Examining the Utility of LA-ICP-MS for Detection of Time-resolved Zinc Exposures and Determination of Hair Growth Rate

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Abstract

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Hair is a unique matrix for metal deposition, as it offers a "timeline" of prior exposures (Stadlbauer et al., 2005). This spatiotemporal relationship of exposures along the hair can make possible the estimation of time and duration of exposures. Metals are known to deposit in the hair, and thus are a group of toxicants whose interventions and treatment could be aided by this timeline of information. Our study aimed to validate Laser Ablation Inductively Coupled Mass Spectroscopy (LA-ICP-MS) for measuring zinc in human hair samples and to use zinc supplementation to determine individual hair growth rates. Twenty-one participants took zinc gluconate supplements two weeks apart, followed by a hair sample collection two weeks later.

This study design aimed to produce two measurable exposures to zinc in the hairs of participants, from which hair growth rates could be calculated. These samples were washed, mounted, and analyzed after several experiments to optimize our analysis method. Although we ultimately were unable to measure hair growth rates using these hair samples, we were able to characterize concentrations of zinc, lead, and mercury in hairs from participants, and determine between and within subject variability in concentrations of those elements. In addition, we identified a suitable washout period to minimize carryover of analyte between successive samples, and we identified helium collision cell gas flows that minimized polyatomic interferences without compromising sensitivity for LA-ICP-MS. This analysis technique benefits the field of environmental health by offering time-resolved exposure information on public health-relevant metals such as zinc, lead, and mercury. This dissertation contributes meaningful method development on this application for LA-ICP-MS.

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I must also thank those in my life who have kept my spirits up when my studies were challenging, cheering me on and helping me feel loved and supported. My Mom, my Dad, Ofir, my friends. My dog Raya.

Contents

List of Tables and Figures

Chapter 1: Background Literature & Specific Aims

Our study attempts to utilize LA-ICP-MS to determine a hair growth rate for an individual, and to do so using a relatively non-toxic, essential metal – zinc—which exists in higher concentrations in the hair relative to trace metals like arsenic or mercury (Rodushkin & Axelsson, 2000). Determining an individual's hair growth rate allows for more precise biomonitoring in that individual, with higher confidence in the timing of an exposure. If applied to rapidly developing individuals like infants or fetuses, the accuracy of these growth rates becomes very important in interpreting what stage of a developmental process may have been affected for exposure to toxic agents.

Several studies have investigated metal exposures over time through hair samples using Laser Ablation Inductively Coupled Plasma Mass Spectroscopy (LA-ICP-MS) technology, but most of them cite an average hair growth rate that they did not themselves measure (Noël et al., 2015). These studies that have estimated times of exposures from LA-ICP-MS metal data have mostly looked at neurotoxic and trace metals like mercury and arsenic.

Biomonitoring for Metal Exposures

Current biomonitoring approaches for metal exposures include sampling blood, urine, hair, teeth, and nails (Heng et al., 2022; Arora et al., 2017; Parizanganeh et al., 2014). Hair is a unique matrix for metal deposition, as unlike the transient concentrations in urine and blood, its concentrations are cumulative (Sulek et al., 2014). This allows the hair shaft to offer a "timeline" of previous exposures, as well as a magnitude of exposure within deposition events (Stadlbauer et al., 2005). An added advantage of hair sampling is its noninvasive collection (Stadlbauer et al., 2005). This may increase willingness of individuals to participate in biomonitoring studies. Lastly, hair is a stable matrix that can be stored at room temperature and without biohazard

precautions (Stadlbauer et al., 2005). This makes sample collection and storage much easier, and thus metal screening more accessible.

Metal biomonitoring in hair has been well established with techniques like acid digestion followed by analysis with Inductively-Coupled Plasma Mass Spectroscopy (ICP-MS) (Stadlbauer et al., 2005). This technique digests the hair in a liquid matrix, and therefore loses the spatio-temporal relationship of metals in the hair. The first attempts to preserve the time-resolved nature of hair exposure data cut each hair into small sections before digesting each piece for ICP-MS analysis (Smith et al., 2015; Gellein et al., 2008). This technique has been applied to analysis of hair cortisol levels, trace metals, and even certain drugs (Smith et al., 2015; Gellein et al., 2008; Kuwayama et al., 2018). However, this method's resolution of exposure data is dependent on the physical segmentation of the hairs, which varies from a few millimeters to centimeters in length (Smith et al., 2015; Gellein et al., 2008; Kuwayama et al., 2018).

The data from LA-ICP-MS is collected continuously along the hair shaft, due to continuous ablation, meaning there is much greater resolution of time-resolved exposures than with microsegmental analysis (Stadlbauer et al., 2005). With an accurate hair growth rate, the time of an exposure can be determined based on the distance between two exposures seen in the hair. This has advantages for treatment of symptoms related to lead or heavy metal exposure, as clinicians could determine the duration of exposures, how long the patient has been exposed, or how long ago the most recent exposure occurred. There are differences in hair growth rates that have been attributed to gender differences, age, and even environmental seasons, but we were not focused on demographic patterns of growth in this study (LeBeau et al., 2011).

Hair growth and physiology

Hair growth occurs cyclically, with the follicle and hair shaft undergoing three phases*:* *Telogen, Anagen*, and *Catagen* (Tobin, 2005). The hair follicle differentiates and *develops in utero*, and begins its cycle of hair growth with a major apoptotic event, characterized by a large decrease in the number of hair follicle cells (Tobin, 2005). Afterward, the hair follicle enters a cycle of growth that starts with a phase called the Anagen phase (Tobin, 2005). The Anagen phase is the longest phase in the cycle, and can average 2-8 years. There is a short period of 2-3 weeks, after the Anagen phase, where the hair shaft decreases in size, this period is known as the *Catagen* phase of the hair growth cycle (Tobin, 2005). The hair within the follicle will then cease to grow, in what is known as the *Telogen* phase (Tobin, 2005). The *Telogen* phase is approximately three months in length, and one in ten hair follicles on the head will be in the *Telogen* phase at a given time (Tobin, 2005). The end of the Telogen phase is sometimes called the *Exogen* phase, and refers to the actual shedding of the hair from the follicle (Tobin, 2005). Once this has occurred, the cycle repeats back with the *Anagen* phase (Tobin, 2005). This cycle of Telogen, Anagen, and Catagen repeats until the end of follicle's life (Tobin, 2005). The scalp hair is estimated to grow 0.35 mm per day (~1 cm per month) during its *Anagen* phase (Tobin, 2005).

Measuring Hair Growth with Internal Biomarkers*:*

There have been previous studies validating LA-ICP-MS technology for detection of metals in hair (Duncan et al., 2021; Li et al., 2022; Noël et al., 2015; Pozebon et al., 2008). There have been some studies that have begun to estimate the time of exposures based on where along the hair shaft the peaks in metal concentration are detected (Li et al., 2022; Pozebon et al., 2008).

Few studies, however, have actually used metals exposure data from hair to estimate hair growth rates (Li et al., 2022; Pozebon et al., 2008).

One study did estimate individual hair growth rate using known time of drug exposure and concentration peaks in the hair analyzed using LA-ICP-MS (Li et al., 2022). In that study, hair samples were analyzed from patients receiving cancer medications with arsenic trioxide. The distance between arsenic peaks in the hair was divided by the time between administration dates, and the mean hair growth rate was 412 micrometers per day. (Li et al., 2022). Assuming 30 days in a month, this would give a growth rate of 1.24 centimeters per month.

Another study had a similar approach and similar results, but with a patient taking a platinum-containing anti-cancer drug, Cisplatin (Pozebon et al., 2008). In this study, LA-ICP-MS was used to analyze hairs from a single patient. The hair growth rate was calculated in the same manner as the rate reported by Li et al (2022), by dividing the distance between peaks in the hair element concentration, by the known time between doses. Pozebon et al (2008) calculated a hair growth rate of 276 micrometers per day. Assuming 30 days in a month, this growth rate translates to 0.828 centimeters per month.

