High-precision Cu isotopic analysis of human dietary Cu sources via multi-collector ICP-mass spectrometry

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Abstract

The disruption of Cu homeostasis is associated with the pathogenesis of many diseases and can result in alterations in Cu isotope fractionation. Changes in the Cu isotope ratio ($^{65}Cu/^{63}Cu$) of body fluids and tissues have been observed in liver disorders, cancers, and other diseases, displaying diagnostic/prognostic potential. However, it is not entirely clear whether certain physiological or lifestyle factors may also influence the bodily Cu isotopic composition,

potentially obfuscating the signature of the pathology. To ascertain whether differences in Cu isotopic composition exist between food products, the isotopic composition of 29 significant dietary Cu sources have been determined for the first time. The general enrichment of Cu and its heavy isotope, ^{65}Cu, in legumes, nuts, and seeds (major Cu sources in plant-based diets) was revealed, suggesting that individuals with plant-based diets may have a bodily Cu isotopic composition offset from that of individuals with omnivorous diets, and require controlling for in study design.

Keywords (max. 6)

Copper; stable isotope ratio; isotope fractionation; MC-ICP-MS; human diet

1. Introduction

The transition metal copper (Cu) is essential for the functioning of organs and is involved in many metabolic processes in humans (Uauy et al., 1998). Copper makes its way into the body primarily through the consumption of foods, but drinking water can account for up to 13% of the average daily intake (Fitzgerald, 1998; Gaetke & Chow, 2003). Once in the body, a small amount of Cu is absorbed in the stomach, but the majority of dietary Cu is absorbed through the small intestine (Bertinato & L'Abbé, 2004; Gaetke & Chow, 2003; Turnlund, 1998; Turnlund et al., 1997) This is largely mediated by high-affinity Cu uptake protein 1 (CTR1 – also known as solute carrier family 31 member 1) following the reduction of Cu^{2+} to Cu^{+} by the STEAP family of cell surface metalloreductases (Bertinato & L'Abbé, 2004; Gaetke & Chow, 2003; Kim et al., 2013; Kuo et al., 2001; J. Lee et al., 2001; Lutsenko et al., 2007; Tsang et al., 2021; Turnlund et al., 1997; Turnlund, 1998). Upon uptake in the small intestine, Cu is bound to a chaperone protein and transported by portal blood to the liver (principal storage site of Cu), where it is absorbed by hepatocytes (Bertinato & L'Abbé, 2004; Gaetke & Chow, 2003; Lutsenko et al., 2007; Turnlund, 1998; Turnlund et al., 1997). Copper is then incorporated into the Cu-dependent ferroxidase ceruloplasmin (major Cu-containing protein in serum) and released into the bloodstream for distribution to the rest of the body (Bertinato & L'Abbé, 2004; Lutsenko et al., 2007; Turnlund, 1998). The European Food Safety Authority (EFSA) defines the adequate intake of Cu as 1.6 mg/day for adult men and 1.3 mg/day for women (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2015). In healthy individuals, excess Cu is removed from the body via biliary excretion, which helps prevent systemic overloading of Cu (Hamza & Gitlin, 2013).

The disruption of Cu homeostasis is associated with the pathogenesis of many diseases and can result in alterations in Cu isotope fractionation, *i.e.* the relative partitioning of the naturally-occurring "heavy" (^{65}Cu) and "light" (^{63}Cu) isotope between two coexisting phases in a system (Bigeleisen & Mayer, 1947; Urey, 1947). Systematic changes in the Cu isotope ratio ($\delta^{65/63}$Cu, see below) in body fluids and tissues have been observed in various liver disorders, cancers, and other diseases, displaying diagnostic and prognostic potential (Costas-Rodríguez et al., 2015; Mahan et al., 2020; Sullivan et al., 2023; Vanhaecke & Costas-Rodríguez, 2021). However, it is unclear whether certain physiological (e.g., obesity, genetic variations) or lifestyle factors (e.g., diet, physical activity) may also influence the Cu isotope ratio in blood fractions (e.g., whole blood, serum/plasma), potentially obfuscating the signature of the pathology (Sullivan et al., 2023). The available evidence indicates that the occurrence of menstruation affects the whole blood and serum Cu isotopic composition, but blood type, timing of the most recent meal, age, and sex (when compared between non-menstruating individuals) do not (Albarède et al., 2011; Jaouen, Gibert, et al., 2013; Jaouen & Balter, 2014; Lauwens et al., 2017; Sullivan et al., 2023; Van Heghe et al., 2012, 2014). One investigation determined the Cu isotopic composition of whole blood from six (three male, three female) omnivorous and six (three male, three female) vegetarian individuals, but this data was inconclusive and the sample size too small to draw any definitive conclusions (Van Heghe et al., 2012).

