



A new anion exchange purification method for Cu stable isotopes in blood samples

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Abstract

The isotopic composition of iron, zinc, copper, and cadmium ($\delta^{56}\text{Fe}$, $\delta^{66}\text{Zn}$, $\delta^{65}\text{Cu}$, and $\delta^{114}\text{Cd}$) are novel and promising tools to study the metabolism and homeostasis of trace metals in the human body. Serum $\delta^{65}\text{Cu}$ has been proposed as a potential tool for diagnosis of cancer in liquid biopsy, and other metals may have similar utility. However, accurate analysis of trace metal isotopes is challenging because of the difficulties in purifying the metals from biological samples. Here we developed a simple and rapid method for sequential purification of Cu, Fe, Zn, and Cd from a single blood plasma sample. By using a combination of 11 M acetic acid and 4 M HCl in the first steps of column chemistry on AG-MP1 resin, we dramatically improve the separation of Cu from matrix elements compared to previous methods which use concentrated HCl alone. Our new method achieves full recovery of Cu, Fe, Zn, and Cd to prevent column-induced isotope fractionation effects, and effectively separates analytes from the matrix in order to reduce polyatomic interferences during isotope analysis. Our methods were verified by the analysis of isotope standards, a whole blood reference material, and a preliminary sample set including five plasma samples from healthy individuals and five plasma samples from cancer patients. This new method simplifies preparation of blood samples for metal isotope analysis, accelerating multi-isotope approaches to medical studies and contributing to our understanding of the cycling of Fe, Zn, Cu, and Cd in the human body.

Keywords $\delta^{56}\text{Fe}$ · $\delta^{66}\text{Zn}$ · $\delta^{65}\text{Cu}$ · $\delta^{114}\text{Cd}$ · Chromatography · Blood

Introduction

Trace metals are involved in a wide range of biological processes across all forms of life. In particular, iron (Fe), zinc (Zn), copper (Cu), and cadmium (Cd) concentrations and stable isotope ratios ($\delta^{56}\text{Fe}$, $\delta^{66}\text{Zn}$, $\delta^{65}\text{Cu}$, and $\delta^{114}\text{Cd}$) have found widespread application in the earth sciences, as tools to understand life processes on both the modern and ancient Earth [reviewed by 1–3]. More recently, the same inductively coupled plasma mass spectrometry (ICPMS) tools used for earth samples such as rocks and seawater have been applied in the medical sciences [4]. This early research has already demonstrated that isotopes can provide new insights into the processes of trace-metal metabolism and homeostasis in the body, and that they have the potential to serve as diagnostic tools. For instance, Albarède et al. [5] used analyses of $\delta^{56}\text{Fe}$ and $\delta^{65}\text{Cu}$ in blood serum and red blood cells to discover new pathways for erythrocyte production not previously accounted for [5]. Balter et al. [6] observed that Cu isotope composition in the serum and red blood cells tended to be lighter for liver

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cancer patients than for healthy individuals, and hypothesized release of Cu from endogenous stores in the patients through the chelation of metallothioneins with light Cu isotopes to be the most likely cause. Low $\delta^{65}\text{Cu}$ values were also measured in serum of breast and colorectal cancer patients [7]. However, Télouk et al. [7] supposed that light Cu isotopes escaping chelation by lactate from cancer cells and excreted into the blood stream were the potential mechanisms accounting for light serum $\delta^{65}\text{Cu}$ in the patients. Though there is no unified model explaining Cu isotopic fractionation in cancer patients yet, the findings suggest a possible diagnostic application. Télouk et al. also found that $\delta^{65}\text{Cu}$ decreased over time if the disease was not cured, suggesting that Cu isotopes could serve as an “early alarm” for cancer development.

A primary hurdle in the further development of such cross-disciplinary methodology is sample purification. Metal stable isotope ratio analysis requires separating the metals of interest, which typically occur at parts-per-million (ppm) to parts-per-billion (ppb) concentrations in blood, from a highly complex biological matrix, all while achieving complete recovery of the metal and complete separation from any other elements which might interfere with ICPMS analysis. The most common and effective purification methods applied in recent studies involve the chromatographic separation of trace-metals on anion-exchange resins, typically AG-MP1 or AG1-x8. Sometimes these methods are developed to purify a single metal for isotope analysis [8, 9], while in other cases, greater sample utilization efficiency and a minimization of sample handling is accomplished by sequentially purifying and eluting several trace metals such as Cu, Fe, Zn, and Cd from an individual sample [e.g., 10–13].

Purification of Cu by anion exchange chromatography has proven particularly challenging. Most modern Cu purification protocols strive to separate Cu from other inorganic elements in high-molarity HCl (7–10 M) on AG-MP1 anion exchange resin with trace amounts of hydrogen peroxide, relying on the slightly stronger binding coefficient of Cu to the resin compared to cations such as Na, Mg, Ca, and others [10, 11, 14, 15]. However, there is a relatively small difference in binding strength of Cu and the interfering elements (when compared, for example, to Fe, Zn, and Cd which all bind very strongly to the resin in high-molarity HCl). Thus, typical Cu purification protocols often require high quantities of resin (1–2 mL) and high aspect ratio columns (lengths ≥ 5 cm) [e.g., 10, 11, 15]. Even with such modifications, it can still be difficult to fully remove interfering elements without also losing some of the Cu, necessitating careful tuning of every individual column [15]. In summary, typical methods for Cu purification are time-consuming, expensive, and prone to sample contamination from resin-blanks.

A distinct approach to purifying Cu in biological samples relies on reduction of Cu(II) to Cu(I). In this method, samples are loaded onto AG1-x8 anion exchange resin in 0.01 M HCl,

with 0.02 M ascorbic acid added as a reductant [16]. While this method seems to work well for the analysis of just $\delta^{65}\text{Cu}$ in biological samples, its application may be limited beyond that. This method would not pair well with methods for purifying Fe, Zn, and Cd because of the use of a reductant which would convert Fe to Fe(II), and because none of these three elements could be separated in 0.01 M HCl. Additionally, because ascorbic acid cannot be purified by distillation, blanks would be expected to be much higher than for acid-only purification protocols.

Here we aim to develop a multi-element purification protocol which is fast and simple and has low blanks of trace metals of interest. We have drawn upon studies done over half century ago which explored trace metal adsorption onto anion-exchange resins in various organic and non-organic solvents including acetic acid, methanol, isopropanol, and hydrobromic acid [e.g., 17–19]. We apply techniques which more effectively separate Fe, Cu, Zn, and Cd from other elements which may interfere with ICPMS analysis, while also saving time and effort compared to current techniques. Our work here is focused on application as a liquid biopsy in cancer care, though we believe that the purification approach outlined will also have widespread application in the earth sciences.

Experimental

Reagents, standards, reference materials, and samples

All sample preparation work was carried out in flow benches with Ultra Low Penetration Air (ULPA) filtration at the University of Southern California. Ultrapure reagents were purchased from the manufacturer (BDH Aristar Ultra HBr and H_2O_2 ; Fluka traceSELECT methanol; BDH ACS grade isopropanol) or purchased in a lower grade and further purified by sub-boiling distillation in a PFA still (BDH Aristar Plus: HCl, HNO_3 , and acetic acid). Ultrapure water (Milli-Q) was used. Labware was acid-cleaned PFA (Savillex) for all steps involving concentrated acids, and acid-cleaned polyethylene for purified sample solutions (LDPE, VWR Metal-free centrifuge tubes). In order to minimize Zn contamination, all clean equipment was handled with polyethylene gloves.

