
SD		0.04				
p (vs. omv)		0.006				

*One outlier of 1.25‰ not included in range. **Only Zn isotope compositions available from diet study.[22]

Table 4: Parameters used for modelling of Zn results in urine and blood serum

	Model 1	**Model 2**	**Model 3**	**Model 4**	**Model 5**
Fractionation system	Closed	Closed	Closed	Open	Open
Fractionation factor (α)	0.99999	0.99998	0.99996	0.99998	0.99990
Initial $[Zn]_{SG\text{-}norm}$ (ng ml^{-1})	1000	1000	1000	800	800
Initial $\delta^{66}Zn$ value (‰)	0.20	0.25	0.30	0.20	0.30

Figure Captions for:

Assessment of coupled Zn concentration and natural stable isotope analyses of urine as a novel probe of Zn status

Rebekah E. T. Moore*, Mark Rehkämper, Wolfgang Maret, Fiona Larner

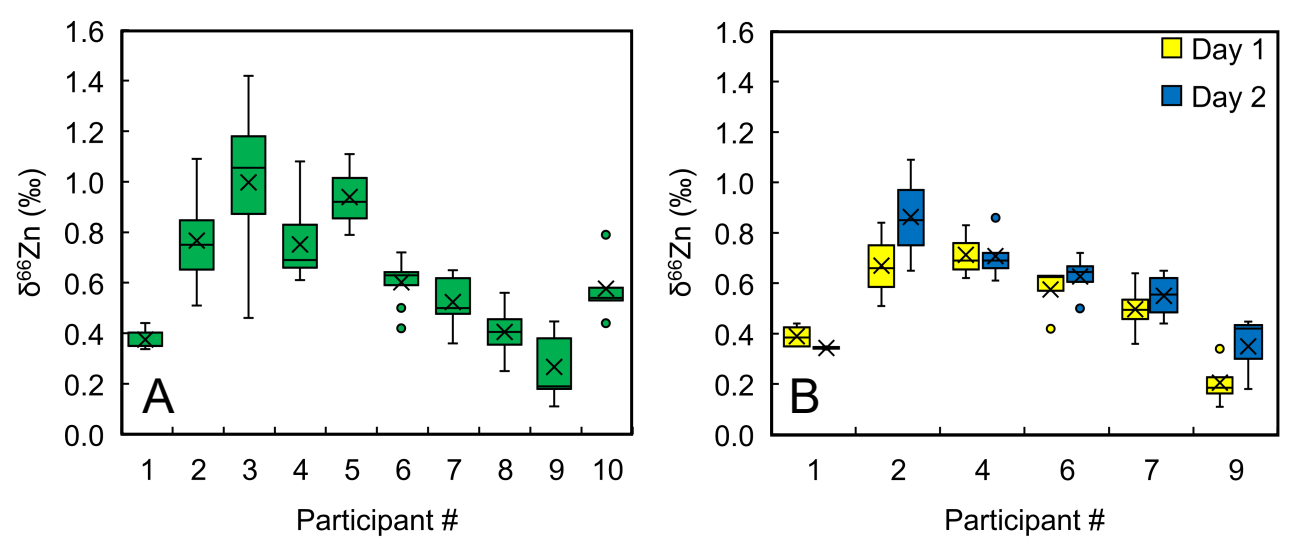
Figure 1: Zinc isotope composition of urine from: (A) all 10 participants, where each participant gave samples over 1 or 2 non-consecutive days (n = 3-10), and (B) the 6 participants who gave samples for 2 non-consecutive days (n = 2-5 per day). Boxes: interquartile ranges, whiskers: first and fourth quartiles, points: outliers, lines within boxes: medians, crosses: means.