The Cu isotopic composition of human dietary Cu sources is yet to be reported, and the extent to which the Cu isotopic composition of ingested Cu may be reflected in the Cu isotopic composition of different compartments within the body is unknown. What little is known is inferred from studies conducted on plants and animals of different trophic levels (Chifflet et al., 2022; Jaouen, Pons, et al., 2013). For example, the Cu isotope ratio of carnivore jaw bones (n = 2) was found to be ~1‰ heavier than both herbivore jaw bones (n = 2) and plants (n = 2) in the Kruger National Park (South Africa) trophic chain (Jaouen, Pons, et al., 2013). An investigation of the Fe isotopic composition of human dietary components found that despite plant-derived foods having a lighter Fe isotopic composition relative to animal tissue (with minimal overlap), there is strong evidence that the blood Fe isotopic composition of vegetarians and omnivores does not differ (von Blanckenburg et al., 2013). This is likely due to the haem molecule, which contains the majority of Fe in both blood and muscle tissues. The absorption efficiency of haem-bound Fe is higher (15 to 35%) than that of non-haem Fe (2 to 20%), and Fe bound to haem passes through the intestinal mucosa without ligand exchange, allowing the preservation of its lower δ^{56}Fe value (von Blanckenburg et al., 2013). Plant Fe is predominantly in the ferric or Fe(III) form but undergoes reduction (favouring the lighter Fe isotopes) in the intestine, resulting in a Fe isotopic composition similar to haem-bound Fe in human blood (von Blanckenburg et al., 2013). Conversely, the relative enrichment of the heavier Zn isotopes in animal-based food compared to fruits and vegetables was found to translate into differences in the blood and urine Zn isotopic composition between omnivores and vegetarians (Costas-Rodríguez et al., 2014).

Given that Cu has been subject to the most investigation amongst non-traditional (Mg, K, Ca, Fe, Zn) stable isotopic systems in the context of biomedicine, it is imperative that steps are taken to elucidate whether diet should be controlled for in the design of studies on Cu isotope ratio variations in human subjects (Sullivan et al., 2022, 2023). As a first step towards addressing this question, the δ^{65}Cu value of 29 significant dietary Cu sources has been determined for the first time (to the best of our knowledge) (Bost et al., 2016; Henriksen & Arnesen, 2023). This data will reveal whether systematic differences in Cu isotopic composition exist between food types, and indicate whether there is the potential for individuals with plant-based diets to have a bodily Cu isotopic composition offset from individuals with omnivorous diets. Further, this data will indicate whether controlled feeding experiments are warranted to define the extent of Cu isotopic composition transfer from diet to the organism *in vivo*. Due to a wide variety of matrix types being analyzed, 12 matrix reference materials (seven previously uncharacterized) were also included to aid with method validation and expand the range of materials characterized for their δ^{65}Cu value.

2. Materials and methods

2.1 Reagents and Materials

Ultrapure water (resistivity > 18.2 MΩ cm) was obtained from a Milli-Q Element water purification system (Merck Millipore, Bedford, MA, USA). Trace metal analysis grade 14 M HNO_3 and 12 M HCl (Fisher Chemicals, Loughborough, UK) were further purified by sub-boiling distillation in a Savillex DST-4000 acid purification system (Savillex Corporation, Eden Prairie, MN, USA). Ultrapure TraceSELECT® 9.8 M hydrogen peroxide and ACS grade acetone were purchased from Sigma Aldrich (Overijse, Belgium). Copper standard solutions were prepared from a Cu standard solution (1,000 mg L^{-1}) from Inorganic Ventures (Christiansburg, VA, USA) and used for quantification purposes. A 1,000 mg L^{-1} Ga standard solution (Inorganic Ventures) was used as an internal standard (final concentration = 10 ng mL^{-1}) in the Cu quantification. A previously-characterized Cu standard solution (Inorganic Ventures) was measured every five samples to monitor the accuracy and precision of the Cu isotope ratio measurements (Hastuti, Costas-Rodríguez, Anoshkina, et al., 2020; Hastuti, Costas-Rodríguez, Matsunaga, et al., 2020; Lauwens et al., 2017; Van Heghe et al., 2012). A wide range of matrix reference materials were characterized to aid with the validation of the Cu quantification and Cu isotope ratio measurements. These include Seronorm™ Trace Elements Serum L-1 reference material (lot 1801802, SERO AS, Billingstad, Norway), DORM-4 (fish protein; National Research Council (NRC), Ottawa, Ontario, Canada), DORM-5 (fish protein; NRC), TORT-3 (lobster hepatopancreas; NRC), BCR-669 (Tuna muscle; Community Bureau of Reference (BCR), the former reference materials programme of the European Commission, Geel, Belgium), BCR-668 (mussel tissue; BCR), BCR-278 (mussel tissue; BCR), BCR-184 (bovine muscle; BCR), NBS 1577c (bovine liver; National Bureau of Standards (NBS), Gaithersburg, MD, USA), Green Algae (*Chlorella sp.*; ANALYTIKA Co. Ltd., Prague, Czechia), BCR-189 (wholemeal flour; BCR), and BCR-191 (brown bread; BCR). Two-mL polypropylene chromatographic columns (Eichrom Technologies, Saint Jacques de la Lande, France) and AG® MP-1M (100-200 mesh) anion exchange resin (chloride form) purchased from Bio-Rad (Temse, Belgium) were used for Cu isolation. Food samples were purchased from grocery stores in the city of Ghent (Belgium) and all available information for each item was recorded (see Supplementary Material).