For the assessment of column elution procedures, Cu, Fe, Zn, and Cd isotope ratios were measured in the reference material Seronorm™ L-3 (human whole blood, lot no. 1509408). Additionally, five plasma samples from five healthy individuals and five plasma samples from three metastatic breast cancer patients (two patients were sampled at two different timepoints) were analyzed. All sampling procedures were approved by the University of Southern California, University Park Institutional Review Board (FWA no. 00007099), and the Scripps Health Green Hospital

Institutional Review Board (FWA no. 00000411). All study participants provided written informed consent. The samples were derived from the standard high-definition single cell analysis (HD-SCA) multi-modality workflow that was previously developed and validated to identify the occurrence of metastatic carcinomas by finding ≥ 5 circulating tumor cells per milliliter of plasma [20].

Sample digestion and purification

For whole blood or plasma samples, approximately 200 μg of sample was digested by heating overnight on a hotplate at 120–130 $^{\circ}\text{C}$ with 3 mL of 14 M HNO_3 and 2 mL of 35% H_2O_2 in a 7 mL PFA vial. The completion of digestion was confirmed by checking that sample solutions had become clear and colorless, and samples were then evaporated to dryness at 120–130 $^{\circ}\text{C}$. Digested samples were amended with ^{57}Fe – ^{58}Fe , ^{64}Zn – ^{67}Zn , and ^{110}Cd – ^{111}Cd double spikes according to published procedures [12], and then purified for Cu, Fe, Zn, and Cd according to the purification protocols discussed below (Table 1).

All purification protocols were tested by adding samples in a volume of 0.5–1 mL in the same solution used as the first eluent meant to wash off the matrix. AG-MP1 resin (100–200 mesh) was used in Bio-Spin® Disposable Chromatography Columns (no. 7326008) with inner diameter of ~ 6.4 mm. After purification, the Cu, Fe, Zn, and Cd elution fractions were collected and evaporated to dryness in 7 mL PFA vials, then refluxed in 1 mL of 14 M HNO_3 and 0.1 mL of 35% H_2O_2 at 160 $^{\circ}\text{C}$ for at least 6 h to decompose any leftover organic matter [21, 22]. The samples were evaporated to dryness again and were re-dissolved with 0.1 M HNO_3 to achieve final concentrations of approximately 50 ppb Cu, 100 ppb Fe, 200 ppb Zn, or 2–6 ppb Cd for isotopic analysis. Additionally, to obtain elemental composition in digested blood and recoveries of purified trace metal samples, a small portion of sample aliquot was taken and diluted in 0.5 M HNO_3 for elemental

analysis. The protocol of sample handling and analysis is shown by a flowchart in Fig. 1.

Instrumentation and mass spectrometry

The elemental analysis was carried out by a Thermo Element 2 ICPMS using a 100 $\mu\text{L min}^{-1}$ Teflon nebulizer, glass cyclonic spray chamber with a PC^3 Peltier Cooled inlet system, standard Ni sampler, and Ni “H-type” skimmer cones at the department of Earth Sciences of University of Southern California. The sensitivity and stability of the instrument was tuned to optimal conditions before analysis. The analysis was conducted with sensitivity around 10^6 counts s^{-1} for 1 ppb In. Both the standard and samples were doped with 1 ppb In to calibrate shift in instrumental sensitivity among standard and sample measurements, and after which elemental concentrations in samples were determined by their signal intensity compared to a 10 ppb multi-element standard.

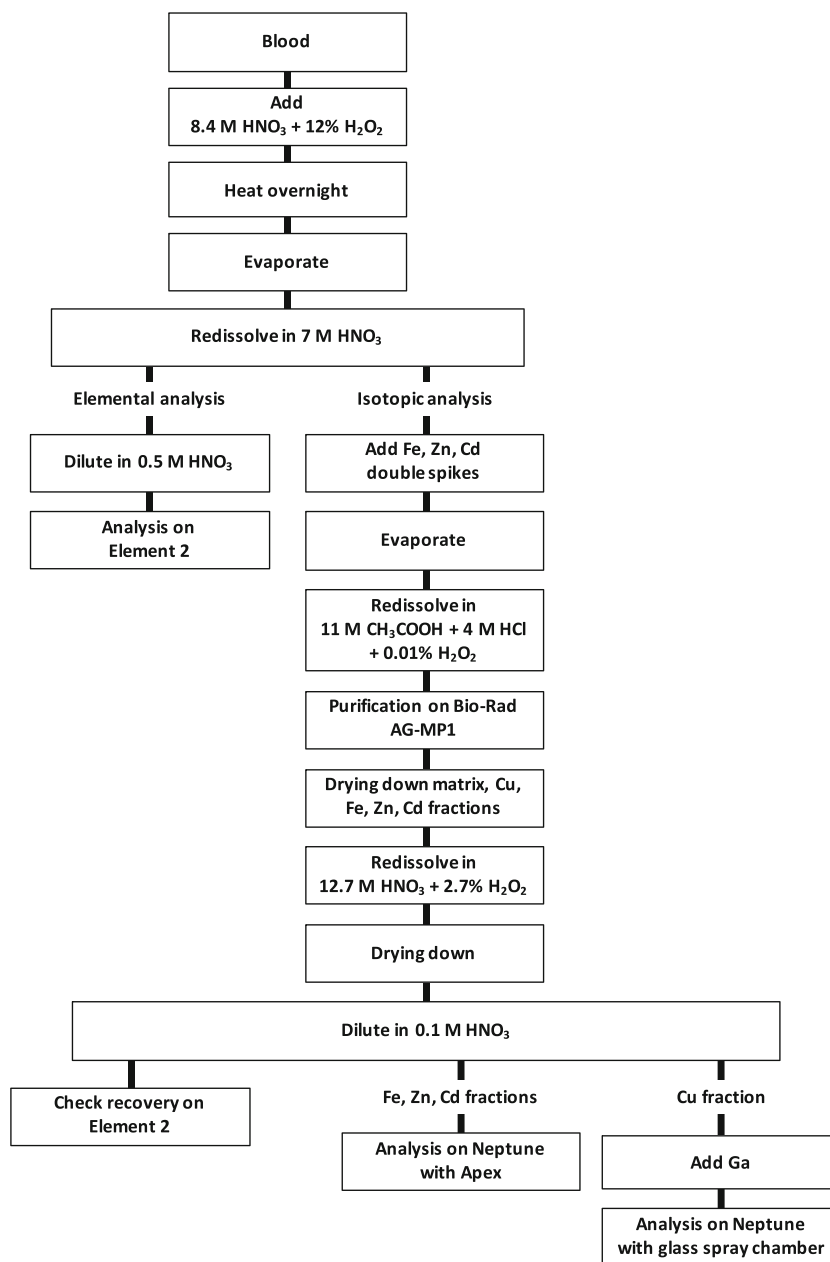
The isotopic composition of Fe, Zn, Cd, and Cu ($\delta^{56}\text{Fe}$, $\delta^{66}\text{Zn}$, $\delta^{114}\text{Cd}$, and $\delta^{65}\text{Cu}$) were measured on a Thermo Neptune Plus MC-ICPMS at the institute of Earth Sciences, Academia Sinica, Taiwan. Fe, Zn, and Cd isotopes were measured using an Apex-IR introduction system. Because Cu more easily sticks to glass surfaces along the long glass flow-path of the Apex leading to high blanks and inaccurate isotope values, Cu isotopes were measured with a double-pass cyclonic spray chamber from which Cu is more easily washed away, allowing for accurate isotope analyses [23]. A Jet sampler and an “x-type” skimmer cones were used to maximize sensitivity. Standards and samples were introduced through a 100–150 $\mu\text{L min}^{-1}$ Teflon or glass nebulizer.

