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An automated chromatography procedure optimized for analysis of stable Cu isotopes from biological materials

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Abstract
An automated ion-exchange chromatography method is developed for the separation of copper (Cu) from biological samples prior to stable, naturally occurring, isotope analysis. The technique does not require Cu to be fully oxidized/reduced into either Cu+ or Cu2+. Distribution coefficients of Cu and other cations to the Cu-specific anion exchange resin enable the effective purification and separation of Cu from complex matrixes using a single, reusable chromatographic column, with the potential to be modified for varying sample types. The automated chromatography system (prepFAST-MC™) can process up to 60 samples per run at a rate of 36 samples per day on a single ion exchange column. Low carryover (<1%) combined with high yields (97 ± 3%) for multiple extractions is observed. Isotopic analyses of the Cu fraction by multi collector-inductively coupled plasma-mass spectrometry, produced accurate Cu stable isotope data (ERM-AE633). The repeatability was assessed to be better than 0.02‰ for pure standard solutions and biological samples, making this method suitable for future applications, such as medical research, that require high throughput for precise isotopic analysis.

Keywords
isotopes, biological, materials, procedure, analysis, chromatography, stable, automated, cu, optimized

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Abstract

An automated ion-exchange chromatography method is developed for the separation of copper (Cu) from biological samples prior to stable, naturally occurring isotope analysis. The technique does not require Cu to be fully oxidized/reduced into either Cu^{+} or Cu^{2+}. Distribution coefficients of Cu and other cations to the Cu-specific anion exchange resin enable the effective purification and separation of Cu from complex matrixes using a single, reusable chromatographic column, with the potential to be modified for varying sample types. The automated chromatography system (prep*FAST*-MC™) can process up to 60 samples per run at a rate of 36 samples/day on a single ion exchange column. Low carryover (<1%) combined with high yields (97±3%) for multiple extractions were observed. Isotopic analyses of the Cu fraction by multi collector-inductively coupled plasma-mass spectrometry, produced accurate Cu stable isotope data (ERM-AE633). The repeatability was assessed to be better than 0.02‰ for pure standard solutions and biological samples, making this method suitable for future applications such as medical research that require high throughput for precise isotopic analysis.

Introduction

Copper is an essential trace element in most aerobic organisms[1] and plays a crucial role in balancing oxidative stress.[2] The binding of Cu with specific ligands as a function of coordination and bond energy results in changes in the ratio of naturally occurring stable isotopes of Cu ($^{65}Cu/^{63}Cu$) on a cellular level.[3,4] Heavy isotopes are anticipated to be enriched in the strongest bonds, as with a decrease of isotope mass, the vibrational frequency decreases as well.[5,6] It is hypothesized that Cu isotopic ratios in blood and various organs should reflect the efficiency of overall body Cu metabolism.[7–11] With the development of multi collector-inductively coupled plasma-mass spectrometry (MC-ICP-MS), high precision Cu isotope ratio measurements of ±0.1-0.2‰ 2 standard errors (2SE) can be achieved[12], enabling the resolution of small natural Cu isotope effects. More recently, increased interest in stable $^{65}Cu/^{63}Cu$ isotope measurements originating from biological source material has led to the application of isotope ratio measurement in medical research, with varying degrees of success.[4,13–19] Previous work was not only able to identify metabolic abnormalities in certain patients with Parkinson's disease[13], but also appears to reflect the state of cancer progression in human serum and gives the ability to isotopically characterize tumor cells.[4,18] In order to develop diagnostic tools and biomarkers based on Cu isotope analysis to specific diseases, such as Parkinson's disease or cancer[13,18,19], automation and the ability to process large sample numbers, is required.[17–19]

Traditional methods for Cu separation from complex sample matrixes for isotopic analysis utilize a variation of ion exchange chromatography methods.[12,17,20] Márechal et al. (1999)[12] used a macroporous anion-exchange resin, where Cu was loaded on the resin in HCl, with H_2O_2 to ensure all Cu was oxidized to Cu^{2+}. Copper is moderately retained at high HCl concentrations, during which the matrix was removed and eluted in the same 7 M HCl + 0.001% H_2O_2 solution, used for sample loading and matrix removal.[12] Several problems may arise in this procedure, such as the potential for incomplete Cu elution, unwanted Cu fractionation, and overlapping of Cu elution with matrix elements, induced by varying sample types. These issues mean that a second column pass and adjustment of the method for each individual application is often required.[20–24] A method by Larner et al. (2011)[20], improved the extraction of Cu from

biological samples by making use of the varying distribution coefficients of Cu^{+} and Cu^{2+} on the AG-1 anion exchange resin.[20] By reducing all Cu present in the sample using L(+)-ascorbic acid to Cu^{+}, the method makes use of the large difference in distribution coefficients between Cu^{+} and other cations (e.g. Ca, Fe), allowing for an improved separation of Cu, compared to Márechal et al. (1999).[20] This approach achieved a repeatability of ±0.15‰ of $^{65}Cu/^{63}Cu$ ratio measurements and was therefore deemed suitable for the isotopic analysis of biological material,[20] with results reported following the standard delta notation which is a unitless expression of the measurement through normalization with a reference material (ERM-AE633).