2.2 Sample preparation

Various procedures were required to prepare food samples ahead of sample digestion. Approximately 1 to 5 g of material was pooled and homogenized, from which at least 0.1 g of material was taken for analysis. Dried nuts, seeds, and legumes were powdered using a mortar and pestle, which was rinsed with ultrapure water between samples. Whole blueberries were thoroughly washed with ultrapure water to remove surface contamination. The inner part of individual apple, banana, carrot, orange, and tomato were taken and homogenized using metal-free implements. Small pieces of meat and seafood samples were taken from the inner part and digested whole. A chicken egg was homogenized through stirring. For tap water, approximately 30 mL was collected after running for two minutes at building S12 on Campus Sterre, Ghent University. Flour, cacao powder, and tap water required no pre-preparation. Samples were transferred (after any necessary preparation) into 15 or 50 mL metal-free centrifuge tubes (Labcon, Langenhagen, Germany). Sample preparation was performed in a class-10 clean room laboratory (PicoTrace, Göttingen, Germany). Between 0.05 and 1 g (wet weight) of samples were weighed into Savillex® PFA beakers and digested using 5 mL of 14 M HNO_3 and 1 mL of 9.8 M H_2O_2 at 110 °C for 24 h. Tap water was preconcentrated by evaporation a hotplate at 90 °C. Following digestion and cooling, the vessels were opened and the samples were evaporated to dryness at 90 °C. If digestion was incomplete, the digestion procedure was repeated after evaporation to dryness. Digested residues were dissolved in 5.5 mL of 8 M HCl.

For Cu concentration determination using ICP-mass spectrometry (ICP-MS), a small aliquot of each digested sample (in 8 M HCl) was taken, evaporated to dryness at 90 °C, and the residue dissolved in 0.3 M HNO_3 to a concentration of approximately 5 ng mL^{-1}. Quantification relied on external calibration. To correct for potential signal drift, matrix effects, and instrument instability during Cu quantification, Ga (10 ng mL^{-1}) was used as an internal standard.

Prior to Cu isotopic analysis, the chromatographic isolation of Cu was performed using the method described by Costas-Rodríguez *et al*. (Costas-Rodríguez et al., 2015). Following this, purified Cu solutions were evaporated to dryness at 90 °C and refluxed in 1 mL of 14 M HNO_3 in closed vessels for 24 hours at 110 °C before being evaporated to dryness again at 90 °C (Maréchal et al., 1999). Refluxing in 1 mL of 14 M HNO_3 and evaporation to dryness was then repeated. The purified Cu fractions were diluted in 0.3 M HNO_3 (to a Cu concentration of 50 ng mL^{-1}) and doped with Ga (to a Ga concentration of 100 ng mL^{-1}). Copper isotopic analysis was carried out using multi-collector ICP-mass spectrometry. A combination of external correction using a standard measured in a sample-standard bracketing (SSB) sequence and internal correction using Ga as an internal standard was used to correct for the mass bias induced by instrumental mass discrimination (Hou et al., 2016; Kidder et al., 2020; Lauwens et al., 2018; Sullivan et al., 2020; Yang et al., 2018). Internal correction was performed according to the revised Russell's law (also referred to as Baxter's approach) (Baxter et al., 2006). The Cu concentration in samples and the bracketing standard used for external correction were each respectively matched to within 10% to ensure accurate isotope ratio measurements (Liu et al., 2014; Yang et al., 2018).

2.3 Instrumentation and measurements

The Cu concentration was determined using an Agilent 8800 tandem ICP-MS (ICP-MS/MS) instrument (Agilent Technologies, Tokyo, Japan). The collision reaction cell was pressurized with a NH_3/He gas mixture (9.93 ± 0.20 mol% NH_3 in He, CRYSTAL mixture, Air Liquide, Brussels, Belgium) and additional pure He (99.9999%, ALPHAGAZ™ 2, Air Liquide).

The ^{63}Cu nuclide was monitored in a mass-shift approach (Q1 = 63, Q2 = 97; reaction product ion monitored: $^{63}Cu(^{14}N^{1}H_3)_2^{+}$), whereas ^{71}Ga was monitored on-mass (Bolea-Fernandez et al., 2021).

Table 1 Agilent 8800 ICP-MS/MS operating conditions.

Instrument settings	
Sample uptake rate	0.35 mL min^{-1}
Plasma gas flow rate	15.0 L Ar min^{-1}
Auxiliary gas flow rate	0.9 L Ar min^{-1}
Nebulizer gas flow rate	~1.12 L Ar min^{-1}
Collision gas flow rate	1.0 mL He min^{-1}
Reaction gas flow rate	3.0 mL NH_3/He (10%/90%) min^{-1}
RF power	1550 W
Sampling cone	Ni tip with Cu base
Skimmer cone	Ni
Data acquisition parameters	
Integration time	1 s
Replicates	10
Sweeps	100
Mass-to-charge ratios monitored	$^{63}Cu^{+} \rightarrow {}^{63}Cu(^{14}N^{1}H_3)_2^{+}$, $^{71}Ga^{+}$ (on mass)

Instrument operating conditions can be found in Table 1.