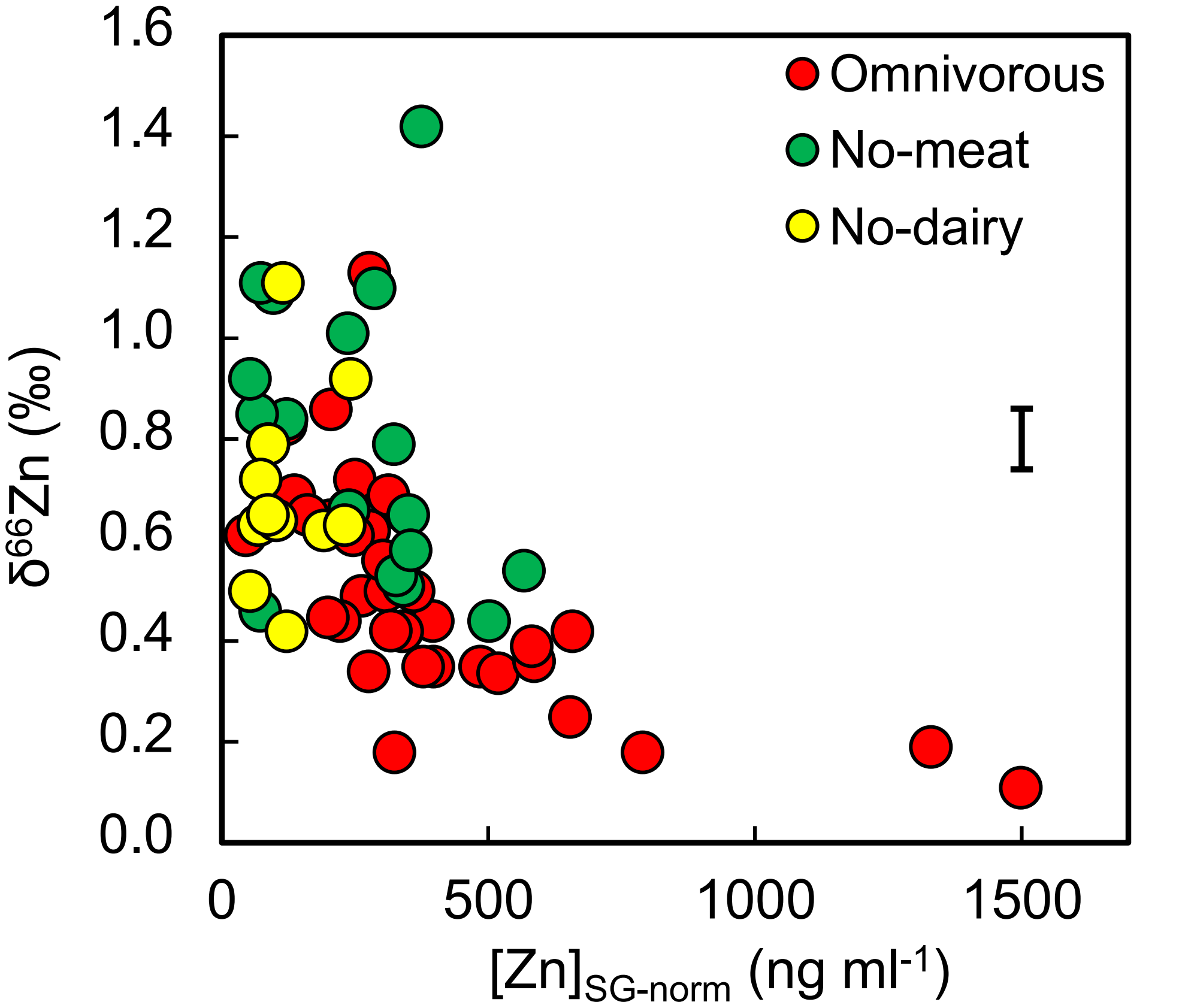
Figure 2: Plot of specific gravity normalised Zn concentrations versus and Zn isotope compositions for urine samples with comparison of diet groups. The error bar represents the average external reproducibility (2 SD) of the δ^{66}Zn data.