The aim of the work presented here is the development and adoption of an automated approach to Cu ion exchange chromatography, optimized for biological materials, to enable high sample throughput, circumventing the manual labor cost associated with traditional methods. The commercially available automated chromatography system prep*FAST*-MC™ (Elemental Scientific, Omaha, USA), has been successfully adopted for the analysis of Ca and Sr in a wide range of matrices[25] and was therefore chosen as a platform. The prep*FAST*-MC™ is a low-pressure automated chromatography system, which uses one reusable column to process samples sequentially, including a cleaning step between each new sample. A highly reusable column, used for the extraction of Cu, enables the simple, reliable, robust and efficient separation of Cu from biological source material on a large scale.

Methods and Materials

Reagents and Materials

Reagents used were Suprapur® HCl, Ultrapur® HCl and Ultrapur® HNO_3 (Merck). Deionized 18.2 MΩ-cm H_2O (Millipore) was used to prepare stock solutions (Table 1).

Perfluoroalkoxy alkane (PFA) vials were used for sample digestion and to dry down solutions. Vials used for MC-ICP-MS analysis were made of high-density polyethylene (HDPE) and for Q-ICP-MS analysis of polypropylene (PP). PFA vials were washed overnight at 100 ºC in 7.5 M HNO_3 and rinsed with H_2O. HDPE and PP vials were rinsed using 0.3 M HNO_3 and H_2O at room temperature.

Samples

Three types of sample were prepared: pure isotopic standard solutions, pseudosamples, and chicken liver. Pure isotopic standard solutions were diluted from the certified reference material solution ERM-AE633 (European Reference Materials, European Commission, Geel, Belgium) and used to initially assess the chromatography's suitability to process isotopic material without inducing isotope fractionation. The concentrations were adjusted to 200 ppb and 500 ppb in 0.3 M HNO_3.

Since there were no biological reference materials, certified for total Cu content as well as Cu isotope composition available, four pseudosamples were prepared from high purity single element standard solutions, with a purity of 99.99% - 99.9995%, (Inorganic Ventures, USA and Elemental Scientific, USA) to simulate, with the exception of carbon, the matrix composition of fully digested biological material. Pseudosamples 1, 2 and 4 imitate the matrix composition of a typical chicken liver

(Table 2) as characterized by the United States Department of Agriculture (Basic Report 05027, National Nutrient Database for Standard Reference Release 28), while pseudosample 3 followed the same proportions as the other pseudosamples, but replaced K with Na, Mn with Hg, Na with Li, P with Se, Se with In and Zn with Ni, due to limited availability of single element standards. In pseudosamples 1 and 2, Cu was added from a high purity single element standard (Inorganic Ventures, USA) to result in a final concentration of 492 ppb whilst in pseudosamples 3 and 4, 492 ppb of Cu was added from the ERM-AE633 isotopically certified solution[26]. Pseudosamples 1 and 2 were exclusively used for the determination of the elution profile, column yield and to assess carry over, while pseudosamples 3 and 4 were used to assess isotopic precision and accuracy of extraction.

Finally, to test the applicability of the method to real biological matrices, two aliquots of chicken liver were prepared. Several livers were purchased from the local butcher, split into several subsamples, and freeze dried at -55 ºC under vacuum for 48 h. Two subsamples were crushed with a chromium alloy rotary disk mill for 30 seconds and homogenized manually using an agate mortar and pestle.

Sample digestion and Cu separation

Biological samples of chicken liver (0.06-0.21 g) and DORM-2 (~0.1 g; Dogfish muscle certified reference material, National Research Council Canada) were weighed out, and then pre-digested in MARSXpress 75 mL PFA vessels in 2 mL of 15 M HNO_3 overnight at room temperature. The following day samples were digested using an MARS5 microwave digestion system. The temperature was ramped to 210 ºC over 30 min and then held constant for 90 min to ensure that all organic carbon was driven off as CO_2.

For quality control purposes, one blank sample and two DORM-2 samples were added to the digestion method. Recovery of elements from the DORM-2 was used to ensure complete digestion of biological sample types.

Upon removal from the microwave, all digestion solution were clear. The digests were then evaporated to dryness under Class 100 cleanroom conditions, refluxed in 2 mL of 0.001 M HCl overnight. To avoid any potential residual particulate matter from entering onto the column, the samples were centrifuged and the supernatant decanted into a clean 15 mL centrifuge tube before loading onto the prep*FAST*-MC™ Cu column.

This new chromatography method was developed and performed on prep*FAST*-MC™ systems[25] at Elemental Scientific (ESI) and the Wollongong Isotope Geochronology Laboratory, University of Wollongong (WIGL, UOW) using a 500 µL Cu column (Part Number: MC-CF-Cu-500). Samples are loaded in 2 mL 0.001 M HCl on the column. The separation protocol uses two reagents, 0.001 M HCl to load and wash the matrix, and 8 M HCl to elute Cu and clean the resin (Table 1). Flow rates and volumes are programmed independently for each step of the method and are syringe-driven, enabling faster flows (3 mL min^{-1}) for cleaning of the column, conditioning and washing off of the matrix, while permitting slower flow rates for the loading and elution steps, where fractionation could potentially occur. This tight control of the flow rates and volumes is a major advantage over conventional gravity-driven and vacuum box methods. This setup enables a high sample throughput of ~36 samples

per 24 h. To avoid unwanted resin degradation of the column between batches of sample processing, it is stored after each use in 0.001 M HCl.