The Cu isotope ratio ($^{65}Cu/^{63}Cu$) was measured using a Thermo Scientific Neptune *Plus* multi-collector inductively coupled plasma-mass spectrometry (MC-ICP-MS) instrument (Bremen, Germany). Instrument operating conditions can be found in Table 2.

Table 2 Neptune MC-ICP-MS operating conditions.

Instrument settings	
Forward power	1200 W
Plasma gas flow rate	15 L Ar min^{-1}
Auxiliary gas flow rate	0.70 to 0.95 L Ar min^{-1}
Nebulizer gas flow rate	~1 L Ar min^{-1}
Sampling cone	Nickel, Jet-type: 1.1 mm orifice diameter
Skimmer cone	Nickel, X-type: 0.8 mm orifice diameter
Sample uptake	0.1 mL min^{-1}
Mass resolution mode	Low
Data acquisition parameters	
Faraday cup configuration – all amplifiers used were equipped with a 10^{11} Ω resistor	L4 (^{63}Cu), L2 (^{65}Cu), H2 (^{69}Ga), H4 (^{71}Ga)
Sensitivity	3.1 V for ^{63}Cu at 50 ng mL^{-1}; 5.8 V for ^{69}Ga at 100 ng mL^{-1}
Signal integration time	4.194 s
No. of integrations, blocks, cycles/block	1, 1, 45
Blank signal (2% HNO_3)	0.001V for ^{63}Cu

Because natural variations in the ratio of ^{65}Cu and ^{63}Cu abundances (^{65}Cu/^{63}Cu) are very small, data are reported in delta (δ) notation (Equation (1)), which denotes the parts per thousand or 'per mille' (‰) difference in the sample relative to the international isotopic standard NIST SRM 976 (National Institute for Standards and Technology, Gaithersburg, MD, USA).

$$\delta^{65}\mathrm{Cu}_{\mathrm{sample}} = \left(\frac{(^{65}\mathrm{Cu}/^{63}\mathrm{Cu})_{\mathrm{sample}}}{(^{65}\mathrm{Cu}/^{63}\mathrm{Cu})_{\mathrm{SRM\ 976}}} - 1\right) \cdot 1{,}000 \qquad (1)$$

3. Results

3.1 Quality assurance/quality control

To quantify the contribution of Cu contamination occurring during sample preparation, a procedural blank was processed under the same conditions with each batch of samples. Total procedural blank Cu contributions represented less than 0.08% of Cu loaded onto the columns for all materials. Post-chromatography Cu recovery was regularly monitored and a quantitative (96.4 ± 9.7%, 1SD), high-purity (matrix element/Cu < 0.1) yield was achieved. The Cu quantification method was validated using human serum (Seronorm™ Trace Elements Serum L-1), fish protein (DORM-5), and lobster hepatopancreas (TORT-3) reference materials (Table S1). Isotopic analysis of the Cu standard solution (Inorganic Ventures, lot C2-Cu02116) and matrix reference materials yielded $\delta^{65}Cu$ values that are in agreement with data from previous studies (Table 3). The homogeneity of samples was assessed through the analysis of multiple aliquots of the same sample (biological replicates) that were processed through the entire analytical procedure separately (Tables S2 and S3). This was performed for banana (Ecuador), blueberry (Peru), and tomato (Spain), and reproducibility was within an acceptable range (0.04 to 0.13‰ 2SD; Table S2) (Hastuti, Costas-Rodríguez, Matsunaga, et al., 2020; Lauwens et al., 2016; Sullivan et al., 2020).

Table 3 δ^{65}Cu value of reference materials.

Reference material	Material type	δ^{65}Cu$_{SRM\ 976}$ (‰)	2SD (‰)	n(*l*)	Literature δ^{65}Cu ± 2SE (‰)	Reference
In-house Cu standard	Pure Cu solution, Inorganic Ventures, VA, USA	0.22	0.04	46	0.23 ± 0.01*	Hastuti, Costas-Rodríguez, Anoshkina, et al., 2020; Hastuti, Costas-Rodríguez, Matsunaga, et al., 2020; Lauwens et al., 2017; Van Heghe et al., 2012
Seronorm serum L-1	Human serum	-0.15	0.13	6(2)	-0.20 ± 0.05	Sullivan et al., 2022
TORT-3	Lobster hepatopancreas	0.35	0.15	10(3)	0.39 ± 0.09	Sullivan et al., 2022
DORM-4	Fish protein	0.48	0.02	3(1)	0.53 ± 0.08	Sullivan et al., 2022
DORM-5	Fish protein	0.13	0.10	10(3)		
BCR-669	Tuna muscle	0.02	0.03	3(1)		
BCR-668	Mussel tissue	0.09	0.02	3(1)		
BCR-278	Mussel tissue	0.10	0.03	3(1)	0.08 ± 0.04	Maréchal et al., 1999
BCR-184	Bovine muscle	0.00	0.04	3(1)		
NBS 1577c	Bovine liver	0.28	0.04	3(1)	0.37 ± 0.02	Sauzéat et al., 2021
Green algae	*Chlorella sp.*	-0.12	0.01	3(1)		
BCR-189	Wholemeal flour	-0.31	0.05	3(1)		
BCR-191	Brown bread	-0.35	0.05	3(1)		