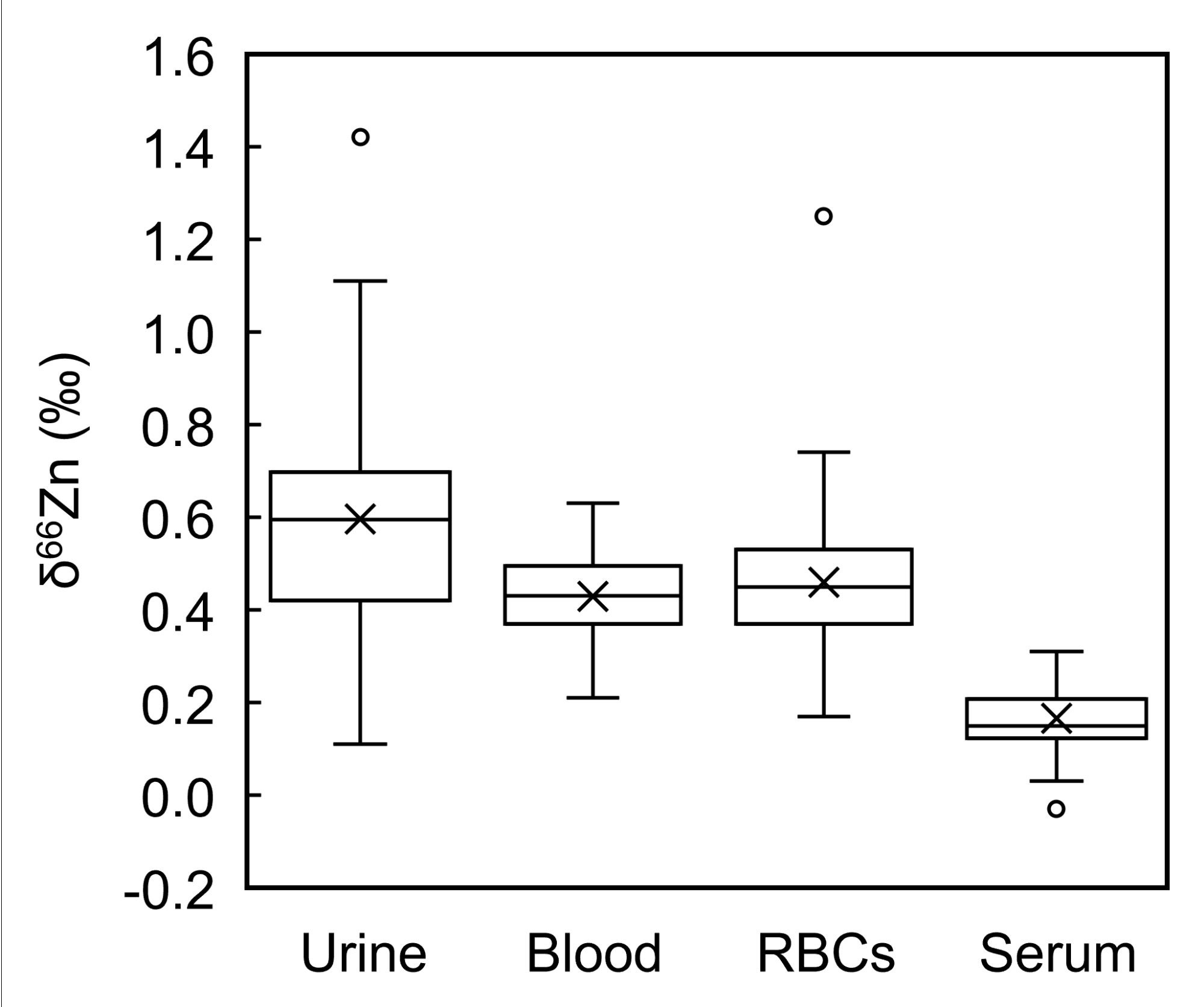
Figure 3: Zinc isotope compositions of urine (n = 60) compared to blood serum (n = 46),[21] whole blood (n = 59)[21,22] and red blood cells (RBCs) (n = 47).[21] Boxes: interquartile ranges, whiskers: first and fourth quartiles, points: outliers, lines within boxes: medians, crosses: means.