Measurements

Elemental Concentrations

Elemental concentration analysis was performed on an iCAP quadrupole-inductively coupled plasma-mass spectrometer at WIGL, UOW and an Element 2 sector field-inductively coupled plasma-mass spectrometer (Thermo Scientific) at ESI. Concentrations were quantified using a multi-element standard external calibration curve. Recoveries of metals from the certified reference material (DORM-2, NRCC) were between 85 and 105% of the expected values (Table 3). A 1 ppb multi-element solution, measured every 6 samples was used to correct for instrument drift, which was typically less than ±1%.

The concentration of major matrix elements in biological samples (Na, Mg, K, Ca, Mn, Fe, Zn, Se, P) and Cu were used to 1) determine recovery of elements from the certified reference material (DORM-2, NRCC) during the digestion process, 2) determine the degree of matrix removal during the column washing steps and 3) determine the Cu recovery in elution cuts. The measured concentrations from 2 and 3 above of pseudosamples 1 and 2 were used to determine elution profiles, column yield and evaluate matrix removal in the eluates.

Copper Isotopic Measurement

After the eluates were collected, they were dried down and refluxed in variable volumes of 0.3 M HNO_3 solution, to dilute them to a target concentration of 100 ppb Cu and doped with a Ni solution, to obtain a final concentration of 250 ppb Ni. This admixed Ni is used as an internal standard to correct mass bias.[12,27] Copper isotope measurements were performed with a Neptune Plus MC-ICP-MS (Thermo Scientific) at WIGL, UOW, using the operating conditions outlined in Table 4. Standard sample and skimmer cones, cyclonic spray chamber and PFA nebulizer with ~100 µL min^{-1} flow rate (Elemental Scientific, Omaha, USA) were used throughout.

The analyses were carried out by static multi-collection with five Faraday cups to monitor masses 60, 61, 62 for Ni and 63, 65 for Cu.[20] Data acquisition was performed over three blocks of 20 cycles of four seconds integration each. Amplifier baseline was run before every block, and a routine instrumental sensitivity of ~35 V ppm^{-1} for ^{63}Cu was achieved. The measurements were corrected for mass discrimination through a combination of internal correction with the admixed Ni applying Russell's exponential law[28] and external normalization using a standard sample bracketing approach, as described in Nielsen et al., 2004[29] and Zhu et al., 2000[30]. The measured $^{65}Cu/^{63}Cu$ isotope ratios were tested for outliers, two standard deviations from the mean (2SD).

The isotopic composition of Cu was expressed using the delta notation ($\delta^{65}Cu$, ‰). It is a dimensionless parameter calculated with equation (1), which represents the normalization of the corrected sample ratio against the ratio of the reference material ERM-AE633 (ref. 26).

$$\delta^{65}Cu=\left[\frac{\left(^{65}Cu/^{63}Cu\right)_{Sample}}{\left(^{65}Cu/^{63}Cu\right)_{ERM\text{-}AE633}}-1\right]\times 1000 \quad (1)$$

The typical measurement repeatability of a NIST SRM-976 standard solution on the Neptune Plus was determined as $\delta^{65}Cu_{ERM\text{-}AE633}$ -0.056±0.007‰ (2SE; n=73), which is in good accordance with published values.[26]

Results and Discussion

Spectral interferences and Matrix removal

High precision Cu isotopic analysis of biological samples can be affected by elements present at high concentrations (e.g. P, Mg, Na). These elements can impede on the five monitored isotopes of Cu and Ni through the formation of polyatomic species[31] (Table 5) and isobaric interferences[32] which can affect mass bias.[32] It is consequently essential that these elements be efficiently removed. With the exception of Fe, which is not an interference-forming element for the observed isotopes, all monitored elements were only present at background levels in the tested samples after passing through the chromatography.

To compile an elution profile, fractions of 1 mL each of the entire chromatography methodology (Fig. 1) and 0.25 mL fractions for the Cu elution (inset Fig. 1) were collected and analyzed. The elution profile served to calibrate the column and optimize the volumes used in the chromatography, to achieve the reproducible collection of matrix-free Cu cuts with high yields (Table 6). The final chromatography volumes were optimized to 2 x 2 mL of 0.001 M HCl for complete matrix removal in the tested samples and 2 x 1.25 mL of 8 M HCl for the Cu elution, resulting in the efficient removal of Na (99.9%), Mg (99.8%), K (100%), Ca (99.9%), Mn (98.6%), Fe (96.6%), Zn (98.5%), P (99.2%) and Se (99.1%) (Fig. 1; Table 6). In the Cu elution fraction, only negligible residual Fe was observed (~40 ng). The high capacity of the resin (3 mg Cu g^{-1}) and ability to operate under flow rates of up to 6 mL min^{-1} makes it ideal for an automated system.

Method validation

Blanks

In trace metal isotope analysis it is crucial that blank concentrations are reduced as much as possible.[32] To achieve this goal, the method was setup to include a resin wash before every conditioning with 3 mL (6 column volumes) of 8 M HCl (Table 1). Processing total procedure blanks alongside the samples monitored the average procedural blank of the method. The average blank contribution was 0.5±0.3 ng Cu (n = 11), equivalent to <0.1% of the amount of Cu processed for sample analysis (~333-990 ng). This is at the lower end of the range reported for blank contributions in other studies, which is between 0.021-3%.[12,14,17,20,33]

Carry Over

Reusing the chromatographic column for high precision isotope ratios requires that carry over from previous samples is negligible. To assess carry over, method blanks were interspersed between samples and processed systematically in every run. Insignificant Cu is retained on the column after the elution as shown above. Blank and

carry over concentrations were not significant to affect isotopic ratios for Cu at the levels observed.