n = number of measurements performed (measurement replicates), *l* = number of separate aliquots of material processed (biological replicates). 2SD = two times standard deviation. 2SE = two times standard error. Data from Sullivan et al., 2022

3.2 Determination of δ^{65}Cu values in biological reference materials

The δ^{65}Cu values of seven matrix reference materials are reported for the first time (to our knowledge) (Table 3). This expands the range of matrix reference materials characterized for δ^{65}Cu to tuna muscle (BCR-669), mussel tissue (BCR-668), bovine muscle (BCR-184), *Chlorella sp.* (Green Algae), wholemeal flour (BCR-189), and brown bread (BCR-191). The latest fish protein reference material from NRC (DORM-5) was also characterized, and yielded a δ^{65}Cu value of 0.13 ± 0.10‰ (2SD) which is different from that of the previous iteration, DORM-4 (0.53 ± 0.10‰, 2SE) (Sullivan et al., 2022). As detailed in their certificates of analysis, both DORM-4 and DORM-

5 were prepared from a commercially available fish protein homogenate and the materials were produced using an enzymatic hydrolysis procedure subsequent to the removal of the bones and the majority of the oil. The production methods slightly differ from this point onwards, with the protein hydrolysate of DORM-5 being flash-pasteurized prior to being spray-dried and sieved using an 850 μm nylon screen before being blended and bottled, whereas DORM-4 did not undergo flash-pasteurization and was sieved using a 297 μm screen (material of screen not specified). The Cu concentration of DORM-4 is also almost 5-fold higher than in DORM-5. The differences in Cu concentration and δ^{65}Cu value between the two reference materials could be due to different sources/batches of fish protein homogenate being used for production and/or material pre-treatment (flash pasteurization and sieving procedures, 297 μm vs. 850 μm).

3.3 Copper concentration

Copper concentrations determined for human dietary Cu sources in this study are wet weight and comparable to those reported in literature (Bost et al., 2016; Cabrera et al., 2003; Ceko et al., 2014; Jorhem & Sundström, 1993). Legumes, nuts, and seeds contained the highest Cu concentrations of any materials and ranged from 1.6 (pea) to 70.2 μg g^{-1} (cacao powder, Germany) (Table S3). However, excluding the two cacao powders and coffee bean sample, which are highly processed during their production, the upper range of the Cu concentration was 21.5 μg g^{-1} (cashew nut). Fruits and vegetables were systematically lower in Cu concentration, ranging from 0.2 (apple) to 1.3 μg g^{-1} (banana, Costa Rica) (Table S2). Bananas from four countries in Central (Costa Rica, Panama) and South America (Colombia, Ecuador) were analyzed and yielded remarkably consistent Cu concentrations (1.1 ± 0.2 μg g^{-1}, 1SD). Blueberries from Chile and Peru were also analyzed and yielded similar Cu concentrations (0.5 and 0.6 μg g^{-1}, respectively). In general, animal-derived food products had similar Cu concentrations to fruits and vegetables, and ranged from 0.2 (salmon) to 1.0 μg g^{-1} (shrimp), but unfertilized chicken egg had a Cu concentration of 8.8 μg g^{-1} (Table S2). Whole wheat flour had an elevated Cu concentration (14.8 μg g^{-1}) relative to white wheat flour (1.2 μg g^{-1}). Although flour samples analyzed in this study were sourced from different producers, the enrichment of Cu and other nutrients in whole wheat relative to white wheat flour is consistent with literature (Ertl & Goessler, 2018). The lowest Cu concentration of the study was observed in tap water (15 ng mL^{-1}) (Table S2).

3.4 Copper isotopic composition

Variations in δ^{65}Cu across food types are visually represented in Fig. 1. The δ^{65}Cu of legumes, nuts, and seeds ranged from -0.18 to 1.00‰, but when coffee bean and cacao powders (products that have undergone significant processing during their preparation) are excluded, the lower end of this range increases to 0.44‰, and a mean value of 0.69 ± 0.18‰ (1SD) is obtained (Table S3). Fruits and vegetables ranged from -0.20 (apple) to 0.57‰ (banana, Panama), with a mean value of 0.05 ± 0.21‰ (1SD; means used for foods with multiple samples measured) (Table S2). In contrast to Cu concentrations in bananas studied being largely invariable, their δ^{65}Cu values varied considerably and ranged from -0.15 (Costa Rica) to 0.57‰ (Panama). The δ^{65}Cu values determined in blueberries were -0.19‰ (Chile) and 0.01‰ (Peru). Animal products display the largest variations in this study and range from -0.32‰ in unfertilized chicken egg to 0.19‰ in rump steak to 0.50‰ in shrimp to 0.89‰ in salmon, displaying an enrichment of ^{65}Cu in higher trophic level consumers (Table S2). White wheat flour displays the lowest δ^{65}Cu value in this study (-0.54‰), and differs greatly from the whole wheat flour (0.03‰) (Table S2). A δ^{65}Cu value of -0.04‰ was determined in tap water (Table S2).