Instrumental settings and data acquisition method of Cu followed Takano et al. [22], and the settings and methods of Fe, Zn, and Cd followed Conway et al. [12]. Briefly, Cd was measured in “low resolution” mode and Cu, Fe, and Zn were measured in “high resolution” mode, using a high resolution slit (25 μm), in order to resolve polyatomic isobaric

Table 1 Protocol for separating Cu, Fe, Zn, and Cd from sample matrix for MC-ICPMS isotopic analysis

Step	Reagent	Volume	Collection
Loading AG-MP1 resin		0.25 mL	
Cleaning resin	2 M HNO_3	2 mL	
Rinsing	Milli-Q	0.5 mL	
Rinsing	1 M HCl	0.5 mL	
Conditioning	11 M CH_3COOH + 4 M HCl + 0.01% H_2O_2	0.4 mL \times 2	
Sample loading	Sample (in 11 M CH_3COOH + 4 M HCl + 0.01% H_2O_2)	0.5–1 mL	Na, Mg, Ca, S, P
Matrix removal	11 M CH_3COOH + 4 M HCl + 0.01% H_2O_2	0.5 mL \times 10	Na, Mg, Ca, S, P
Cu elution	5 M HCl + 0.01% H_2O_2	0.4 mL \times 16	Cu
Fe elution	1 M HCl	0.2 mL \times 14	Fe
Zn elution	2 M HNO_3 + 0.1 M HBr	0.2 mL \times 12	Zn
Cd elution	2 M HNO_3	0.2 mL \times 10	Cd

Fig. 1 Flowchart of the sample handling and analysis protocol described in the paper



interferences. For Fe measurements, four Fe isotopes ^{54}Fe , ^{56}Fe , ^{57}Fe , and ^{58}Fe were simultaneously measured in each analysis. Potential isobaric interferences from ^{54}Cr and ^{58}Ni were corrected within the run by simultaneously monitoring ^{53}Cr and ^{60}Ni . $\delta^{66}\text{Zn}$ was calculated from ^{64}Zn , ^{66}Zn , ^{67}Zn , and ^{68}Zn signals, along with ^{60}Ni which was used for correcting the interference from ^{64}Ni . $\delta^{114}\text{Cd}$ was calculated from ^{110}Cd , ^{111}Cd , ^{112}Cd , and ^{114}Cd , using ^{105}Pd and ^{117}Sn measured simultaneously to correct the interference from ^{110}Pd , ^{112}Sn , and ^{114}Sn . $\delta^{65}\text{Cu}$ was based on ^{63}Cu and ^{65}Cu signals, along with ^{69}Ga and ^{71}Ga to account for instrumental mass bias. Data collection consisted of 30 4.2 s cycles. Pure 0.1 M HNO_3 solution was measured every 4–6 samples to

determine background signals, which were then subtracted from sample intensities. Standards were also measured every 4–6 samples routinely to correct potential systematic drift in each occasion. Isotope ratios are expressed in per mil (‰) relative to the average of the standards NIST 976 Cu, IRMM-14 Fe, Alfa Aesar-ETH Zn, and NIST 3108 Cd, respectively, using delta notation calculated as follows;

$$\delta^{65}\text{Cu} = \left[\frac{(^{65}\text{Cu}/^{63}\text{Cu})_{\text{sample}}}{(^{65}\text{Cu}/^{63}\text{Cu})_{\text{NIST SRM 976}}} - 1 \right] \times 10^3$$

$$\delta^{56}\text{Fe} = \left[\frac{(^{56}\text{Fe}/^{54}\text{Fe})_{\text{sample}}}{(^{56}\text{Fe}/^{54}\text{Fe})_{\text{IRMM-14}}} - 1 \right] \times 10^3$$

$$\delta^{66}\text{Zn} = \left[\frac{(^{66}\text{Zn}/^{64}\text{Zn})_{\text{sample}}}{(^{66}\text{Zn}/^{64}\text{Zn})_{\text{AA-ETH}}} - 1 \right] \times 10^3$$

$$\delta^{114}\text{Cd} = \left[\frac{(^{114}\text{Cd}/^{110}\text{Cd})_{\text{sample}}}{(^{114}\text{Cd}/^{110}\text{Cd})_{\text{NIST SRM 3108}}} - 1 \right] \times 10^3$$

Mass bias correction

We used a double spike technique to obtain precise Fe, Zn, and Cd content and isotopic composition in samples [12]. The technique corrects the artificial isotopic fractionation on sample Fe, Zn, and Cd potentially caused by purification protocols and mass spectrometry. Isotopic fractionation of Cu, Zn, Fe, and Cd could occur if there were significant losses of the metals through heating or evaporation. To ensure that this does not occur, we have previously performed tests by adding double spikes into pure Zn, Fe, and Cd standard (in diluted nitrate acid solution) before or after evaporating the solutions, and then measured their elemental concentrations and isotopic compositions to determine that losses and isotopic fractionations during evaporation are negligible. In brief, the technique utilizes four isotopes in the sample-spike mixtures to calculate three independent ratios (e.g., $^{68}\text{Zn}/^{64}\text{Zn}$, $^{67}\text{Zn}/^{64}\text{Zn}$, $^{66}\text{Zn}/^{64}\text{Zn}$), which allows for the calculation of three unknowns, including the sample-spike ratio, procedural/instrumental isotopic fractionation, and the isotopic fractionation of a sample relative to the standard, assuming all fractionation follows the mass dependence law. A data reduction scheme following the iterative approach of [24] was used to calculate the unknowns and then to obtain sample isotope ratios. Detailed information concerning the preparation, concentration, and isotopic composition of the double spikes are described in Conway et al. [12]. Because Cu has only two isotopes, a double spike technique is not available for the isotopic analysis. To avoid potential isotopic fractionation by chromatographic separation on Cu samples, Cu recovery must be $\sim 100\%$. Instrumental mass bias was corrected using Ga added at roughly twice the concentration of Cu [25].

Result and discussion

Here we describe the development of our new purification protocol, describing a series approaches sorted generally in order from the least effective approaches, through approaches with intermediate applicability, and ending with the protocol which we believe is best suited to the analysis of $\delta^{65}\text{Cu}$ in blood samples. Next, we discuss linking the Cu purification protocol with simultaneous purification of Fe, Zn, and Cd to create a single combined method. Finally, we present the

isotope analyses for various standards and blood samples purified with this new method and consider analytical precision and accuracy of the new method.