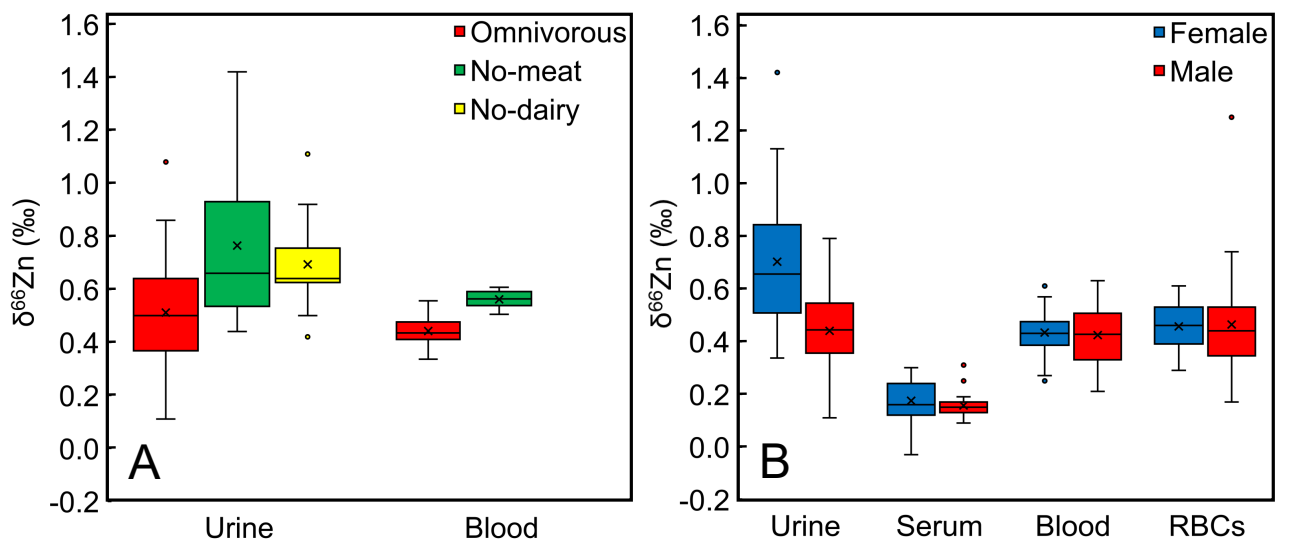
Figure 4: Variation of Zn isotope compositions with gender and diet. (A) Urine: $n_{omnivorous}$ = 42, $n_{no\text{-}meat}$ = 15, $n_{no\text{-}dairy}$ = 11, blood: : $n_{omnivorous}$ = 6, $n_{no\text{-}meat}$ = 6.[22] (B) Urine: n_{female} = 36, n_{male} = 24, whole blood: n_{female} = 31, n_{male} = 28,[21,22] red blood cells (RBCs): n_{female} = 25, n_{male} = 22,[21] and serum: n_{female} = 25, n_{male} = 21.[21] Boxes: interquartile ranges, whiskers: first and fourth quartiles, points: outliers, lines within boxes: medians, crosses: means.