Column yield and column life

Cu can fractionate during the ion exchange process and is most likely to occur during loading or eluting off the column.[21,34] High yields ensure that all the Cu is retained and released by the resin at the appropriate time, thereby eliminating any potential fractionation. Our assessed yield was determined as 97±3 (2SD)% (Table 7) which is in good agreement with Cu yields from commonly applied methods: 100±6% (ref. [12]) and 100±2% (ref. [20]). The yield remained high across different pseudosample matrices and no apparent systematic change in Cu isotope ratios was observed with yields of less than 100% (R^2=-0.004) (Fig. 2). This suggests that Cu does not readily fractionate on the column to induce systematic changes in the isotopic composition of the sample.

It is recommended to only process samples through the chromatography, which were digested with methods able to completely drive off the organic carbon present in biological samples (e.g. microwave digestion methods). Initial experiments with a digestion method using small vessels in a household microwave[35] resulted in partial digestions. Attempts to process these samples via the chromatography led to column degradation and low yield as a function of the partial digestion. This problem was resolved through the application of the above-described microwave digestion method. Incomplete digestion can lead to (1) Cu being complexed in the matrix and therefore not readily held on the resin, resulting in low yields, and potential Cu fractionation; (2) incompletely digested organic matter accumulating on the column. The accumulation of organic matter could lead to a reduction of available binding sites and associated reduction in the resin's capacity. It was found that repeated flushing of the column with 8 M HCl and 15 M HNO_3 did not result in the visual removal of the accumulated organic matter retained on the resin, with yields remaining low, even for pure standard solutions and it had to be exchanged.

Achieving continuously high Cu yields and observing negligible Cu isotope fractionation from the various sample types indicated no obvious resin degradation for the processing of the samples (n>50) for this study. It is expected that column end-of-life-behavior will result in a reduction in Cu yields. If the reduction in Cu yields leads to fractionation of Cu on the column is not clear at this point. It is recommended that column performance is monitored by systematically processing a pure synthetic pseudosample doped with an isotopic reference material every five samples. By doing so, column quality parameters, such as Cu recovery, matrix removal, and isotope fractionation due to resin exhaustion are monitored.

Repeatability of isotope ratio values

ERM-AE633: A pure 250 ppb ERM-AE633 solution was repeatedly processed and analyzed to test the overall accuracy, precision, and repeatability of the chromatography. A δ^{65}Cu value (-0.01±0.01‰ (2SE; n=20)) for 20 consecutive replicates processed on the same column (Table 7 and Figure 3), was determined to be in accordance with recommended values.[26] The mean value of the measurements demonstrates high accuracy, while the low two standard error indicates high precision.

Pseudosamples: Matrix effects were investigated with pseudosamples that approximate samples of biological origin. Pseudosamples 3 and 4, spiked with the isotopic reference material (~990 ng $Cu_{ERM\text{-}AE633}$), were each processed 5 times. No fractionation during the automated chromatographic process was observed (Table 7,8 and Figure 4), with very good precision, for pseudosample 3 ($\delta^{65}Cu$ = -0.01±0.02‰; 2SE) and pseudosample 4 ($\delta^{65}Cu$ = -0.03±0.02‰; 2SE). These results show that organic free matrix samples are easily processed with high precision and accuracy.

Biological samples: The method was finally tested for its suitability to process real biological samples of unknown isotopic composition. Seven subsamples of the two aliquots of chicken liver were processed, interspersed with two aliquots of pseudosample 4, and the Cu isotope composition analyzed (Fig. 5) in a random order. The results of the pseudosample indicate that the between-batch variability is negligible compared to previous analyses and that the repeatability of Cu isotopic measurements within the two batches of liver tissue was very good. Batch 1 and 2 yielded average $\delta^{65}Cu$ of 0.51±0.02‰ (2SE; n=3) and 1.06±0.01‰ (2SE; n=4), respectively (Table 7). The precision for the analysis of biological samples is similar to or better than previously published values.[13,18–20,36] Metals and metal isotopes have been shown to be heterogeneously distributed in organ tissues[3,37–39], suggesting that the difference in the two sample clusters can be explained by natural variability of Cu isotopes in the bulk chicken liver tissue. As the samples were purchased from a wholesale butcher, it was not possible to control for general sources of heterogeneity of the samples such as sex, age or diet of the chickens. The concentrations of Cu in the samples from both aliquots did not vary significantly (Batch 1 16.2±0.4 ppm; Batch 2 17.5±3.3 ppm). Comparison with published measurements of sheep and mice livers, show a similarly large spread of Cu isotope compositions: mice liver 0.05 to 0.79‰ (n=10) and sheep liver -1.38 to -0.75 ‰ (n=4) with a reproducibility of <0.05‰.[3]

Significance of automation

Improved knowledge of the role that metalloproteins play in biology and medicine has led to the establishment of the discipline of medical isotope metallomics.[5] Significant pilot studies were able to demonstrate the potential for metal stable isotope analysis as a medical diagnostic tool. Bone loss was traced via Ca isotope levels in blood and urine[40,41], cancer disease progression was traced via Cu and S isotopes in blood plasma[4,18] and breast cancer cells identified via Zn isotopes[19]. One issue that is common to all the previously mentioned studies is that they are based on small samples sizes and sample processing with each specific method can take weeks if not months. It was recently proposed that 'new technology needs to be developed that increases sample analysis rates and makes high precision isotope analyses accessible (…)'[42]. While initial attempts at simplifying sample processing and analyzing unprocessed sample matrix straight away were encouraging[43], this approach will most likely stay restricted to lower complexity biological samples, such as urine. In order for the discipline to grow and move on from the pilot study-phase, it is crucial to develop methods that allow for high-throughput sample processing. By application of these new sample processing-strategies, sample populations should be increased by a factor of 50[42], overcoming the issue of often low statistical significance in a clinical setting.