Cu purification

The following Cu elution protocols differ primarily in the amount of AG-MP1 resin used, and the eluent used to load the column and elute the “primary” matrix, which we define as alkali metals (monovalent atomic cations such as Na), alkali earth metals (divalent atomic cations such as Mg), and molecular anions such as P. In all cases, 0.01% H_2O_2 is added to keep Fe and Cu oxidized as Fe(III) and Cu(II), respectively. And in all cases, Cu is eluted with 5 M HCl, a concentration of HCl which easily elutes Cu, while Fe, Zn, and Cd are strongly retained on the resin.

10 M HCl; 0.25 mL resin; Fig. 2A The challenges associated with previous purification methods are illustrated by our attempt to purify Cu from other elements in concentrated HCl. Approximately 3 mL of eluent is necessary to elute the primary matrix elements, yet at that point over 90% of the Cu was eluted as well. As discussed in the “Introduction,” poor separation of Cu from primary matrix elements in HCl has been overcome in previous methods by using columns with high quantities of resin and high aspect ratios. However, such modifications greatly increase the time required to run columns, and they still may not effectively purify Cu unless each column is individually calibrated.

9 M isopropanol + 3.5 M HCl; 0.25 mL resin; Fig. 2B Effective binding of Cu(II) to anion exchange resins in mixtures of HCl and isopropanol were observed by Fritz and Pietrzyk [17]. We find that this eluent is able to effectively separate Cu from alkali metals and alkali earth metals. This method provided even better separation of Ti and Mn from Cu than the acetic acid-based methods described below, though this was not considered a major advantage for processing blood samples because the amounts of Ti and Mn in blood are so low. There are several disadvantages to this method. The method is time-consuming because the isopropanol-based eluent flows slowly through the resin, approximately half as fast as the 11 M acetic acid eluent, which make the protocol require 2 h to complete. The isopropanol evaporates quickly so that the eluent composition may change over the course of a single run, and occasionally the eluent evaporated from the columns during a run leaving the resin dry, after which significant Cu loss was noted. Finally, the isopropanol-based eluent dissolves salts less easily than acid-based eluents, requiring larger volumes to dissolve samples which have a high salt concentration.

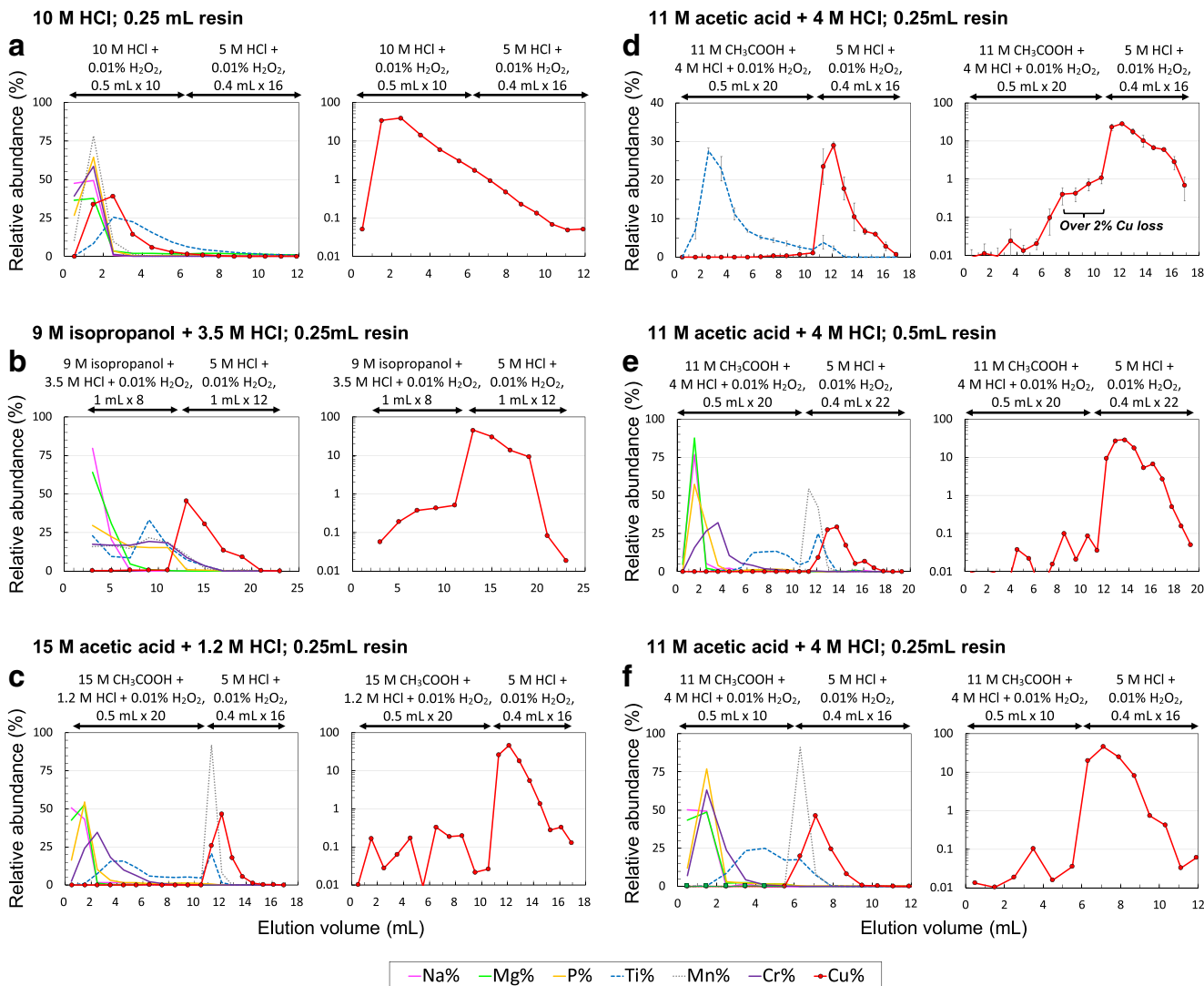


Fig. 2 A series of Cu elution protocols which differ in the amount of AG-MP1 resin and the solution used, including an HCl-based purification similar to published methods (A), an isopropanol-based eluent (B), a 15 M acetic acid eluent which is slow (C), a 11 M acetic acid eluent with high eluent volumes (D), a 11 M acetic acid eluent on a larger resin volume (0.5 mL; E), and the final optimized protocol (F). Results are

15 M acetic acid + 1.2 M HCl; 0.25 mL resin; Fig. 2C Anion exchange chemistry in acetic acid-based solutions was previously studied by Korkisch and Hazan [18] and Klakl and Korkisch [19]. Here we find that a solution with 15 M acetic acid and 1.2 M HCl is very effective at separating Cu from the primary matrix. Ti is not completely separated, and Mn is not separated at all, but as discussed above, this should not be a problem for blood samples which contain little of these elements. The only disadvantage of this method is that it takes around 2 h to complete, which is also time-consuming. A larger volume of eluent (approximately 10 mL, compared to 5 mL for the “final version”) is required to remove all of the Cr. Also, the high acetic acid concentration means that the eluent flow slowly, approximately half as fast as 11 M acetic acid.

presented for the recovery of multiple elements (left) and recovery of Cu in a log scale (right). Experiments were performed with 0.5 mL of the eluent with 100 ng of a multi-element mixture, except for B in which elements were dissolved in 3 mL eluent, and D which contained 50 ng Ti and Cu. Error bars in D reflect duplicate experiments

11 M acetic acid + 4 M HCl; 0.25 mL resin; Fig. 2D Decreasing the concentration of acetic acid created a less viscous eluent which flowed more quickly. This method also had the best separation of Ti, though 27% was still eluted in the Cu fraction. However, when running a full 10 mL of eluent, approximately 2% of the Cu was lost with the primary matrix, which could bias the final $\delta^{65}\text{Cu}$ analyses if there is isotopic fractionation on the column.