Noel et al. (2015) successfully analyzed zinc concentrations in grizzly bear hair using LA-ICP-MS and successfully corresponded increased zinc levels with seasonal fish consumption, supporting the time-correlated position of these peaks (Noël et al., 2015). However, an estimated hair growth rate of 1.5 cm per month was used from literature and not calculated in this study (Noël et al., 2015).

Zinc was also part of the suite of metals analyzed via LA-ICP-MS in studies by Steely et al. (2007) and Sela et al. (2007). Steely et al. (2007) analyzed single hairs with LA-ICP-MS to determine variability of zinc (Zn64), lead (Pb208) and arsenic (As75) along the hair shaft. Steely

et al. (2007) found that zinc and lead both had identifiable peaks and variation above baseline hair signal, although this was not mapped to any specific exposure nor was it converted to concentration. Sela et al. (2007) also successfully analyzed hairs for zinc, as well as lead, copper, uranium, chromium, and iron. Sela et al. (2007) compared the concentrations determined via LA-ICP-MS with liquid-introduction ICP-MS and reported ranges for each element from their study as well as from literature. For zinc, the average concentration in this study was found to be 34.7 μg/g, although the published range referenced by Sela et al. (2007) for zinc is between 40 and 327 μg/g. These two studies confirm detectable concentrations of zinc in human hair and provide additional confidence in the feasibility of zinc peak determinations with LA-ICP-MS.

These studies showcase current literature on LA-ICP-MS analysis for metal exposure in hair, and the use of controlled dosing to elucidate hair growth rates. These studies highlight the large variation in hair growth rates being reported in LA-ICP-MS literature, but also the success of attempts to measure growth rate from individuals' hair strands.

Zinc Metabolism and Deposition

Zinc is a low-toxicity element known to be deposited into the hair shaft, making it a possible metal supplement that can safely be used for the purpose of establishing individual-specific hair growth rates (Ogawa et al., 2016). Zinc daily intake is estimated between 5.2-16.2 mg/day. (ATSDR, 2005; Fryar et al., 2021). The lowest observable adverse effect level (LOAEL) for zinc is 60 mg/day – based on chronic exposures (NAS, 2001). Zinc is also an essential element our body naturally absorbs and utilizes for essential processes (Wegmüller et al., 2014).

Zinc is absorbed mostly through the intestinal tract, and is incorporated into many proteins (Wegmüller et al., 2014). Zinc is absorbed by active transport across the walls of the

small intestine (Hambidge et al., 2005). Because of this, zinc transport proteins can become saturated, limiting the maximum amount of zinc absorbed at a given time (Hambidge et al., 2005). A review paper by Hambidge et al. (2005) discussed a dose-response curve of zinc from a healthy adult study given zinc in the fasted state (from Tran, et al (2004)), and compared these values with a composite dose-response curve from the Food and Nutrition board (2001), showing mean values from 10 studies used to determine Recommended Daily Intake (RDI) values. The maximum absorbed zinc in the dose response curve from Tran et al (2004) was 13 mg, and was from an ingested dose of 30 mg of zinc (Hambidge et al., 2005). From the other dose-response curve, a maximum absorbed level of zinc of 6.9 mg per day was derived (Hambidge et al., 2005). The authors suggested that the difference between the estimated maximum absorbed zinc and the experimental maximum absorbed zinc could be due to dietary factors of participants. It was not clear, from the Food and Nutrition Board (2001) summary nor Hambidge et al (2005) if the composite dose-response curve had all participants dosed with or without a meal. One study used in the Food and Nutrition Board's dose response curve used the zinc content within meals to provide doses (Hunt et al, 1992).

Another inhibitor of zinc absorption seems to be previous zinc supplementation (Hambidge et al., 2005). Hambidge et al (2005) and Tran et al. (2004) both cite Tran et al's pilot study where they found decreased fractional zinc absorption on the second day of consecutive zinc supplementation with a dose of 20 mg of zinc sulfate.

Hambidge et al. (2005) concludes the discussion of zinc absorption and saturation kinetics by recommending that any study investigating zinc supplement absorption should take care to select participants that do not have significant levels of zinc already in their diet. A

summary of zinc supplementation literature relevant to our study is summarized below in Table 1.

Pharmacokinetics of Ingested Zinc in Plasma

Table 1 Summary of Zinc Supplementation Studies in Literature

Citation	Form(s) of Zinc	Duration of Study	Frequency of Supplementation	With/Without Meal	Dose(s) (mg)	Relevant Findings
Tran et al., 2004	Zn sulfate	15 weeks	Once (ea. Dose)	Without meal	2, 5, 10, 15, 20, 30	Fractional zinc absorption was measured using two zinc radioisotopes. A dose-response curve shows an asymptotic response of zinc absorption to ingested zinc. This levelling-off occurs between 20 and 30 mg of ingested zinc doses.
Hunt et al., 1992	Zn from food	9 weeks (7 of dosing)	Daily	With meal	14 (men), 7.8 (women)	This study used meals with controlled amounts of zinc and radio-isotope tracer to determine amount of zinc absorbed, as well as amount excreted, to determine overall zinc balance.
Hunt et al., 2008	Zn from Food	4 or 8 weeks of dosing	Daily	With meal	Ranged between 4.3 – 21.0 with diet	This study found measured zinc absorption from meals with high phytate and low phytate meals. Higher phytate meals inhibited absorption of dietary zinc.
Siepmann et al., 2005	Zn gluconate, Zn oxide	2 x 14 days	Daily	Without meal	20, 17.4	Zinc gluconate and Zinc oxide showed similar time periods of elevated plasma zinc, but zinc gluconate showed significantly higher maximum concentration.
Henderson et al., 1996	Zn acetate	3 days	Once	Without meal	10, 25, 50, 100	Plasma zinc is elevated for about 4 hours after dosing, with an additional elevation 16-24 hours after dosing. Plasma concentration increases as ingested dose increases, but levels off at doses above 50 mg.

Citation	Form(s) of Zinc	Duration of Study	Frequency of Supplementation	With/Without Meal	Dose(s) (mg)	Relevant Findings
Lowe et al., 2004	Zn sulfate	86 days	Daily	With meal	12.2 (66)*	Dietary zinc depletion shows significant decrease in plasma zinc concentrations and decrease in zinc pool. Plasma zinc returned to baseline after repletion, whereas zinc pool did not quite return to baseline.
Wegmuller et al., 2014	Zn citrate, Zn gluconate, Zn oxide	61 days	Once - per compound	Without meal	10 ea.	Absorption of zinc oxide was significantly lower than zinc citrate and zinc gluconate. The absorption was comparable between zinc citrate and zinc gluconate.
Lauwerys et al., 1983	Zn gluconate	8 weeks	Daily - 5 days per week	Without meal **	60	In a group of metal workers, serum zinc levels were found to be elevated significantly compared to a placebo group after 4 weeks of dosing. However these elevated levels were no longer significantly higher than the placebo group at 8 weeks.
Barrie et al., 1987	Zn picolinate Zn citrate Zn gluconate	4 x 4 weeks	3 times per day (summing to 50 mg)	Without meal	50 ea	Zinc picolinate and zinc gluconate show elevated serum concentrations after 4 weeks, however, only zinc picolinate had significant elevation.
Piacenza et al., 2023	Zn aspartate Zn sulfate Zn gluconate	6 months	Once – per compound	Without meal	2 mg ea.	Zinc aspartate and zinc gluconate showed significantly higher fractional zinc absorption compared to zinc sulfate. Fractional absorption of zinc aspartate was significantly higher than zinc gluconate.

Citation	Form (s) of Zinc	Duration of Study	Frequency of Supplementation	With/Without Meal	Dose(s) (mg)	Relevant Findings
Dirajlal-Fargo et al., 2019	Zn gluconate	16 weeks	daily	Not detailed	45, 90	Zinc serum concentrations increased from 74 to 91 µg/dL in the 45 mg group and 73 to 100 µg/dL in the 90 mg group.
Sian et al., 1993	Zn (elemental) as Zn67, Zn68, and Zn70 isotopes.	3 days	Daily	With a meal (low and high phytate) and without a meal.	1, 3, 5, 10	Fractional zinc absorption was measured using zinc radioisotopes. Significantly higher zinc absorption when zinc was ingested with only water compared with high phytate and low phytate meals.