4. Discussion

4.1 Enrichment of ^{65}Cu in nuts, seeds, and legumes

Despite the δ^{65}Cu values of human dietary Cu sources analyzed in this study spanning 1.54‰ (-0.54‰ in white wheat flour to 1.00‰ in chickpea), the only clear trend that emerges across food types is the elevated δ^{65}Cu value of nuts, seeds, and legumes (excluding cacao powder and coffee bean), which range from 0.44 to 1.00‰ (Fig. 1). The Cu concentrations also tends to be elevated in these food products, ranging from 1.6 to 21.5 μg g^{-1}. However, no overall relationship exists between Cu concentration and δ^{65}Cu. Although certain Cu sources can have considerable isotopic variability (discussed below) and just one sample from each of the nuts, seeds, and legumes was analyzed, the likelihood of a diverse range of nuts, seeds, and legumes centering around a relatively narrow range of elevated δ^{65}Cu values (0.69 ± 0.18‰, 1SD) by chance is low. Due to nuts, seeds, and legumes being major protein (and Cu) sources in vegetarian and vegan diets, and being both enriched in Cu and its heavy isotope (^{65}Cu), it is possible that such diets may result in elevated bodily δ^{65}Cu values relative to omnivorous diets (Mariotti & Gardner, 2019). However, another dimension of this reasoning is in the differences in Cu bioavailability between plant- and animal-based Cu sources: plant-based diets have slightly reduced Cu bioavailability resulting from the presence of fibers and phytates (purportedly), but their higher Cu content (up to more than twice that of omnivorous diets) has consistently been shown to sufficiently compensate for this (~20% more Cu absorbed) (Abdulla et al., 1981; Bost et al., 2016; Donovan & Gibson, 1996; Hunt & Vanderpool, 2001; Turnlund, 1983; Turnlund et al., 1985). It must be noted that although phytates have been reported to inhibit absorption of Mg, Ca, Fe, Mn, and Zn in studies involving humans, this is not the case for Cu (Bohn et al., 2008). There is even evidence that phytates may actually enhance Cu bioavailability (D.-Y. Lee et al., 1988). The enrichment of ^{65}Cu in nuts, seeds, and legumes may be due to the chelation of Cu^{2+} by phosphate groups in phytate, and the large discrepancy between white and whole wheat flour Cu content (1.2 vs. 14.8 µg g^{-1}, respectively) and δ^{65}Cu values of (-0.54 vs. 0.03‰, respectively) may reflect the separation of the germ and fiber- and nutrient-rich bran from endosperm (Fujii et al., 2014; Kremer et al., 2020). As a refined flour, white wheat flour also contains less phytates (Febles et al., 2002). That said, further data is required to conclude whether this is a systematic effect, and not one resulting from differences in growth conditions (e.g., soil type, climate, among others). With a plausible mechanism for the enrichment of ^{65}Cu in individuals with nut-, seed-, and legume-heavy diets, the question remains whether there is a transfer of the dietary Cu isotopic composition into consumers (see 4.2).

INSERT FIGURE 1

4.2 Influence of diet on δ^{65}Cu values in animal model and trophic level studies

To assess whether dietary Cu isotopic compositions can be transferred into consumers, the available evidence from animal models and trophic level studies can be examined. Three studies have included investigations of Cu isotope ratio variations across different organs and fluids of similarly aged mice (3 to 5 months) and found that despite the different δ^{65}Cu values of their food (0.15 ‰, 0.33‰, and 0.55‰), their organ and biofluid δ^{65}Cu values were in remarkable agreement and showed no specific trends. (Fig. 2) (Balter et al., 2013; Costas-Rodríguez et al., 2019; Moynier et al., 2022). Interestingly, from the four compartments for which data from the three studies were complete (kidney, brain, muscle, and liver), the δ^{65}Cu of the diets was only conserved in the liver, the principal storage site of Cu. Drinking water can significantly contribute to overall dietary Cu intake, but the $\delta^{65}Cu$ value of water was characterized in one study only, so it is not possible to factor this into the overall $\delta^{65}Cu$ value of the diets (Costas-Rodríguez et al., 2019). Based on the limited data available, it remains inconclusive as to whether the dietary Cu isotopic composition is conserved elsewhere in the body following ingestion.