11 M acetic acid + 4 M HCl; 0.5 mL resin; Fig. 2E Using the same eluent as described above, but increasing the resin volume to 0.5 mL solved the problem of premature Cu elution, though Ti was slightly less well separated.

Table 2 Recovery of various elements in different elution steps of the trace metal separation protocol

Elements	11 M CH ₃ COOH +4 M HCl + H ₂ O ₂ 6 mL		5 M HCl+ H ₂ O ₂ 6.4 mL		1 M HCl 2.8 mL		2 M HNO ₃ + 0.1 M HBr 2.4 mL		2 M HNO ₃ 2 mL	
	(Matrix)		(Cu fraction)		(Fe fraction)		(Zn fraction)		(Cd fraction)	
	AVE	±SD*	AVE	±SD	AVE	±SD	AVE	±SD	AVE	±SD
Cu	0.1	±0.1%	99.8	±0.1%	0.1	±0.1%	0.0	±0.0%	0.0	±0.0%
Fe	0.1	±0.1%	0.2	±0.4%	98.8	±0.3%	0.7	±0.3%	0.1	±0.0%
Zn	0.1	±0.0%	0.3	±0.6%	0.7	±1.5%	98.9	±2.1%	0.0	±0.0%
Cd	0.0	±0.0%	0.1	±0.2%	0.1	±0.1%	3.7	±1.1%	96.1	±1.4%
Na	100.0	±0.0%	0.0	±0.0%	0.0	±0.0%	0.0	±0.0%	0.0	±0.0%
Mg	99.5	±0.4%	0.2	±0.3%	0.1	±0.1%	0.2	±0.3%	0.0	±0.0%
Ca	99.9	±0.1%	0.1	±0.1%	0.0	±0.1%	0.0	±0.0%	0.0	±0.0%
Al	99.0	±1.0%	0.2	±0.1%	0.5	±0.5%	0.2	±0.2%	0.1	±0.2%
Ni	100.0	±0.0%	0.0	±0.0%	0.0	±0.0%	0.0	±0.0%	0.0	±0.0%
V	99.6	±0.2%	0.4	±0.2%	0.0	±0.0%	0.0	±0.0%	0.0	±0.0%
Cr	99.4	±0.7%	0.5	±0.7%	0.0	±0.0%	0.0	±0.0%	0.0	±0.0%
P	98.5	±0.8%	0.8	±0.5%	0.5	±0.5%	0.1	±0.1%	0.1	±0.1%
S	91.1	±0.4%	8.8	±0.4%	0.1	±0.1%	0.0	±0.0%	0.0	±0.0%
Ga	0.0	±0.0%	0.0	±0.0%	99.5	±0.1%	0.4	±0.1%	0.1	±0.0%
Mo	3.6	±3.9%	27.1	±4.1%	46.9	±3.2%	21.3	±4.8%	1.1	±0.3%
Sn	0.0	±0.0%	0.0	±0.0%	0.0	±0.1%	98.7	±0.5%	1.3	±0.5%
Ti	71.2	±3.1%	27.3	±3.0%	0.8	±0.1%	0.5	±0.1%	0.2	±0.0%
Mn	0.2	±0.5%	99.8	±0.5%	0.0	±0.0%	0.0	±0.0%	0.0	±0.0%

*Values are calculated from results of five replicate tests

Ultimately, this method was not chosen because using more resin requires more time for all steps of purification, from resin cleaning through elution of Cu, Zn, Fe, and Cd.

11 M acetic acid + 4 M HCl; 0.25 mL resin; Fig. 2F This method was chosen as the optimal protocol. The time required to complete the purification was only around 1 h. Good separation of the primary matrix elements was achieved after 3.5 mL of eluent was passed through the column (3 mL of fresh eluent, plus the 0.5 mL of eluent in which the sample was loaded). Using 5.5 mL of eluent removed ≥ 99% of Na, Mg, P, and Cr from both multi-element standards and blood samples (Tables 2 and 3). Only 73% of the Ti and none of the Mn were removed with the matrix; however, when this method was tested on human whole blood and plasma samples, less than 0.5 ng Ti and 3 ng Mn were present in samples containing 100 ng or more Cu, amounts which are not expected to interfere with $\delta^{65}\text{Cu}$ measurements. Using 5.5 mL of eluent also allows for effective separation of Cu from primary matrix elements even with possible slight differences in eluent composition or column loading from day-to-day or between users. Less than 1% of Cu was eluted with the primary matrix, allowing for complete recovery of the Cu in 6.4 mL of 5 M HCl.

Fe, Zn, and Cd purification

The Cu purification protocol retains Fe, Zn, and Cd on the column, allowing them to also be purified from the same sample using a single combined column procedure. Based on techniques originally developed to purify Fe, Zn, and Cd from seawater [12], we simply modified the volumes of eluents

Table 3 Recovery of Cu, Fe, Zn, and Cd and interfering elements for Cu obtained from applying the trace metal separation protocol to whole blood and plasma samples

Fraction	Elements	Whole blood ($n = 3$) <i>Seronormi™ Trace Elements L-3</i> AVE ± SD	Plasma ($n = 10$) <i>From 10 donors</i> AVE ± SD
Cu	Cu	99.7 ± 0.1%	99.5 ± 0.1%
	Na	0.1 ± 0.1%	0.3 ± 0.3%
	Mg	0.4 ± 0.3%	1.4 ± 1.8%
	Ca	1.6 ± 0.4%	1.9 ± 1.3%
	P	0.1 ± 0.1%	0.6 ± 0.8%
	S	0.2 ± 0.0%	0.3 ± 0.5%
Fe	Fe	99.7 ± 0.2%	98.1 ± 0.6%
Zn	Zn	99.6 ± 0.1%	98.6 ± 0.7%
Cd	Cd	93.8 ± 3.5%	–

used, in order to account for differences in resin volume and column geometry. We found that effective purification was achieved with 2.8 mL of 1 M HCl to elute Fe, 2.4 mL of 2 M HNO₃ + 0.1 M HBr to elute Zn, and 2 mL of 2 M HNO₃ to elute Cd (Table 1). The application of HBr is based on Strelow [26] which has found that Cd can be retained in anion resins in 2 M HNO₃ + 0.1 M HBr eluent but Zn cannot. It is explained that Cd has higher tendency to form stable bromide complexes than Zn in the eluent [26]. This method was tested on both a multi-element standard and human blood samples (Fig. 3). These tests demonstrated both that potential interfering elements were removed, and that the analytes of interest were completely recovered (Tables 2 and 3).