*This was intravenous and given to only 3 participants, during the repletion period of this study.

**This study's supplementation occurred only 15 minutes before lunch, so although this is not taken with food, a meal may still have impacted absorption.

The human body on average contains 2.5-3 grams of zinc, however, most of this is inaccessibly stored in our bones (Lowe et al., 2004). Homeostatic levels of zinc in our body are maintained by regular ingestion of zinc at sufficient amounts, and the accessible "exchangeable zinc pool" is quickly depleted, as shown in a study by Lowe et al. (2004). Lowe et al. (2004) measured changes in this "exchangeable zinc pool" using radioisotope tracers and by measuring zinc fluctuations in urine, feces, and plasma. The study was designed with three periods of zinc intake: a 16 day baseline period where a daily intake of around 12 mg of zinc was ingested, then a 41 day zinc depletion period where daily zinc intake was around 0.25 mg, and then finally a 29 day repletion period where about 12 mg of zinc was given, and in 3 participants intravenous zinc was also given twice during the repletion period (Lowe et al., 2004). Lowe et al (2004) found that over a 41 day period of zinc depletion, the size of this exchangeable zinc pool fell by 36%. During this time period, zinc plasma also fell 70%, with a 20% decrease in concentration in just the first 11 days of depletion (Lowe et al, 2004). Lowe et al (2004) did report that plasma zinc went back to baseline for subjects during the repletion period, but the total body zinc was not fully recovered. This signified a rapid drop in available zinc in this "exchangeable zinc pool" with a drop in dietary zinc intake and supported the idea that plasma zinc is a good measure of total body available zinc until repletion is attempted (Lowe et al., 2004).

A study done by Siepmann et al. (2005) gave twelve male participants 20 mg of zinc gluconate and 17.4 mg of zinc oxide once daily for 14 days. On the 14th day, the researchers measured plasma zinc levels for 24 hours at 2-4 hour intervals. The time it took for zinc plasma concentration to peak was measured as t_max, with both supplements' t_max at 2 hours after dosing (Siepmann et al., 2005). This study also observed elevated serum zinc for 4 hours after dosing, and a second modest elevation of plasma zinc 16-24 hours after dosing (Siepmann et al.,

2005). Maximum concentrations were not reported for this second elevation in zinc, but the researchers hypothesized that it may have been due to a "rebound effect", where reabsorption of excreted endogenous zinc was occurring during this second elevation of plasma zinc (Siepmann et al., 2005). Zinc gluconate plasma concentrations were higher than that of zinc oxide dosing, although there was also a slight difference in the doses of zinc gluconate and zinc oxide: 20 mg and 17.4 mg, respectively (Siepmann et al., 2005).

Henderson et al. (1996) measured zinc plasma concentrations hourly, in participants dosed with a single dose of 10-100 mg elemental zinc as zinc acetate. Subjects fasted before being dosed with zinc acetate. Zinc plasma levels were elevated for about 5-6 hours after dosing, and Henderson at al., concludes a linear absorption of zinc from 10 mg to 50 mg, with a small increase from 50 to 100 mg of zinc acetate (1996).

Zinc Supplements

Many forms of zinc supplements exist and have been tested for their bioavailability from oral ingestion. Studies have found comparable absorption between zinc gluconate, zinc sulfate, and zinc citrate (Wegmüller et al., 2014). Additionally, Siepmann et al. (2005) found evidence that zinc gluconate is absorbed better than zinc oxide. These studies help elucidate the most bioavailable forms of zinc supplementation.

Serum zinc concentrations were measured for a group of metal workers given 60 mg of zinc gluconate every day for 8 weeks (Lauwerys et al., 1983). This study measured significantly elevated serum zinc levels after 4 weeks of supplementation compared to the placebo, however, they only measured serum zinc levels at 0, 4, and 8 weeks, not daily (Lauwerys et al., 1983). After 4 weeks, supplemented zinc serum mean concentration was 103 µg/100 mL, while placebo zinc serum mean concentration was only 90 µg/100 mL (Lauwerys et al., 1983).

Some studies have found forms such as zinc picolinate have higher absorption than zinc gluconate (Barrie et al., 1987). This study gave participants 50 mg of zinc in the form of zinc picolinate, zinc citrate, and zinc gluconate (Barrie et al., 1987). Each supplement was given for a total of 4 weeks, daily (Barrie et al., 1987). Hair zinc was determined for these experiments and both zinc gluconate and zinc picolinate showed an increase in hair zinc from 154 ppm to 157 ppm, for zinc gluconate, and from 154 ppm to 162 ppm for zinc picolinate (Barrie et al., 1987). However, only zinc picolinate gave significantly elevated results (Barrie et al., 1987). This study also showed no significant elevation in zinc serum concentrations, however these serum concentrations were not taken more than once a day, so short elevations in serum zinc may not have been observable (Barrie et al.,1987).

In comparison with zinc aspartate, zinc gluconate, and zinc sulfate, Piacenza et al. (2023) found highest fractional absorption by zinc aspartate. This study used very low doses of zinc (2 mg) but does support zinc gluconate as a well absorbed dose (Piacenza et al., 2023). Piacenza et al (2023) found significantly higher fractional absorption of zinc for zinc gluconate (~20%) compared to zinc sulfate (~9%), and significantly higher fractional absorption of zinc aspartate (~35%) compared to both zinc sulfate and zinc gluconate. No other studies were found that used zinc aspartate for supplementation. The fractional zinc absorption was measured 48 hours after administration of the zinc dose, and was calculated using a ratio of zinc isotopes in the urine (Piacenza et al., 2023).

Although certain papers cite forms like zinc picolinate and zinc aspartate as slightly more bioavailable than zinc gluconate, supplementation studies seem to use zinc gluconate frequently (Dirajlal-Fargo et al., 2019; Barrie et al., 1987; Lauwerys et al., 1983; Siepmann et al., 2005; Wegmüller et al., 2014) and the use of zinc picolinate or zinc aspartate in zinc treatments have

not been found outside of the few studies previously cited (Barrie et al., 1987; Piacenza et al., 2023).

Specific Aims

The literature described above indicates that hair growth rates can be determined by measuring peaks in metal concentration in individual hairs, following supplementation with those metals. However, the existing studies all took place in patients who were ill and were being treated with toxic metals (arsenic) or received high concentrations of metals via injection (e.g. gadolinium) (Li et al, 2022; Duncan et al., 2012). Noel et al. (2015), who did analyze for zinc in grizzly bear hair using laser ablation, did not attempt to determine individual hair growth rates for the grizzly bears, instead using an estimated rate of 1.5 cm per month. This results in a gap in current literature in which no low-toxicity metals have been used to determine individual hair growth rates in healthy individuals.

Cumulatively, there is evidence supporting the feasibility of zinc supplement-induced peaks providing individualized hair growth rates. Figure 1.0 shows a visual representation of our hypothesized time-resolved zinc exposures in hair, which we hope to translate into a time series of zinc concentration in the hair using LA-ICP-MS technology.

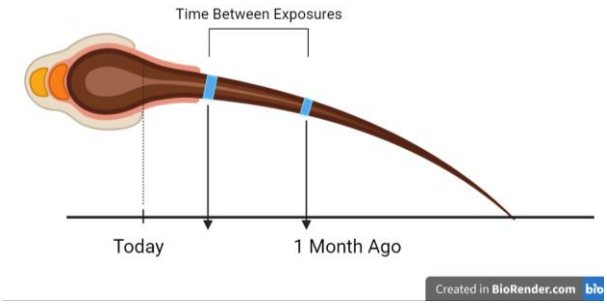

Figure 1 Time Resolved Exposures - Conceptual Model. Created with Biorender.com

Our long-term goal is to standardize a protocol by which LA-ICP-MS can be used to detect metal exposures in hair and to provide an estimated time and magnitude of exposure. LA-ICP-MS technology maintains the time-resolved signatures of exposure incorporated within hair and provides a sufficiently sensitive method of analysis to detect the small magnitude deposition in the hair shaft of metals exposures.