Conclusion

A new automated chromatography method is presented, which enables the quick and efficient separation of Cu from biological material, resulting in a clean Cu fraction in a discrete volume. Copper yields were high for matrix matched pseudo- and real biological samples and the method did not induce fractionation of the Cu isotopes. Pure standard, matrix matched samples, and biological tissues were processed and analyzed with a precision of ≤0.02‰. This is better than previously reported: ±0.05-0.3‰[3,13,18–20,44] in biological samples. The methodology is suitable to resolve small natural stable Cu isotope effects, such as those observed in biological samples, which have a range of ~3‰.[20,45]

Compared to previously described, manually executed methods[12,20], this automated approach has several distinct advantages: (1) by utilizing the prep*FAST*-MC™ automated platform, it enables the unsupervised processing of over 30 samples per 24h, and at the same time reduces user-induced errors. This presents a major leap forward in terms of sample throughput, as manual methods typically enable only 10-30 samples to be processed per week; (2) the application of a highly specific Cu resin removes the need to rely on reducing/oxidation agents[12,20], to retain Cu on the resin; (3) there is no requirement of a cleaning step (for example with $HClO_4$ [20]) to remove residual organic matter from the introduced reducing agent. The automated approach is characterized by low blank contribution and high sample throughput with very good precision and repeatability of Cu isotope ratio measurements of biological samples. In order to enable easier comparison of future development and refinement of Cu chromatography methods, an international biological reference material should be characterized.

The method presented herein represents an important milestone with regards to the automation of chromatography procedures for the application of isotope ratio analysis in biological samples. Future application and refinement of the method will facilitate new area of biomedical research as a result of the ability to process very large sample sets, commonly found in clinical studies, with comparative ease.

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Tables

Table 1 – Chromatographic steps for the automated separation of Cu (500 µL Cu Column)

Step	***Purpose***	***Volume (mL)***	***Flowrate (µL min^{-1})***	***Reagent***
1	Clean Column	2x1.5	3000	8 M HCl
2	Condition Column	2x3	3000	0.001 M HCl
3	Load Samples	2	400	0.001 M HCl
4	Elute Sample Matrix	2x2	3000	0.001 M HCl
5	Elute Cu fraction	2x1.25	1000	8 M HCl

479

Table 2 - Typical chicken liver (Basic Report 05027, National Nutrient Database for Standard Reference Release 28, United States Department for Agriculture)

Element	***Unit***	***Value per 100 g***	***Std. Error***	***N***
Ca	mg	8	1.040	4
Mg	mg	19	0.403	4
P	mg	297	8.109	4
K	mg	230	13.720	4
Na	mg	71	5.542	4
Zn	mg	2.67	0.045	4
Mn	mg	0.255	0.014	4
Se	µg	54.6	8.247	4
Cu	mg	0.492	0.102	4
Fe	mg	8.99	0.403	4

480

Table 3 – Recovery of metals from DORM-2 certified reference material (NRCC)

Element	*Unit*	*Certified Value*	*2SD*	*Measured Value*	*2SD*	*Recovery (%)*	*N*
Co	mg kg^{-1}	0.182	0.031	0.164	0.081	90.4	4
Cu	mg kg^{-1}	2.34	0.16	2.381	0.110	101.7	4
Fe	mg kg^{-1}	142	10	133	19	93.8	4
Ni	mg kg^{-1}	19.4	3.1	16.5	1.6	84.9	4
Zn	mg kg^{-1}	25.6	2.3	26.9	1.8	105.1	4

Table 4 - Operating conditions for the Neptune Plus MC-ICP-MS	
RF Power	1200 W
Cool gas	17 L min^{-1}
Auxiliary gas	0.7 L min^{-1}
Sample gas	1 L min^{-1}
Sensitivity for Cu, Ni	~35 V ppm^{-1}
Sample Uptake Rate	100 µL min^{-1}

481

Table 5 - Selection of major isobaric and matrix interferences for Cu and Ni isotope measurements for biological samples (May et al., 1998; Larner et al., 2011)

Element	*Mass*	*Interferences*
Cu	63	$^{23}Na^{40}Ar^{+}$, $^{23}Mg^{38}Ar^{+}$, $^{26}Mg^{37}Cl^{+}$, $^{31}P^{16}O_2^{+}$, $^{47}Ti^{16}O^{+}$
	65	$^{25}Mg^{40}Ar^{+}$, $^{32}S^{33}S^{+}$, $^{33}S^{16}O_2^{+}$, $^{49}Ti^{16}O^{+}$, $^{130}Ba^{2+}$
Ni	60	$^{23}Na^{37}Cl^{+}$, $^{24}Mg^{36}Ar^{+}$, $^{44}Ca^{16}O^{+}$
	62	$^{23}Na_2{}^{16}O^{+}$, $^{24}Mg^{38}Ar^{+}$, $^{26}Mg^{36}Ar^{+}$, $^{31}P_2^{+}$, $^{46}Ti^{16}O^{+}$