Blanks

Reagent blanks were estimated from the measured blanks for each of the pure chemicals, and the reagent composition. Reagent blanks were less than 1 ng for Cu, Fe, and Zn and less than 0.002 ng for Cd and Ga (Table 4). Procedural blanks were similar to reagent blanks for Cd and Ga, suggesting no additional contamination during the column purification process. However, Cu, Fe, and Zn procedural blanks were roughly an order of magnitude higher than reagent blanks, suggesting some sort of contamination. Possible sources of this blank

Table 4 Accumulated blanks from the reagents used in column chemistry, and procedural blanks for the full trace metal separation protocol

Elements	Reagent (<i>n</i> = 3) <i>AVE</i> ± <i>SD</i> (ng)	Procedural (<i>n</i> = 5) <i>AVE</i> ± <i>SD</i> (ng)
Cu	0.044 ± 0.006	0.20 ± 0.06
Ga	< 0.002	0.001 ± 0.001
Fe	0.37 ± 0.05	1.7 ± 0.3
Zn	0.64 ± 0.07	9.8 ± 0.2
Cd	< 0.001	< 0.001

include supplies (e.g., resin, column, pipette tips) or operation (e.g., during column purification or during evaporation). These blanks are insignificant except for Zn, where procedural blank can be as high as ~ 10 ng. Assuming typical Zn concentrations of 9 ppm in whole blood, ~ 110 uL of blood would be needed to overwhelm blanks (less than ~ 1% blank contribution).

Isotope analysis

The accuracy of our analytical conditions was verified by determining the isotope composition of two international

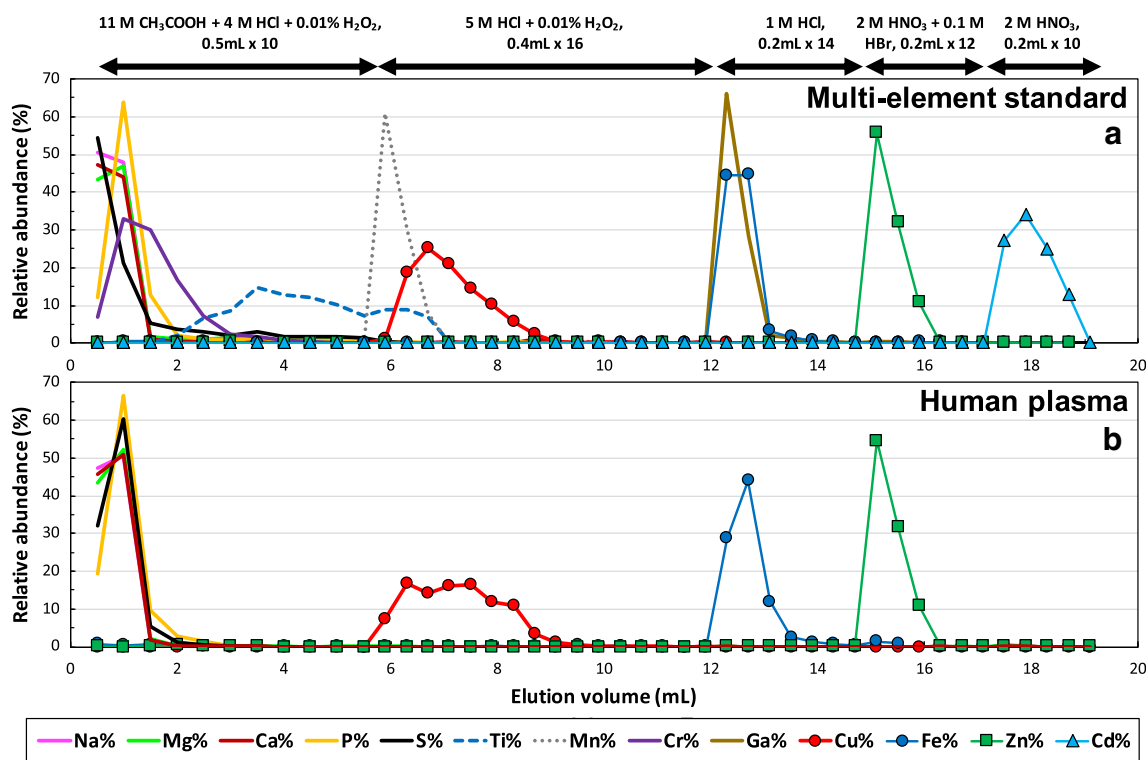
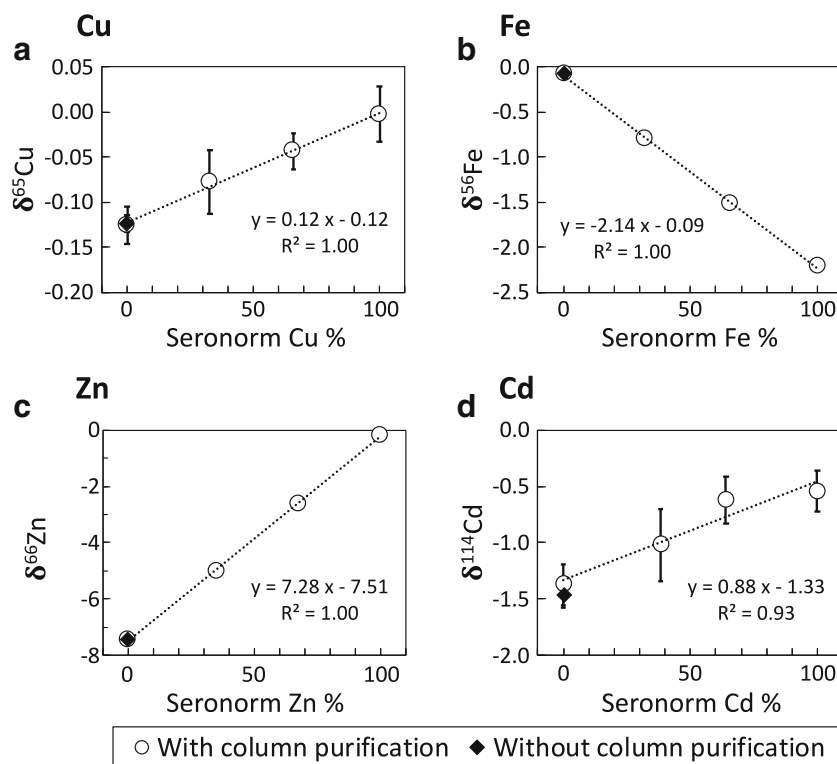


Fig. 3 Elution curves for the final optimized separation method on a multi-element standard (A) and a human plasma sample (B). The standard contains the elements listed in Fig. 1A with mass of 100 ng in 0.5 mL, and the plasma sample contains 114,628 ng Na, 794 ng Mg,

1112 ng Ca, 7298 ng P, 107295 ng S, 64 ng Cu, 33 ng Fe, and 46 ng Zn. The results of Ti, Mn, Cr, Ga, and Cd of the plasma are not shown because their masses in the sample were lower than 1 ng and could therefore not be measured accurately