Our primary hypotheses are that (i) LA-ICP-MS technology will accurately detect varying zinc concentrations in the shafts of hair collected from study participants, and (ii) the locations of the bands of higher zinc concentrations will correspond with zinc supplementations and can be used provide an estimated growth rate for each individual hair. If successful, this project will fill the current research gap that exists by extending the application of LA-ICP-MS to determine hair growth using zinc metal concentrations in the hair. This purpose has two primary specific aims.

1. Firstly, we aim to determine the feasibility of measuring zinc hair concentrations using LA-ICP-MS. We hypothesize that zinc supplementation will give adequate peaks in zinc hair concentrations that can be detected by LA-ICP-MS.
2. Secondly, we will determine hair growth rate using the time-resolved zinc concentrations measured by LA-ICP-MS. The dates of zinc supplementation, integrated with the distance between peaks on the hair shaft, can calculate an average growth rate of hair

In addition, these samples afford the opportunity to examine components of variability (between-hair within-subject, and between-subject) for multiple metals in human hair samples. Therefore, our third study aim is:

3. Examine components of variability (between-hair within-subject, and between-subject) for multiple metals in human hair samples.

This research will set forth a model for non-invasive time-resolved exposure monitoring for metals, and provide a protocol by which future research can accomplish the task of measuring hair growth rate for an individual. The development of these protocols will increase confidence in LA-ICP-MS and hair analysis as a biomonitoring tool, and the major strength of LA-ICP-MS for hair biomonitoring is its ability to provide time-resolved exposure information.

Chapter 2: Methods:

Our study aims to detect peaks of zinc concentration in hair samples, subsequent to intentional ingestion of a dietary supplement containing 60 mg of zinc as zinc gluconate. The study design is illustrated in Figure 2, below.

Our study (#00017870) was approved by the University of Washington's Institutional Review Board (IRB) and all participants were consented upon enrollment in the study.

Study Location

Our study was carried out at the Roosevelt One building (4225 Roosevelt Way NE, Suite 100). Participants visited this location for supplementation and hair collection visits. Hair samples were also analyzed with the LA-ICP-MS instrument at this location.

Recruitment and Enrollment of Participants

Subjects were recruited from the local Seattle, WA area. A study poster was approved by the IRB and was displayed inside the Roosevelt One building (4225 Roosevelt Way NE, Suite 100). The study was also posted on the Institute of Translational Health Sciences' human subjects' recruitment website alongside its recruitment criteria. Interested participants were instructed to call a research-study-specific phone number for a screening call. The following exclusion criteria were used in the screening of potential participants:

1) Physician-diagnosed zinc deficiency (may have altered zinc metabolism)
2) Diabetes (may have altered zinc metabolism) (*Office of Dietary Supplements - Zinc*, 2022)
3) Currently pregnant or planning to become pregnant (may have altered zinc metabolism) (*Office of Dietary Supplements - Zinc*, 2022)
4) Currently taking zinc supplements (zinc-containing daily vitamins or homeopathic medications for colds with zinc)

5) Persons using zinc-containing shampoos (e.g. anti-dandruff shampoos)
6) Persons using denture adhesives that contain zinc (*Office of Dietary Supplements—Zinc*, 2022).
7) Have eaten oysters in the last month (oysters contain 28-32 mg of zinc per serving) (*Office of Dietary Supplements—Zinc*, 2022).
8) Persons defined as "heavy drinkers" per NIAA definition: For men, consuming more than 4 drinks on any day or more than 14 drinks per week. For women, consuming more than 3 drinks on any day or more than 7 drinks per week (*Drinking Levels Defined | National Institute on Alcohol Abuse and Alcoholism (NIAAA)*, n.d.). Ethanol consumption reduces zinc absorption (Bode & Christian Bode, 2003).
9) Currently taking any of these medications which interfere with zinc update or metabolism: (*Possible Interactions with: Zinc | Complementary and Alternative Medicine | St. Luke's Hospital*, n.d.).
    i. Thiazine diuretics (reduce zinc absorption)
    ii. Amiloride (Midamor) (increases blood zinc levels)
    iii. Angiotensin-converting enzyme (ACE) inhibitor blood pressure medications (decrease zinc blood levels)
    iv. tetracycline antibiotics or penicillamine (zinc may reduce absorption of these drugs)
    v. quinolone antibiotics (interact with zinc)
    vi. Cisplatin (chemotherapy drug)
    vii. immunosuppressant medications such as Prednisone (Zinc may counteract these medications)
    viii. Non-steroidal Anti-Inflammatory Drugs (NSAIDS) (Zinc may decrease effectiveness)

We also required participants to have currently growing hair on their head, read and speak English fluently, and be 18 years or older. Participants were consented upon meeting these eligibility criteria and prior to beginning any supplementation.

Zinc Supplementation:

Because of relatively high concentrations of endogenous zinc, we decided to use a dose of zinc on the high-end of what we saw in literature. Doses of 50-60 mg of zinc were given in multiple studies (Barrie et al., 1987; Lawerys et al., 1983; Henderson et al., 1996). We chose zinc gluconate as our supplement form because it was readily available from a supplement manufacturer (*Zinc 30 mg Tablets | Support Healthy Immune System | Nature Made®, n.d.*), showed frequency of use in previous zinc dosing studies, and good bioavailability. We decided on the 60 mg dose to maximize the blood zinc concentration and thus maximize the chances of zinc deposition above background levels in the hair. Although some studies used doses higher than 60 mg, we felt this was the highest dose we wanted to give participants, as the LOAEL for zinc is 60 mg/day – based on chronic exposures (NAS, 2001). We felt that our study's two doses were unlikely to elicit adverse effects similar to chronic, daily ingestion of such zinc levels.

Mild symptoms of zinc supplementation were reported in zinc supplementation studies, which included nausea and vomiting, and sometimes stomach cramps (Solomons et al., 2011; Siepmann et al., 2005; Henderson et al. 1996).

Our literature search found studies where zinc supplements were given with a meal or some amount of food (Hunt et al., 2008), however, we also found evidence that components of food like phytate and copper can inhibit zinc absorption (Hambidge et al., 2005). Thus, we decided it would be best to limit food immediately before and after the zinc supplement to prevent inhibition of absorption. We instructed participants to not eat within one hour of their

zinc supplementation visits, to optimize their absorption of zinc. This was communicated in the consent form overview of the study as well as during appointment reminders so participants were

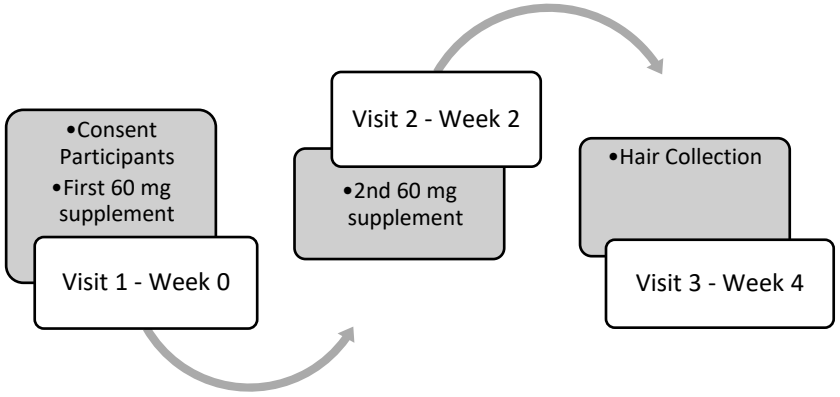

Figure 2 Study Timeline for Participants

reminded in advance of their visits.