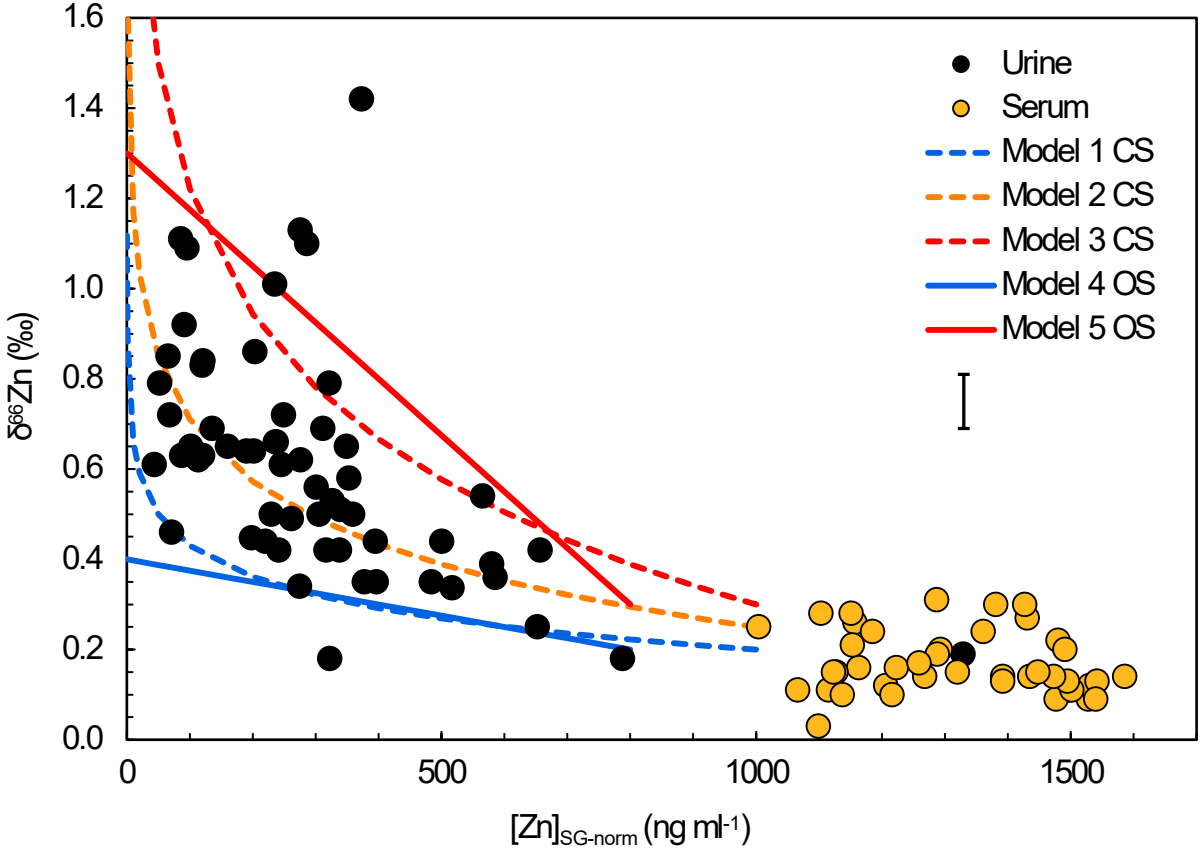
Figure 5: Plot of Zn concentrations vs. isotope compositions for urine (this study) and blood serum[21] from healthy people. CS: closed system fractionation, OS: open system fractionation. The error bar represents the average external reproducibility (2 SD) of the δ^{66}Zn data.


δ66Zn (‰)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
1 2 3 4 5 6 7 8 9 10
Participant #
Day 1
Day 2
B
1 2 4 6 7 9


Omnivorous
No-meat
No-dairy
δ66Zn (‰)
[Zn]SG-norm (ng ml-1)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
δ66Zn (‰)
Urine
Blood
RBCs
Serum


Omnivorous
No-meat
No-dairy
δ66Zn (‰)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
A
Urine
Blood
Female
Male
B
Urine
Serum
Blood
RBCs


Urine
Serum
Model 1 CS
Model 2 CS
Model 3 CS
Model 4 OS
Model 5 OS
δ66Zn (‰)
[Zn]SG-norm (ng ml-1)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
500
1000
1500