Fig. 4 Mixtures of whole blood reference material (Seronorm™ Trace Elements L-3) mixed with various amount of metal standards ($\delta^{65}\text{Cu}$ (A), $\delta^{56}\text{Fe}$ (B), $\delta^{66}\text{Zn}$ (C), and $\delta^{114}\text{Cd}$ (D)). Circles reflect sample-standard mixtures or purified through column chemistry, while the diamond represents the original metal isotope standard



standards for each element on the same day. Cu standard Wako Cu was $+0.26 \pm 0.01$ ‰ compared to Cu standard NIST SRM 976 (2σ , $n = 36$); NIST SRM 3126a Fe was $+0.32 \pm 0.02$ ‰ compared to IRMM-014 (2σ , $n = 16$); AA-ETH Zn was -0.29 ± 0.01 ‰ compared to Lyon JMC (2σ , $n = 8$); and BAM 1012 Cd was -1.31 ± 0.06 ‰ compared to NIST SRM 3108 (2σ , $n = 8$). These values are statistically consistent with reported values previously [22, 27, 28].

The analytical reproducibility for the reported isotopic composition of each sample was obtained by combining the internal precision of both sample and standard measurements [12]. The precision of single measurement (~ 30 cycles) ranged from ± 0.03 to ± 0.07 ‰ for Cu, from ± 0.03 to ± 0.06 ‰ for Fe, and from ± 0.02 to ± 0.04 ‰ for Zn. Analytical uncertainties for Cd were much larger, ranging from ± 0.18 to ± 0.32 ‰, which was attributed to the small amounts of Cd present in samples (1 to 3 ng).

The accuracy of our purification method was evaluated by isotope standard addition experiments in which a whole blood samples were mixed with varying amounts of a standard of known isotope composition, then analyzed for $\delta^{65}\text{Cu}$, $\delta^{56}\text{Fe}$, $\delta^{66}\text{Zn}$, and $\delta^{114}\text{Cd}$. Additionally, we have measured $\delta^{65}\text{Cu}$, $\delta^{56}\text{Fe}$, and $\delta^{66}\text{Zn}$ on ten blood plasma samples taken from healthy donors or breast cancer patients, both in order to validate our methods and to demonstrate the potential value of utilizing multiple trace metal isotope systems in medical research.

Isotope standard addition

Our measurements of stable isotope ratios in mixtures of the whole blood standard Seronorm L-3 and pure metal standards provide three ways to test that our methods are reliable (Fig. 4, Table 5). First, the internal analytical errors are similar for pure standards, standards processed through the full purification protocol, and mixtures with whole blood, suggesting a lack of isobaric interferences from either blood matrix or column contamination. Second, mixing between the pure metal standards and the whole blood produces a nearly-linear array of isotope values, as would be expected by isotope mass balance for mixing between two end-members (R^2 values between 0.93 and 1.00). Finally, we find that each of the end-members has an expected isotope composition. Standards which have been processed through the purification chemistry have the same measured isotope composition as pure unprocessed standards. The Seronorm L-3 sample measured here had slightly different stable isotope ratios than those reported previously for Seronorm L-3 (-0.37 ± 0.08 ‰, $+0.24 \pm 0.09$ ‰, and -0.20 ± 0.05 ‰ compared to previously reported values for $\delta^{65}\text{Cu}$, $\delta^{56}\text{Fe}$, and $\delta^{66}\text{Zn}$, respectively), however we attribute this to the fact that samples were from a different lot with different metal concentrations, and thus they may have had genuinely different isotopic compositions (Table 5). All values were within the range previously measured in human blood (see the following section).

Table 5 Elemental and isotopic results for Cu, Fe, Zn, and Cd in a reference material (Seronom™ L-3, lot no. 1509408), trace metal standards, and their mixtures with two mixing ratios

Seronom to standard mixing ratios	[Cu] ± 2σ (ppm)	δ ⁶⁵ Cu ± 2σ (‰)	[Fe] ± 2σ (ppm)	δ ⁵⁶ Fe ± 2σ (‰)	[Zn] ± 2σ (ppm)	δ ⁶⁶ Zn ± 2σ	[Cd] ± 2σ (ppb)	δ ¹¹⁴ Cd ± 2σ
100% standard (unprocessed) ^b		-0.12 ± 0.01‰ (34)		-0.07 ± 0.02‰ (12)		-7.45 ± 0.01‰ (16)		-1.46 ± 0.12‰ (2)
100% standard (processed) ^b		-0.12 ± 0.02‰ (5)		-0.07 ± 0.04‰ (1)		-7.44 ± 0.03‰ (1)		-1.37 ± 0.18‰ (1)
33% Seronom-67% standard	6.41 ± 0.03	-0.08 ± 0.03‰ (3)	905 ± 6	-0.79 ± 0.04‰ (1)	23.5 ± 1.8	-5.02 ± 0.03‰ (1)	28.7 ± 5.5	-1.02 ± 0.32‰ (1)
67% Seronom-33% standard	3.19 ± 0.02	-0.04 ± 0.02‰ (3)	439 ± 3	-1.51 ± 0.04‰ (1)	12.3 ± 1.0	-2.61 ± 0.03‰ (1)	17.2 ± 3.3	-0.62 ± 0.21‰ (1)
100% Seronom	2.10 ± 0.01	0.00 ± 0.03‰ (4)	288 ± 2	-2.21 ± 0.05‰ (1)	8.32 ± 0.65	-0.18 ± 0.03‰ (1)	11.0 ± 2.1	-0.54 ± 0.18‰ (1)
Certified values for lot no. 1509408	2.08 ± 0.42	-	301	-	9.42 ± 1.90	-	9.9 ± 2.0	-
Reported Seronom™ L-3 with different lot no.	0.703 ± 0.030 ^c	+0.37 ± 0.07‰ ^d , +0.36 ± 0.08‰ ^e	435 ± 32 ^c	-2.45 ± 0.07‰ ^d	4.77 ± 0.28 ^c	+0.02 ± 0.04‰ ^d	11.3 ± 0.6 ^c	-

^a Measurement times for individual samples^b Standard solution without and with processing with column protocol^c Certified values for lot no. OK0337^d Lot no. OK0337 [10]^e Lot no. 1112691 [29]