The participants' first in-person meeting included the consent process, enrollment, and zinc supplementation, if the participant enrolled. Zinc supplementation occurred twice, once upon enrolling in the study, and a second time two weeks later. The participants were given two tablets of 30 mg zinc as zinc gluconate, to be consumed with water in front of the study technician – a total of 60mg of Zinc. The tablets were obtained from a commercial supplier of dietary supplements (*Zinc 30 mg Tablets, n.d.*). This dose is equal to zinc's LOAEL (*Office of Dietary Supplements - Zinc*, 2022).

Two weeks after their initial visit to the Roosevelt One building, participants return to the Roosevelt One building to receive their second dose of zinc supplements. The participants were given two tablets of 30 mg zinc as zinc gluconate, to be consumed in front of the study technician, with water.

After an additional two weeks (i.e. 4 weeks after initial enrollment) study participants returned to the Roosevelt One building a third time for collection of the hair samples. A small tuft of ~ 10-20 hairs was cut from the scalp, in the back of the head, using ceramic scissors.

Participants' hairs were stored in Ziplock bags prior to analysis. Upon completing the study, participants received a fifty-dollar e-gift card as compensation for their time and participation.

Table 2 Participant Dates of Zinc Supplementation and Hair Collection

Participant ID	Supplementation Date #1	Supplementation Date #2	Hair Collection Date	Time between supplementations (days)	Total time of participation (days)
1	7/27/2023	8/10/2023	8/28/2023	14	32
2	7/31/2023	8/14/2023	8/29/2023	14	29
3	7/31/2023	8/14/2023	8/28/2023	14	28
4	8/14/2023	8/28/2023	9/11/2023	14	28
5	8/03/2023	8/17/2023	8/31/2023	14	28
6	8/02/2023	8/16/23	8/29/23	14	27
7	8/10/2023	8/24/23	9/7/23	14	27
8	8/4/2023	8/18/23	8/31/23	14	27
9	8/8/2023	8/22/2023	9/5/23	14	28
10	8/8/2023	8/22/2023	9/5/2023	14	28
11	8/10/2023	8/23/2023	9/6/2023	13	27
12	9/19/2023	10/3/23	10/17/2023	14	28
13	9/15/2023	9/29/2023	10/13/2023	14	28
14	9/29/2023	10/13	10/30/2023	14	31
15	10/2/2023	10/16/2023	10/30/2023	14	28
16	9/7/2023	9/21/2023	10/5/2023	14	28
17	9/8/2023	9/25/2023	10/6/2023	17	28
18	9/28/2023	10/12/2023	10/26/2023	14	28

Participant ID	Supplementation Date #1	Supplementation Date #2	Hair Collection Date	Time between supplementations (days)	Total time of participation (days)
20	9/20/2023	10/4/2023	10/18/2023	14	28
21	9/19/2023	10/2/2023	10/16/2023	13	27
22	10/6/2023	10/20/2023	11/3/2023	14	28

There was one additional participant not listed in Table 2 who enrolled and received their first supplement, but then stopped communicating with study staff. This participant was notified of their removal from the study and our inability to offer compensation for their partial participation. They gave no reason for discontinuing communication.

Hair Collection Procedure

A Ziplock baggie was labelled with the participant ID (on a piece of lab tape). The baggie was turned inside out, where it was set on a clean paper towel. A section of scotch tape about 2-3 inches in length was cut with ceramic scissors. One end of the tape was folded over to create a small, 1-2 cm tab (this helped in removing the tape from the baggie later). This tape was laid sticky side up on a clean paper towel, ready to stick to a hair sample. For later LA-ICP-MS analysis, preserving the orientation of the hairs was crucial. Keeping the proximal end free of the tape, and well-marked, allowed for the relevant hair section to be free of tape residue but still identifiable. The hairs also needed to be removable, without damaging the hair or disrupting the storage of other hairs.

Wearing gloves, the study staff isolated 10-20 hairs on the back of the participant's head. Once these strands were identified, ceramic scissors were used to cut the strands as close to the scalp as possible. These strands' distal ends were stuck to the tape, with the most proximal 3-4" section of hair remaining above the tape. Excess hair, at least 5" from the proximal end, was

trimmed off of the distal end of the hair sample if the hair was too long to be manageable. For short-haired participants, the study staff tried to keep as much of the proximal end above the tape as possible, at least 1.5 inches. Wide-tipped plastic tweezers were available if they were needed to help manage the hair samples.

The tape holding the hair sample was then stuck to the Ziplock while it was still inside out. The Ziplock baggie was then flipped right side out again, and the proximal end of the hair (the end that was closest to the scalp) was then labelled on the Ziplock baggie. The ceramic scissors and wide-tipped tweezers were cleaned with a Kimwipe and methanol after use.

The labelled Ziplock baggies were stored in the lab in a black plastic box at room temperature. The hairs stayed in this clean, dry box in the lab until washing and preparation of the sample for analysis.

Washing the Hair Samples

The Ziplock baggie containing the participant sample was opened and turned inside out so that the hairs and their tape are accessible. This Ziplock was secured with lab tape to a clean surface. The proximal and distal ends were identifiable by the labelling on the Ziplock bag.

~8-10 inches of waxed, unscented dental floss (Reach brand) were laid out on a clean surface. An overhand knot was tied loosely in the floss. The hairs are pulled through this loop with plastic tweezers and then secured as the knot is tightened around them. 3-5 cm of the proximal end of the hairs was exposed below the floss knot and tape. If a short hair sample was being cleaned, the tape may have been removed and the knot slid closer to the distal end of the hairs to keep 3-5 cm below the knot. A second knot was tied on top of the first knot, once it was

tight and in the correct place along the hairs. Once the knots were tightened and the hairs were secured, the tape was peeled back to free the distal hair ends.

Cleaning of the hair samples was based on cleaning procedures developed in previous publications (Duncan et al., 2021; Hasegawa et al., 2020; Reiss et al., 2016). A 15 mL centrifuge (Corning) was filled with 10 mL of acetone and the tied hairs were placed in the tube. The tube was then filled with an additional volume of 1-4 mL of acetone once the hair was inside, so that the hairs were submerged. The cap of the centrifuge tube was screwed on, trapping the dental floss tails in the threads to secure the hairs. The centrifuge tube was labelled with the participant ID and "P" and "D" for hair orientation. The tubes were placed in a plastic test tube rack in the sonicator; this held them upright. These hairs were sonicated for 30 minutes. Once sonication was complete, the hairs and floss were removed from the centrifuge tube. These hairs were laid on a paper wipe (Kimwipe) in a chemical fumehood to dry.

Once the hairs were dry, they were transferred to clean 15 mL centrifuge tubes (Corning) for storage, labelled with their participant ID and "P" and "D" to maintain the hair's proximal and distal orientation, respectively. The hairs were stored at room temperature in the laboratory until they were mounted for analysis.

Hair Mounting for Laser Ablation:

This protocol is based off of a method previously developed and used in a publication by Duncan et al., 2021. Petrographic slides (Beta Diamond Products) were labelled on their back with the participant ID, and the proximal and distal ends of the slide. All hairs were to be mounted in the same direction, matching this labelling. Double stick tape (Scotch 3M, ½") was laid down on the slide, followed by a strip of packing tape (Office Depot Brand Heavy Duty

Shipping Packing Tape, 1.89"), adhesive side up. The packing tape is stickier than the double-stick tape, which is why this layering system was used.

Each hair to be mounted was then removed from its floss knot in its centrifuge tube. Starting with the proximal end, the strand of hair was pressed firmly into the tape. Any excess hair on the distal end that was longer than the slide was trimmed off. Plastic tweezers were used to help in maneuvering the hair on the slide and pressing it down. All 5 hairs for each participant were mounted on a single slide. The back side of the slide was marked with a permanent marker (Sharpie) about 2 cm from the proximal end of each hair. This line can be seen from the camera on the laser, and allows the operator to know they have ablated 2 cm from the proximal end.