482

Table 6 - Removal of matrix elements included in the pseudosamples

	Sample	***Cu***	***Na***	***Mg***	***K***	***Ca***	***Mn***	***Fe***	***Zn***	***Se***	***P***
Pseudosample 1 (n=8)											
Loaded (ng)		980	142000	38000	460000	16000	510	17980	5340	—	—
Elution (ng)		927	28.2	6.6	44.4	21.0	4.0	437.7	0.7	—	—
Removal (%)		94.61±4.94%	99.98±0.01%	99.97±0.01%	99.98±0.01%	99.87±0.02%	99.21±0.02%	97.57±0.7%	99.99±0.01%	—	—
Pseudosample 2 (n=10)											
Loaded (ng)		991	23829	69807	209241	34405	861	32667	9984	—	—
Elution (ng)		977.5	24.0	24.0	29.4	72.0	0.6	1807.8	14.4	—	—
Removal (%)		98.64±2.19%	99.90±0.04%	99.97±0.01%	99.99±0.01%	99.79±0.03%	99.93±0.08%	94.47±0.3%	99.86±0.06%	—	—
Pseudosample 3 (n=5)											
Loaded (ng)		984	—	32777	—	—	—	17981	6384	205819	118276
Elution (ng)		944.5	—	8.0	—	—	—	930.0	231.0	26.3	1525.1
Removal (%)		96.00±3.94%	—	99.98±0.11%	—	—	—	94.83±0.45%	96.38±0.63%	99.99±0.01%	98.71±0.04%
Pseudosample 4 (n=5)											
Loaded (ng)		995	—	49401	516031	—	972	48113	9530	136	571747
Elution (ng)		943.5	—	288.3	512.0	—	31.9	260.8	229.4	2.5	1599.8
Removal (%)		94.82±2.62%	—	99.42±0.12%	99.9±0.01%	—	96.72±0.68%	99.46±0.06%	97.59±0.44%	98.16±0.13%	99.72±0.07%

483

Table 7 - Cu isotope data obtained for pure standard solutions, pseudo samples and biological samples (‰, 2 std error)

Type of sample	***Standard***	***N***	***^{65}Cu (‰)***	***2SE***
250ppb NIST-976	ERM-AE633	73	-0.056	0.007
250ppb ERM-AE633	ERM-AE633	20	-0.01	0.01
Pseudosample 3	ERM-AE633	5	-0.01	0.02
Pseudosample 4	ERM-AE633	5	-0.03	0.02
Chicken liver aliquot 1	ERM-AE633	3	0.51	0.02
Chicken liver aliquot 2	ERM-AE633	4	1.06	0.01

484

Table 8 - Cu recovery from pseudosamples, as well as isotope analysis of select samples (‰, 2 std error)

Pseudosample	***N***	***Containing Elements***	***Cu Recovery***	***Isotope ratio*** $\delta^{65/63}Cu_{ERM\text{-}AE633}$	***Reported (Accepted, Ref. 26)***
1	8	Ca, Fe, Mg, Zn, Cu, Se, P, K, Na, Mn	96±2%	NA*	
2	10	Ca, Fe, Mg, Zn, Cu, Se, P, K, Na, Mn	99±2%	NA*	
3	5	Ca, Fe, Mg, Se, Na, Li, Hg, Cu, Ni, In	95±2%	-0.01±0.02	0.00±0.94 (0.00±0.05)
4	5	Ca, Fe, Mg, Zn, Cu, Se, P, K, Na, Mn	96±4%	-0.03±0.02	

NA*: Pseudosample 1 & 2 did not contain Cu-isotopic reference material and were therefore not analysed for their isotopic composition.