INSERT FIGURE 2

As discussed earlier, there is evidence (albeit limited) that mammals up the trophic chain are enriched in ^{65}Cu (Jaouen, Pons, et al., 2013). This also extends to the planktonic food web, for which the $\delta^{15}N$ tracer was used to evaluate the trophic level and it was shown that higher trophic levels are enriched in ^{65}Cu (Chifflet et al., 2022). Therefore, although emerging evidence supports the preferred transfer of ^{65}Cu up the trophic chains, this still remains to be demonstrated in animal models and studies involving the analysis of blood fractions from human subjects with plant-based and omnivorous diets. Controlled animal feeding experiments conducted by Tacail *et al.* demonstrated that the dietary K isotopic composition is the primary control of inter-individual variability (healthy subjects); this study could potentially serve as a basis for a future investigation of the influence of diet on the bodily Cu isotopic composition (Tacail et al., 2023). Potentially complicating matters, legumes, nuts, and seeds have a similarly heavy Cu isotopic composition to higher trophic level consumers (shrimp, salmon), but the risk of this influencing the bodily Cu isotopic composition is small due to the very minor dietary Cu contribution of fish and seafood in most diets (Bost et al., 2016).

4.3 Variability in the Cu isotopic composition of the Cavendish banana

This study was inspired by the research of Von Blanckenburg *et al*. and Costas-Rodríguez *et al*., which characterized the Fe and Zn isotopic composition (respectively) of human dietary components (Costas-Rodríguez et al., 2014; von Blanckenburg et al., 2013). Costas-Rodríguez *et al*. characterized single samples of food products, whereas Von Blanckenburg *et al*. incorporated the study of source-specific variability of the Fe isotopic composition in food types – sometimes analyzing two or three samples from a particular food. The results were remarkably consistent (typical range of 0.1 to 0.2‰ or less), with few cases of differences greater than this. However, only information on the supermarket and producer were provided. Without the country of origin, it is not possible to ascertain the extent to which the growing conditions of these products differed, and whether that can affect isotope fractionation. To gain information on how large variability in the Cu isotopic composition can be for a single dietary component and assess whether the δ^{65}Cu determined from a single sample can be deemed representative of a food type, the δ^{65}Cu value of bananas from four countries were determined. The banana was specifically selected for this as predominantly one type (Cavendish banana from the Cavendish subgroup of the AAA banana cultivar group) is produced and consumed worldwide. Specifically, Cavendish bananas account for 47% of global banana production and 99% of bananas intended for international export to developed countries (*Banana Facts and Figures*, 2024). This ensures that inter-sample genetic variability is minimized and the variables are limited to differences in growing conditions. However, numerous factors can potentially alter the Cu isotopic composition of produce, such as climate, soil, slurry/manure spreading, the use of Cu compounds as bactericides or fungicides, and the Cu emissions from nearby smelting and casting industries (Bost et al., 2016; Sullivan et al., 2022). The bananas from the two South American countries, Ecuador and Colombia, had similar δ^{65}Cu values (0.38 ± 0.05‰ (2SD) and 0.32 ± 0.04‰ (2SD), respectively), whereas the bananas from the two neighbouring Central American countries, Costa Rica and Panama, differed by 0.72‰ (δ^{65}Cu = -0.15 ± 0.05‰ (2SD) and 0.57 ± 0.05‰ (2SD), respectively). Due to the numerous factors that could alter growing conditions, it is difficult to invoke a particular cause for this discrepancy, although anthropogenic activities have been linked to elevated δ^{65}Cu values (Sullivan et al., 2022).

5. Conclusions

The δ^{65}Cu value of 29 dietary Cu sources was determined for the first time (to the best of our knowledge). The data reveal the general enrichment of Cu and its heavy isotope, ^{65}Cu, in unprocessed legumes, nuts, and seeds (major Cu sources in plant-based diets), with Cu concentrations ranging from 1.6 (pea) to 21.5 µg g^{-1} (cashew nut) and δ^{65}Cu values ranging from 0.44 (black bean) to 1.00‰ (chickpea), with a mean of 0.69 ± 0.18‰ (1SD). These Cu concentrations and δ^{65}Cu values are considerably elevated relative to the fruits and vegetables, flours, and tap water studied (mean > 0.6‰). There is some overlap with the Cu isotopic composition of higher trophic level consumers (shrimp δ^{65}Cu = 0.50‰; salmon δ^{65}Cu = 0.89‰), but with Cu concentrations of 0.2 (salmon) and 1.0 µg g^{-1} (shrimp), these represent relatively minor Cu sources in most diets. Taken together, this suggests that there is potential for individuals with plant-based diets to have a bodily Cu isotopic composition that is offset from individuals with omnivorous diets. Ultimately, this data indicates that controlled animal feeding experiments are warranted to define the extent of Cu isotopic composition transfer from diet to the organism *in vivo*. This will reveal whether diet must be controlled for in the design of studies on Cu isotope ratio variations in human subjects.

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Conflicts of interest

There are no conflicts to declare.