Analysis of Samples:

Prior to the analysis of any hair samples, Standard Reference Material of trace elements in glass (SRM 612, metals in glass; National Institutes of Standards and Technology, NIST) was analyzed to ensure stability of the LA-ICP-MS response, and gelatin standards were used to create a calibration curve for the elements of interest. The use of gelatin as a standard matrix was developed by Hasegawa et al. (2020). The NIST SRM 612 glass was used as a quality control standard because of its known concentration of metals, however gelatin standards were also used as they are more similar to the hair matrix we analyzed, and would provide a standard used with the same laser parameters and energy to the hairs. Duncan et al (2021) previously described use of these spiked-gelatin standards for quantification.

Gel standards spiked with known concentrations of metals, including zinc, ranged in concentration from 0 ppm to 1000 ppm. We applied a linear regression model to the data from these gels, which gave a conversion equation from ICP-MS signal (in counts per second) to concentration in parts per million (ppm). Some calibration curves were calculated using linear

regression in Rstudio in order to do a 1/x weighting, to help improve performance at lower concentrations (Rstudio Team, 2020). The weighted linear regression was done using the stats package in R (R Core Team, 2023).

Hair samples were analyzed using an LA-ICP-MS system that consisted of a New Wave Research (NWR) Neodinium YAG Laser 213 and Agilent 7900 inductively-coupled mass spectrometer. The laser parameters used for the NIST 612 glass standard, gel calibration standards, and hair samples are shown in the table below (Table 3). These parameters were previously used by Duncan et al (2021). A 10 second warm-up time was included at the start of each run, so that data collection on the ICP-MS begins before ablation has started. This allows for background instrument signal to be collected for comparison with ablation signal. A 30 second washout period was included after each sample run, to minimize carryover between samples (see Appendix B).

A number of experiments were run to decide on additional experimental parameters, see Appendices A through D.

Table 3 Laser Parameters

Run Type	**Laser Velocity (µm/s)**	**Spot size (µm)**	**Frequency (Hz)**	**Laser Energy (%)**
NIST 612 Glass	20	100	20	50
Calibration Gel Standard	100	30	20	60
Sample Hair	100	30	20	60

Helium gas is run at a flow rate of 800 mL/min through the laser, carrying the ablated sample to the ICP-MS. The makeup gas is argon, introduced to the sample line between the laser

and the ICP-MS with a flow rate of 1.07 L/min. Helium was also used as a collision cell gas to minimize potential interference from polyatomic species, and set to 2.0 mL/min (see Appendix C).

Based on our literature review that estimated an elevated plasma zinc period of 4-6 hours following acute ingestion of zinc supplements, and an estimated hair growth rate of 0.35 mm per day, the amount of hair growth during elevated zinc blood levels following acute zinc supplementation is estimated to be between 0.058 mm – 0.088 mm of hair growth (Henderson et al., 1996; Siepmann et al., 2005; Tobin, 2005). The LA-ICP-MS parameters for our study must have a resolution smaller than 58–88 micrometers in order to detect these peaks in zinc concentration.

The ICP-MS acquisition parameters to match this resolution are described in Appendix D with greater detail. These calculations, including the velocity with which the laser travels over the hair during ablation, yielded an ideal ICP-MS total sampling time of 0.179 seconds in order to acquire five data points within an elevated zinc peak. Taking previously collected hair data, we determined the ratio of the ablated hair signal to background instrument signal, creating a "signal-to-noise" ratio. This ratio helps determine how detectable an element's concentration is above background noise from the instrument. A longer dwell time for detection of a single element increases the sensitivity of the instrument for that element (Wilschefski & Baxter, 2019), at a cost of poorer spatial resolution. We determined that elements with lower signal to noise ratios in our hair samples would benefit from a longer dwell time, as this gives the ICP-MS detector more time to detect a signal above background counts. On the other hand, elements with high signal to noise ratios would require less dwell time to detect a strong signal, because these elements generate a strong signal with much higher counts than the background. Thus, individual

dwell times were selected for each element to help maintain a strong signal as we decreased the total sampling time, as shown in Appendix D. The average signal from previously-run calibration curves, as well as the signal-to-noise ratios from hair samples, were used to determine the individual dwell times for each element. (Refer to Appendix D for addition details).

Although there are examples of previous LA-ICP-MS studies using sulfur as an internal standard for hair analysis and normalizing to this element, consistent with Duncan et al. (2021) we decided against normalization for our samples. (Refer to Appendix E for additional details)

Data Analysis:

For analysis of the hair samples, lines are programmed on the laser software ActiveView2 to track along the length of the hair shaft for approximately 2 cm. The average hair growth rate cited by previous literature varies significantly between studies, with Tobin (2005) citing an average of ~1 cm per month, Li et al. (2022) measuring 1.24 cm per month, and Pozebon et al. (2008) determining a growth rate of 0.828 cm per month. We based our protocol on an estimate of 1 cm per month, and estimated that the hair growth of participants is unlikely to exceed 2 cm per month. So, this length gives us confidence that we are not missing exposure data distal to the end of our line scan, and likely provides hair data from growth before the supplementation began, in addition to the period of supplementation.

Processing the raw signal time series produced by the ICP-MS was done with R programming language in R studio (R Core Team, 2023; RStudio Team, 2020). The first step is to convert from duration of ablation (in seconds) to distance along the hair (in millimeters), from duration of ablation (in seconds). This conversion was done using the equation below:

$$\frac{[(Time\ (sec) - 10\ sec) * (100\ \mu m/sec)]}{1000\ \mu m/mm} = Distance\ (millimeters)$$

The Time [sec] variable from the dataset was converted into distance (in millimeters along the hair shaft) using the velocity of the laser as a conversion factor (100 micrometers/sec) and after removal of a 10 second warm up time. All hairs were ablated from proximal to distal end, so this converted distance represents the distance along the hair shaft from proximal to distal ends.

The first step in processing was isolating the time series data correlating with laser ablation and separating it from the warm up and cool down data. After isolating the relevant data, the next step was creating a new variable "Distance (mm)" that is calculated based on time and laser velocity and represents the distance along the hair shaft from proximal to distal end. The next step was to remove signal spikes due to instrumental noise from the data series. We decided a sliding median function would help eliminate elevated signal due to instrumental noise and still preserve elevated signal due to real elemental variation in the hair. This function, from the package "Slider" smooths the time series as a result (Vaughan, 2023). Below you can see the raw hair data series (in green) overlayed with the data series after the 3-point sliding median function has been applied (in black). The elevated zinc signal between 15 and 20 mm along the hair shaft is preserved, but some single-point peaks are eliminated at around 7 mm and 15 mm.

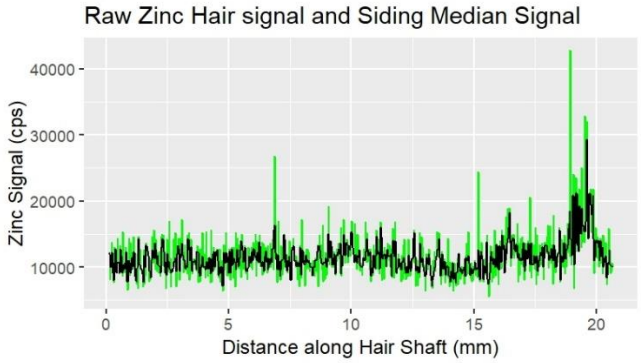

Figure 3 Smoothing the Hair Data

In order to generate accurate zinc concentrations from the data, the average of the smoothed background instrument signal (i.e. the instrument response prior to turning on the laser) was subtracted from the smoothed ablation signal, thus giving a signal that more accurately represented the zinc signal found only in the hair or the gelatin. This adjustment of signal was done to both the calibration gel data and the hair data for consistency and accuracy of the calibration curve.