Figures

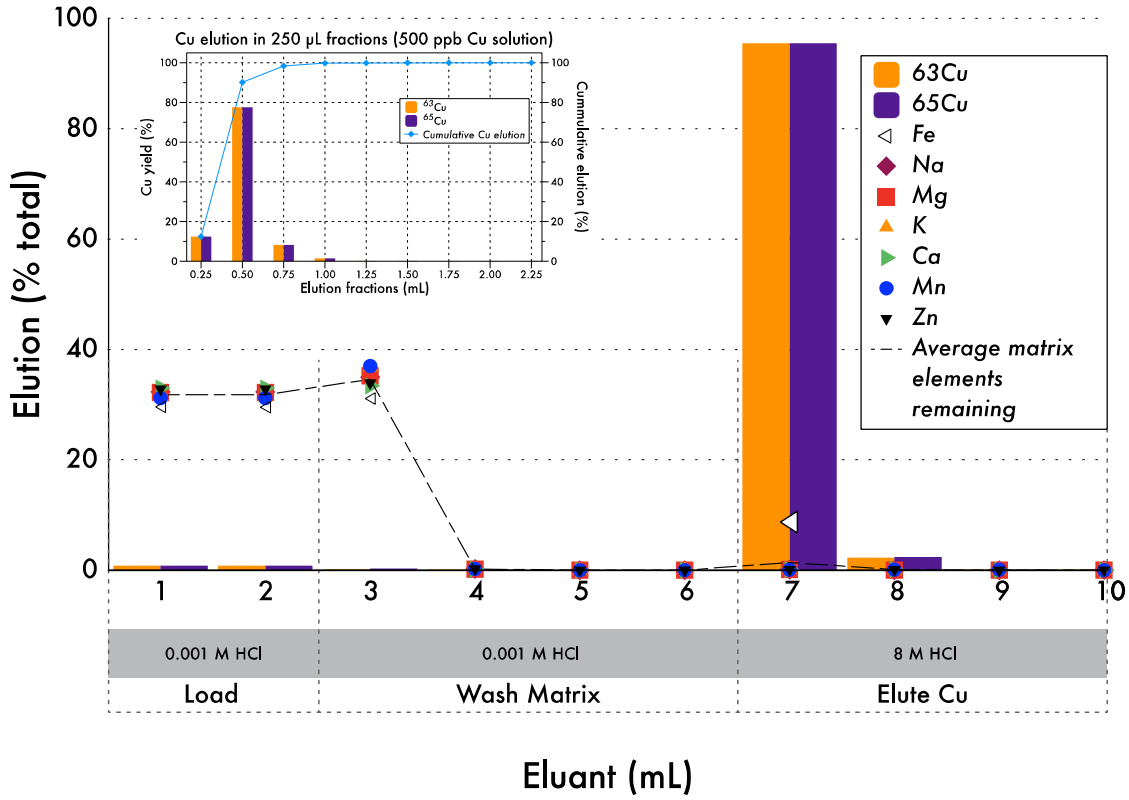

Figure 1. Cumulative elution profile for the method, performed on pseudosample 2. The entire method was split up into 1 mL steps that were individually analyzed to generate the main elution curve. The insert depicts the elution step at a higher resolution of 250 µL fractions performed on a pure 500 ppb ERM-AE633 solution. Cu is well separated from all major matrix elements.

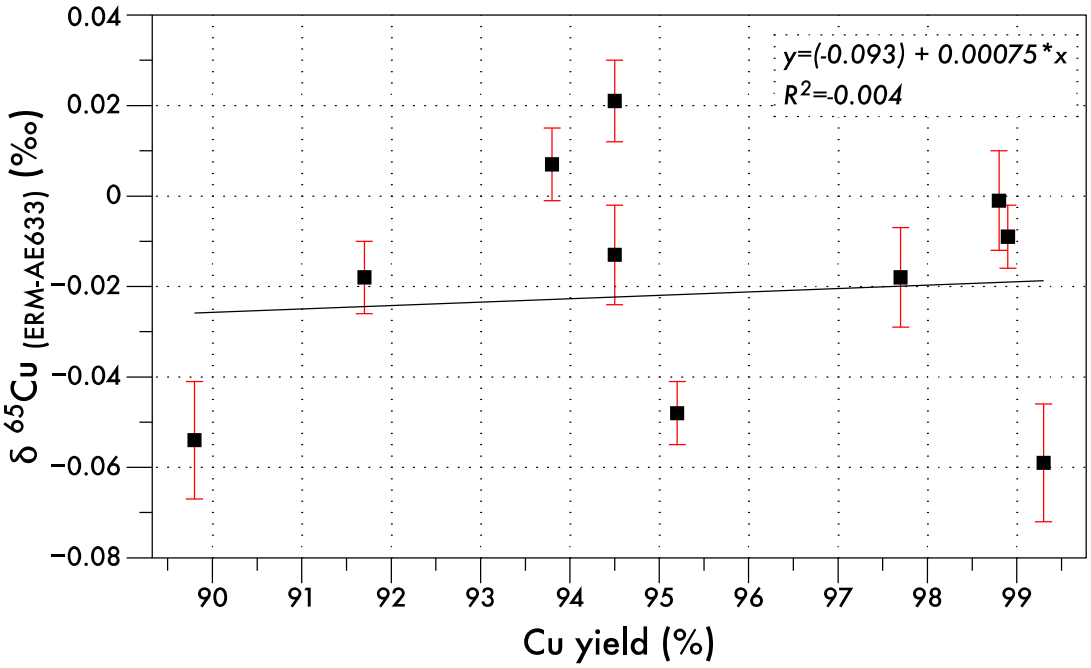

Figure 2. Scatterplot showing no correlation of Cu yield and Cu isotope measurements of pseudosamples 3 and 4, indicating that yields of less than 100% can potentially be sufficient to produce high precision isotopic measurements.

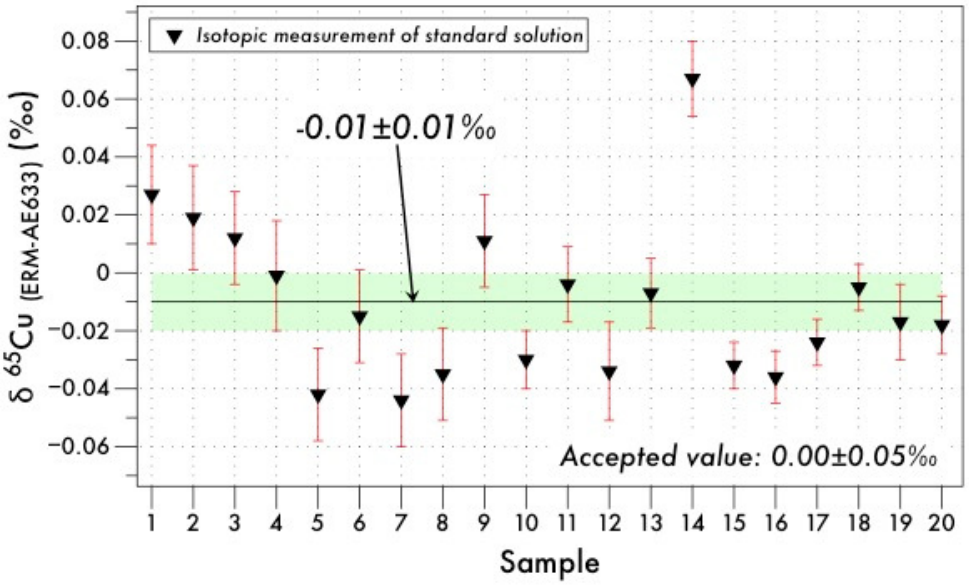

Figure 3. Repeatability of a pure 250 ppb $Cu_{ERM\text{-}AE633}$ solution spiked with Ni after processing through the column. (Accepted value 2SD, N=60, Ref. 26).