Author contributions: CRediT

Conceptualization, F.V., K.V.S., K.M. and M.C.-R.; Methodology, F.V., K.V.S., K.M. and M.C.-R.; Software, K.V.S.; Validation, K.V.S., K.M., M.C.-R., E.B.-F.; Formal analysis, K.V.S.; Investigation, K.V.S. and K.M.; Resources, F.V.; Data Curation, K.V.S. and K.M.; Writing—Original Draft Preparation, K.V.S. and K.M.; Writing—Review & Editing, F.V., K.V.S., M.C.-R.

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Supplementary materials to:

High-precision Cu isotopic analysis of human dietary Cu sources via multi-collector ICP-mass spectrometry

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Table S1 Copper concentration of matrix reference materials

Reference material	**Experimentally determined Cu concentration**			**Certified/reference Cu concentration**	
	Mean	**1SD**	**n(*l*)**	**Mass fraction**	**Expanded uncertainty (*k* = 2)**
Seronorm™ Trace Elements Serum L-1 (ng mL^{-1})	1,265		1(1)	1,128	227
DORM-5 (mg kg^{-1})	3.32	0.46	2(2)	3.30	0.07
TORT-3 (mg kg^{-1})	514	59	2(2)	497	22

1SD = standard deviation; n = number of measurements performed (measurement replicates), *l* = number of separate aliquots of material processed (biological replicates).

Table S2 Copper concentration and isotopic composition of vegetables, fruits, wheat, animal products, and tap water.

Food	**Provenance**	**Cu (μg g^{-1})**	**$\delta^{65}Cu_{SRM\ 976}$ (‰)**	**2SD (‰)**	**n(*l*)**
Vegetables					
Carrot	Belgium	0.5	0.33	0.04	3(1)
Potato	France	0.9	0.06	0.02	3(1)
Fruits					
Banana	Panama	0.9	0.57	0.01	3(1)
Banana (Bio)	Ecuador	0.9	0.38	0.05	3(1)
Banana	Colombia	1.2	0.32	0.04	6(2)
Banana	Costa Rica	1.3	-0.15	0.05	3(1)
Blueberry	Peru	0.6	0.01	0.08	6(2)
Blueberry	Chile	0.5	-0.19	0.10	3(1)
Orange	Spain	0.3	0.10	0.08	3(1)
Tomato (vining)	Spain	0.4	-0.18	0.13	6(2)
Apple (Jonagold)	Belgium	0.2	-0.20	0.05	3(1)
Wheat					
Whole wheat flour	Heverlee, Belgium	14.8	0.03	0.02	3(1)
White wheat flour	Belgium (Delhaize Group SA)	1.2	-0.54	0.02	3(1)
Animal products					
Salmon	Atlantic Ocean	0.2	0.89	0.07	3(1)
Shrimp (*Penaeus vannamei*, peeled, cooked)	Atlantic Ocean	1.0	0.50	0.03	3(1)
Rump steak	Belgium (Delhaize Group SA)	0.7	0.19	0.05	3(1)
Chicken egg (medium, free-range, unfertilized)	Belgium	8.8	-0.32	0.04	3(1)
Water					
Tap water	Ghent University, Belgium	15*	-0.04	0.02	3(1)

n = number of measurements performed (measurement replicates), *l* = number of separate aliquots of material processed (biological replicates). 2SD = standard deviation multiplied by two. *Reported in ng mL^{-1}. The typical precision on Cu concentration results as obtained with ICP-MS/MS was between 3 and 5%.

Table S3 Copper concentration and isotopic composition of nuts, seeds, and beans.

Food	**Provenance**	**Cu (µg g^{-1})**	**$\delta^{65}Cu_{SRM\ 976}$ (‰)**	**2SD (‰)**	**n(*l*)**
Nuts and seeds					
Sesame seed	Belgium (Delhaize Group SA)	15.8	0.77	0.04	3(1)
Sunflower seed	Romania	18.1	0.68	0.03	3(1)
Cashew nut	Ivory Coast	21.5	0.57	0.03	3(1)
Almond	California, USA	7.2	0.56	0.05	3(1)
Coffee bean	Café Progreso, Uganda, Honduras, Colombia, Guatemala, Peru	20.7	-0.18	0.04	3(1)
Cacao powder	Non-EU / produced in Germany	70.2	0.25	0.05	3(1)
Cacao powder	Jacques Belgian Chocolatier / Van Houten, Belgium	33.9	-0.07	0.08	3(1)
Legumes					
Chickpea	Italy	8.7	1.00	0.04	3(1)
Kidney bean	China	11.0	0.83	0.02	3(1)
Pea	Kenya	1.6	0.71	0.05	3(1)
Black bean	China	7.1	0.44	0.03	3(1)

n = number of measurements performed (measurement replicates), *l* = number of separate aliquots of material processed (biological replicates). 2SD = standard deviation multiplied by two. The typical precision on Cu concentration results as obtained with ICP-MS/MS was between 3 and 5%.

Highlights

- Copper isotopic composition of 29 dietary Cu sources determined for the first time
- Legumes, nuts, and seeds were enriched with Cu and its ‘heavy’ isotope, ^{65}Cu
- Significant variation of Cu isotopic composition in banana from various locations
- Copper isotopic composition of 7 matrix reference materials reported for first time