For the calibration curves, there were also endogenous, unknown, concentrations of the target metals in the gelatin matrix that contributed to the spiked standards' overall signal, which, if unaccounted for, would lead to a less-accurate signal vs ppm calibration curve. We thus subtracted the average signal of the blank gel, or "zero ppm" gel from the average signal of the spiked gels, in order to adjust for this unknown amount of signal. The final calibration curves for each element are listed below:

Table 4 Calibration Curves for Sample Analysis

Element	Equation	R squared	Calibration gel Range	Limit of Detection (ppm)	Linear Model
Zinc (Zn66)	y = 83.3x - 1080	0.959	0 – 1000 ppm	5	Linear regression
Lead (Pb208)	y = 3050x + 78.2	0.999	0 – 50 ppm	0.05	Linear regression
Manganese (Mn55)	y = 484x - 299	0.999	0 – 50 ppm	1	Linear regression
Mercury (Hg202)	y = 1302x +215	0.986	0.05 – 50 ppm	0.05	Linear regression with 1/x weighting
Arsenic (As75)	y = 50.4x + 4.93	0.987	0.05 – 50 ppm	0.05	Linear regression with 1/x weighting
Cadmium (Cd111)	Y = 108x -5.84	0.997	0.1 – 50 ppm	0.1	Linear regression with 1/x weighting

Linear regression with 1/x weighting was done with R in Rstudio using the lm() function in the stats package (Rstudio Team, 2020; R Core Team, 2023). These calibration curves were applied to the hair data in order to graph the concentrations in parts per million (ppm) for each element. The hair was also processed in the same way the calibration gels were, with a sliding median function and subtraction of background instrument signal. We calculated limits of detection for all of our metals of interest using our calibration curve, and defined our Limit of Detection (LOD) as the lowest detectable gelatin in our calibration curves for each metal, which were clearly above our blank gelatin standards.

For our third aim, we examined all six elements of interest and their calibration curves to determine and compare hair concentrations between and among participants. For the three elements with detection frequencies > 50% (Zn, Pb, and Hg) we ran a one-way ANOVA to compare the amount of variation for these elements' concentrations between participants and within participants. This was done with R in R studio, using the aov() command in the stats R package (Rstudio Team, 2020; R Core Team, 2023). We also ran a repeated-measures ANOVA to compare the amount of variation for each metal between and within hairs. This was done using the rstatix package (Kassambara, 2023b).

Chapter 3: Results

Aim 1:

Determine the feasibility of measuring zinc hair concentrations using LA-ICP-MS. We hypothesize that zinc supplementation will give adequate peaks in zinc hair concentrations that can be detected by LA-ICP-MS.

Zinc Hair Time Series:

Calibration curves allowed us to convert the data collected on participants' hairs into concentration, using a linear regression equation. Once we had the final calibration curve for zinc we could process the hair time series as follows:

To look for the presence of zinc peaks corresponding with the zinc supplementations, we graphed all of the hairs for each participant. For a peak to be attributed to the zinc supplementation, the peak should be approximately 5 data points wide or wider (based on our literature search and analysis parameters). A peak associated with zinc supplementation would also be expected to appear across multiple hairs from the same participant and at relatively the same position along the hair shafts.

The zinc concentrations along hairs from Participant 1 through 22 are pictured below.

Zinc Concentrations Along Hairs

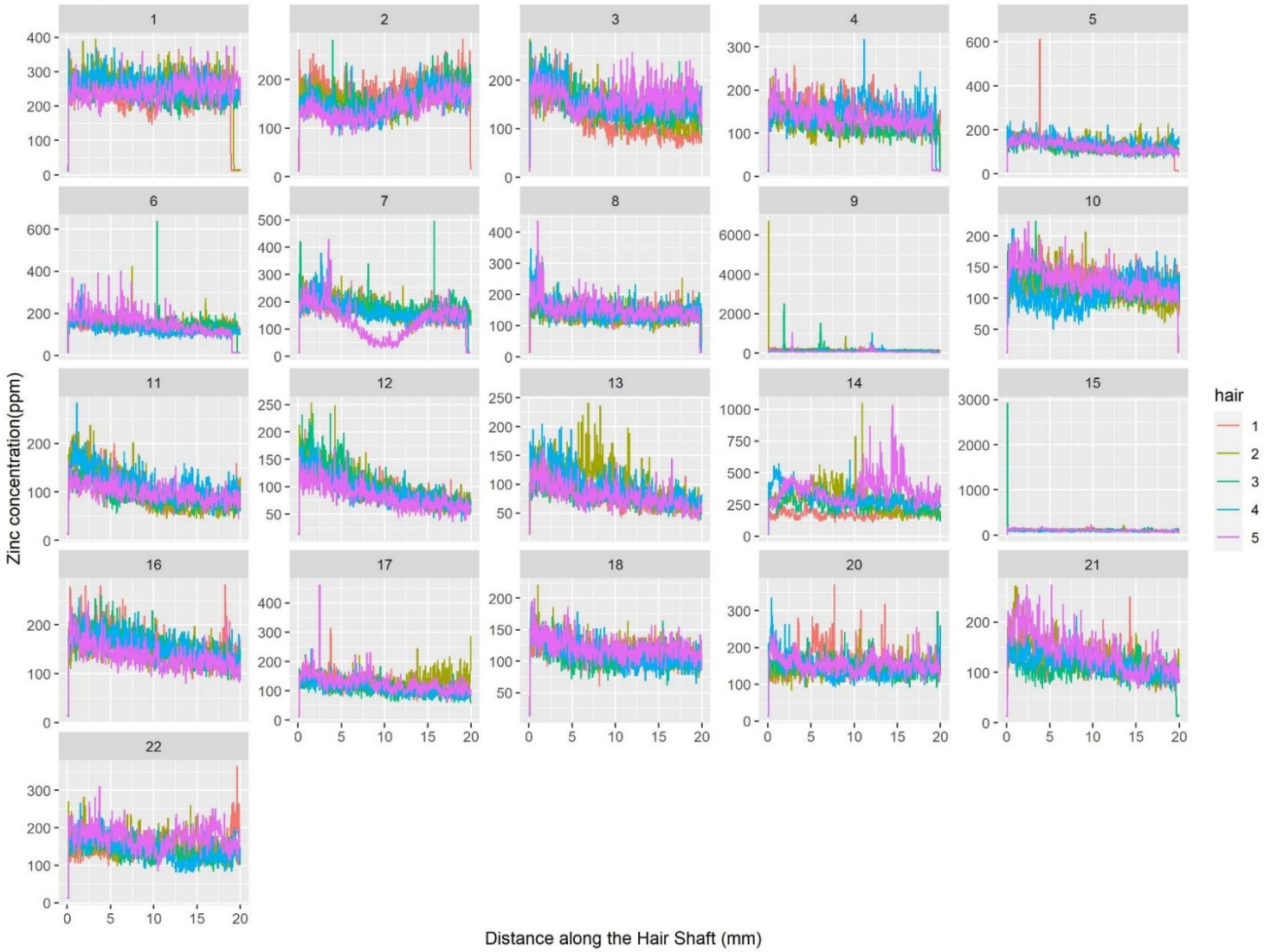

Figure 4 Zinc Hair Results

Visual inspection of Figure 4 does not show consistent evidence of zinc peaks that could be ascribed to ingestion of the zinc supplement. Most samples do not show clear peaks at all (e.g. participants 3, 10 and 18). For several participants zinc peaks were present in one hair, but not in the other hairs from that participant (e.g. participants 4 and 5). Participants 9 and 15 did have one or more extremely large zinc peaks that are outside a feasible range of zinc concentrations in hair. Although these peaks survived our smoothing and data processing, these peaks are presumed to not be due to real variation of zinc levels in the hair. Graphs of just participant 9 and 15 hairs can be found in Appendix F with adjusted axes to allow for variation in the rest of the data to be visible.

In total, there were 5 hairs analyzed per participant, for a total of 105 hairs. The average zinc concentrations are summarized by hair and by participant in Appendix G. Based on the results seen in these time series, we do not see consistent peaks across multiple hairs. Because any variation that can be seen in the hairs does not match our expected peak location along the hair, consistency among hairs, or peak width, we cannot attribute it to the supplementation events from our study.

Aim 2:

Determine hair growth rate using the time-resolved zinc concentrations measured by LA-ICP-MS. The dates of zinc supplementation, integrated with the distance between peaks on the hair shaft, can calculate an average growth rate of hair.