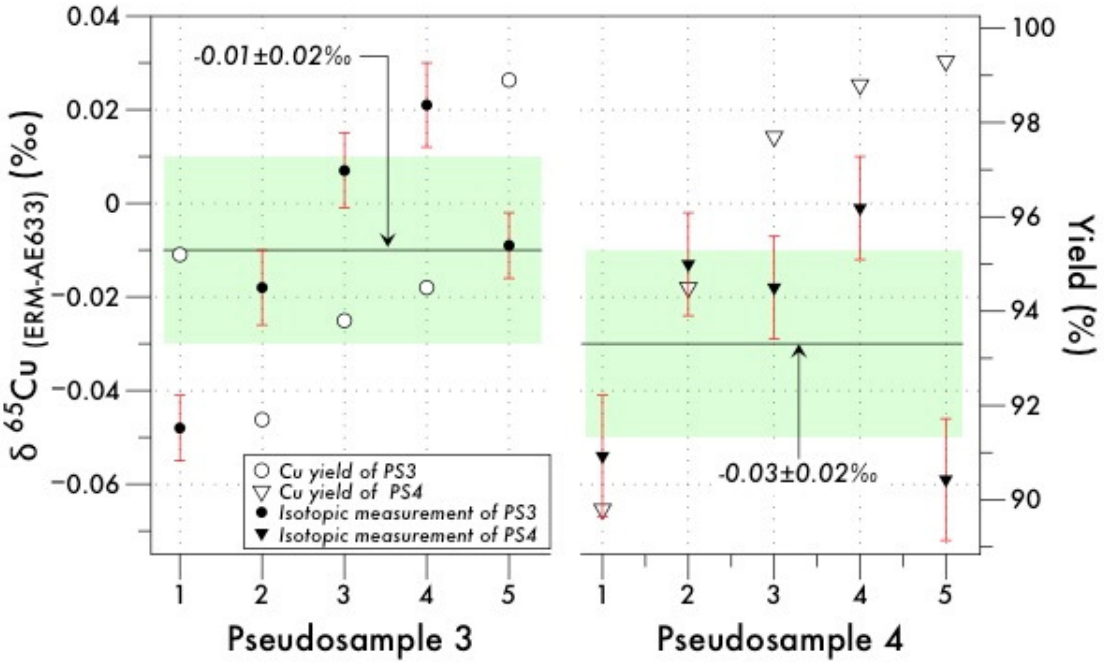

Figure 4. Repeatability of Cu isotope measurement for a sequence of randomly ordered analyses of pseudosamples 3 and 4, demonstrating a high degree of repeatability. Pseudosamples contained Cu from the ERM-AE633 certified reference material with an expected $\delta^{65}Cu_{ERM\text{-}AE633}$ of 0.00±0.94‰.[26]

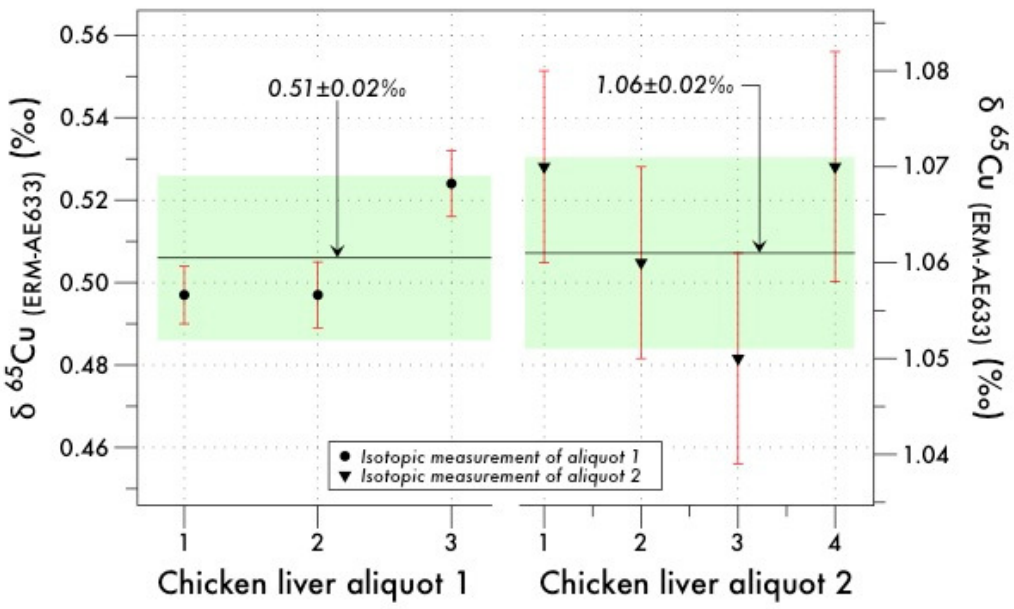

Figure 5. Repeatability of Cu isotope measurements for a sequence of randomly ordered analyses of chicken liver aliquot samples.