Plasma samples

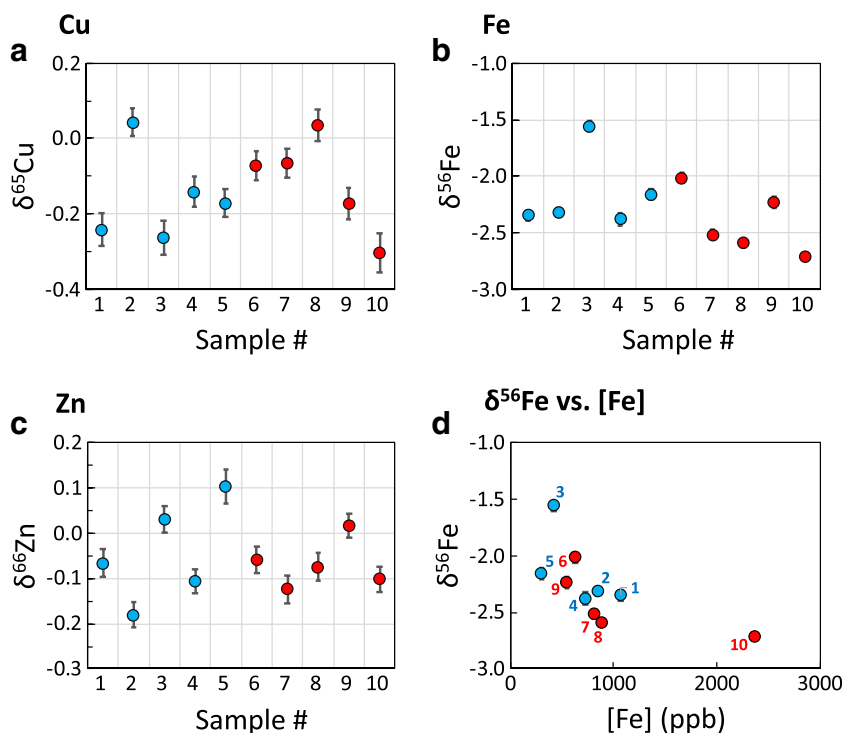
Plasma samples from five healthy individuals and three metastatic breast cancer patients were analyzed for the elemental and isotopic composition of Cu, Fe, and Zn (Fig. 5). Cd concentrations in the plasma samples were too low to yield accurate δ¹¹⁴Cd. To our knowledge, the data is the first to combine multiple metal isotope tracers to investigate the interactions between the isotopes and their potential for cancer diagnosis/prognosis, though paired δ⁶⁵Cu and δ³⁴S have been reported [6]. For all three isotope systems, the values reported here are similar to values previously reported in the literature. Télouk et al. [7] showed that δ⁶⁵Cu from breast and colorectal cancer patients was -0.51 ± 0.52‰ (n = 86) and -0.40 ± 0.29‰ (n = 17), respectively, which are statistically lighter than that of the healthy donors, -0.26 ± 0.40‰ (n = 53). However, Balter et al. [6] did not find any statically difference in serums between liver cancer patients and healthy individuals (with δ⁶⁵Cu of -0.01 ± 0.56‰, n = 21 and 0.10 ± 0.45‰, n = 19, respectively). In a different study, Costas-Rodríguez et al. [30] found that liver cirrhosis patients had lower serum δ⁶⁵Cu than healthy individuals (-0.78 ± 0.72‰, n = 25 vs. -0.29 ± 0.27‰, n = 29). Our δ⁶⁵Cu values for breast cancer patients and healthy donors are -0.12 ± 0.26‰ and -0.16 ± 0.24‰, suggesting that δ⁶⁵Cu is not effectively distinguishing between healthy donors and patients, at least with this small preliminary dataset.

Previous studies have suggested that there is likely to be less difference in serum δ⁶⁶Zn between cancer patients and healthy donors, with δ⁶⁶Zn values of -0.4 to +0.4‰ and -0.4 to +0.3‰ for healthy donors and cancer patients, respectively [data compiled by 4]. Overall, our samples ranged from -0.2 to +0.1‰, which is comparable with published data. The values in breast cancer patients and healthy donors are statistically indistinguishable, as previously observed (-0.07 ± 0.11‰ vs. -0.04 ± 0.22‰).

To the best of our knowledge, there are no studies of Fe isotopes in cancer patients. Published serum δ⁵⁶Fe values show large isotope fractionations compared to most natural materials, ranging from -3.6 to -0.4‰, which is likely to be affected by erythropoietic pathways [5]. Our δ⁵⁶Fe values range from -2.7 to -1.6‰, which are consistent with this range. Between cancer patients and healthy individuals, there is no significantly δ⁵⁶Fe difference (-2.41 ± 0.57‰ vs. -2.15 ± 0.69‰). In addition, we found that there is a negative relationship between plasma Fe content and δ⁵⁶Fe. A similar relationship was observed in a recent study of chronic kidney disease patients with iron deficiency anemia, who had significantly heavier serum δ⁵⁶Fe (δ⁵⁶Fe = -1.36 ± 0.82, n = 11) compared to a reference population (δ⁵⁶Fe = -2.67 ± 0.34, n = 23) [31].

While our preliminary δ⁶⁵Cu, δ⁶⁶Zn, and δ⁵⁶Fe values largely overlap between healthy individuals and cancer

Fig. 5 Stable isotope composition of blood plasma from five healthy individuals (blue circles) and three metastatic breast cancer patients (red circles), including $\delta^{65}\text{Cu}$ (A), $\delta^{56}\text{Fe}$ (B), $\delta^{66}\text{Zn}$ (C), and $\delta^{56}\text{Fe}$ compared to Fe concentration (D). Samples 9 and 10 were collected from the same patient at different timepoints, with the latter taken when the patient had substantial diarrhea



patients, there may be trends observed over time for the single patient for whom two samples were analyzed. Samples 9 and 10 were collected from the same patient at different timepoints, with the latter taken when the patient had substantial diarrhea. All three stable isotope values show a significant negative shift between samples 9 and 10 ($-0.47 \pm 0.07\text{‰}$, $-0.48 \pm 0.06\text{‰}$, and $-0.12 \pm 0.04\text{‰}$ for $\delta^{65}\text{Cu}$, $\delta^{56}\text{Fe}$, and $\delta^{66}\text{Zn}$, respectively). Additional samples from this patient at other times, as well as comparison to temporal variability in $\delta^{65}\text{Cu}$, $\delta^{66}\text{Zn}$, and $\delta^{56}\text{Fe}$ for other patients and healthy individuals, may help to distinguish normal variability from changes in metal stable isotopes which can be attributed to specific medical conditions.

Conclusions

We have developed a new method for sequential purification of Cu, Fe, Zn, and Cd from a single blood sample for determination of stable isotope ratios. The method successfully recovers the metals of interest, while effectively separating these metals from other matrix elements which might interfere with analysis. The most notable improvement in this method compared to previous techniques is the ability to easily purify Cu. The acetic acid-based chromatography described here has a higher distribution coefficient for Cu compared to previous HCl-based

techniques, allowing for more effective separation of Cu from matrix.

This new method has the potential to accelerate research on metal stable isotopes in medicine. Faster and simpler column chemistry enables higher-throughput sample handling, potentially allowing for medical samples to be easily processed by the hundreds to thousands. In addition to being faster, the Cu purification techniques described here should be more robust to small methodological changes such as slight differences in resin or eluent volumes, or eluent concentration. Finally, pairing this Cu purification technique with purification of Fe, Zn, and Cd opens the door to the simultaneous study of several isotope systems.

With additional work, this method may be adapted to other areas of isotope geochemistry. Cu isotope analysis in seawater, marine particles and geological samples may also benefit from the improved acetic acid purification, especially in combination with methods to separate Cu from Mn and Ti.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

All sampling procedures were approved by the University of Southern California, University Park Institutional Review Board (FWA no. 00007099), and the Scripps Health Green Hospital Institutional Review Board (FWA no. 00000411). All study participants provided written informed consent.

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