Unfortunately, without clear zinc peaks in the hairs from participants in this study, we are unable to determine hair growth rate and produce results for this aim.

Aim 3: Components of variability Across 6 metals

Examine components of variability (between-hair, within-subject, and between-subject) for multiple metals in human hair samples.

Although we were unable to find zinc peaks consistent with the study's supplementations, we were able to quantify zinc concentrations within and across participants. We were also interested in characterizing other metals in the hairs, namely lead (Pb208), mercury (Hg202), cadmium (Cd111), arsenic (As75) and manganese (Mn55).

The calibration curves for the 5 elements other than zinc can be found in Table 4, and the limits of detection for each are summarized below, together with summary statistics describing levels of the 6 elements in our hair samples, and detection frequencies.

Table 5 Average Metal Concentrations in ppm and Comparison with Literature Values

Element	LOD	Min conc.*	Max conc.	Average conc.	% Participants Above LOD	Literature Mean**	Literature Range**
Zn	5	87	275	142	100%	142	68 - 198
Pb	0.05	0.0728	1.53	0.191	57%	0.960	0.22 - 7.26
Hg	0.05	0.0783	2.49	0.665	90%	0.261	0.053 - 0.927
Mn	1	1.76	1.76	0.757	5%	0.560	0.080 - 2.41
As	0.05	0.0603	0.166	0.0549	33%	0.085	0.034 - 0.319
Cd	0.1	0.117	0.117	0.0729	5%	0.058	0.010 - 0.356

*The minimum concentration for participants above the LOD
**These values are published by Rodushkin & Axelsson (2000)

The average concentrations of all six metals across all 21 participants is shown in Table 5. For calculating this average concentration, if a hair concentration for a particular participant was below our limit of detection for that element, then that hair's average was replaced with the value of the LOD for that metal divided by the square root of two. The overall averages for all participant's hairs listed in Table 6 include these adjusted values for below-LOD concentrations.

From this table, we can see that over half of our participants had average hair concentrations above our limits of detection for zinc, lead, and mercury. Thirty three percent of our participants had arsenic concentrations above our limit of detection, but only five percent of our participants had manganese and cadmium concentrations above what we could detect. To view average concentrations by participant, see Appendix G.

For zinc, we see that our limit of detection was lower than all of the average hair concentrations, and all of our participant average concentrations fell within the range of standards included in our calibration curve. For lead, mercury, cadmium, manganese, and arsenic, we see that some participants had average concentrations below our limit of detection. For ANOVAs and characterization of participants' variability in metal concentrations, we included zinc, lead, and mercury, but did not look at arsenic, manganese, and cadmium because less than 50% of participants had levels above our limits of detection for each metal.

Variation Between and Within Participants

We can visualize both the variation between and within participants, as well as between and within hairs, with a box and whisker plot for each particular metal, as illustrated in Figure 5.

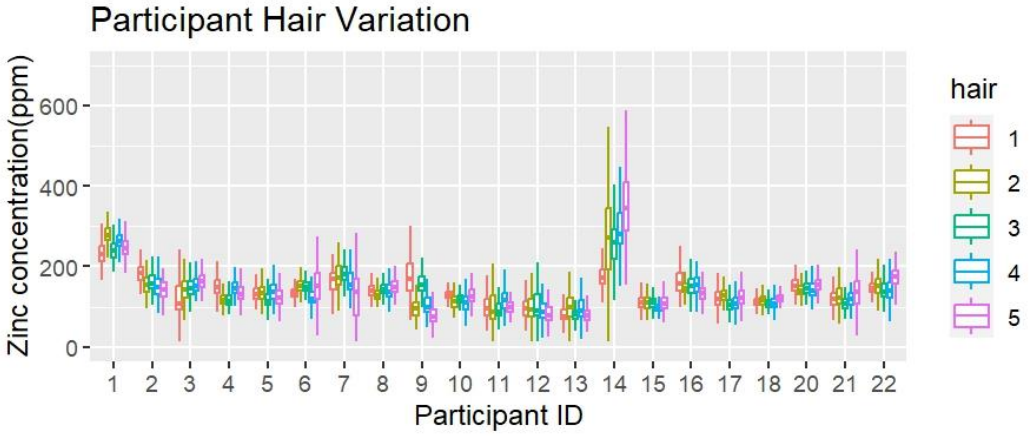

Figure 5 Zinc Within- and Between- Participant Variation

The boxplot above was generated using the R package "ggplot2" (Wickham, 2016). The box visualizes the median hair zinc concentration, as well as the 25^{th} and 75^{th} percentile concentrations (Wickham, 2016). The "whiskers" visualize data within 1.5 times the interquartile range (Wickham, 2016). Outliers are hidden, for better visualization of the of the boxplots, but not excluded from the calculations of quartiles and medians (Wickham, 2016).

We ran a one-way ANOVA to compare the variability in metal concentrations within and between participants in Rstudio using the R package Stats (Rstudio Team, 2020; R Core Team, 2023). An ANOVA has two important assumptions about the data it assesses: one, the data is normally distributed, and two, each observation is independent of one another (*F Statistic / F Value*, n.d.). When aggregating all of the hairs' average concentrations and running a Shapiro-Wilke's test in R using the rstatix package, the distribution of all 105 hairs was not normally distributed for zinc ($P = 4.01x10^{-10}$), lead ($P = 4.36x10^{-18}$), and mercury ($P = 5.55x10^{-9}$), indicated by P values less than an alpha value of 0.05 (Kassambara, 2023b). However, we plotted all 105 hairs' concentrations in a Q-Q plot for zinc, lead, and mercury concentrations using the packages ggplot2 and ggpubr to visualize any deviation from normal distribution (See Appendix H) (Wickham, 2016; Kassambara, 2023a). We see a small portion of hairs deviating from normal distribution for zinc and lead and slightly more hairs deviating from non-normal distribution for mercury. We went ahead with the ANOVA for all three metals, noting that a small number of hairs were not normally distributed for zinc and lead and mercury may have a more significant non-normal distribution.

Table 6 Between- and Within-Participants ANOVA

Element	Type	DF	Variability (Sum Squares)	Variance (Mean Square)	Total Sum Squares	% Variability due to Between Participants	F Statistic	P Value
Zn	Between Groups	20	199900	9995	235769	84.78 %	23.4	<2e-16
	Within Groups	84	35900	427				
Hg	Between Groups	20	39.7	1.98	40.9	96.98 %	135	<2e-16
	Within Groups	84	1.24	0.0147				
Pb	Between Groups	20	10.9	0.545	14.3	75.98%	13.3	<2e-16
	Within Groups	84	3.44	0.0410				

DF – Degrees of Freedom

The Sum of squares characterizes variation in the data, for between and within participants (*13.2 - The ANOVA Table | STAT 415*, n.d.). The Mean Squares value is the variance between participants, or average variance within a participant, and is found by dividing the Sum of squares by the Degrees of Freedom (DF) (*13.2 - The ANOVA Table | STAT 415*, n.d.). Assessment of significance from our ANOVA test is done using both the P value and the F statistic (*F Statistic / F Value*, n.d.). The F statistic is a ratio of the variance between participants and the average variance within participants (*F Statistic / F Value*, n.d.). Our null hypothesis for this statistical test is that the variation between groups and the variance within groups will not be significantly different. This would mean that the groups themselves are not significantly different. Our alternative hypothesis states that at least two participants have significantly

different metal concentrations in their hair than one another. For a significance threshold of 0.05 (alpha = 0.05) and degrees of freedom for between and within group comparisons 20 and 84 respectively, the F statistic is 1.6968 (*Critical Value Calculator,* n.d.). Because all of our F statistics are above this value, and our P value is smaller than 0.05, we can reject the null hypothesis, and support a conclusion that our results include at least one participant with concentrations significantly different than the other participants. For these one-way ANOVAs, all of the metals we analyzed have F statistics above the F critical value, so we can reject our null hypothesis for all of them. This finding is what we would expect, as hairs on the same head are expected to be more similar in metal deposition than hairs from different heads of participants.

The Sum of Squares between and within participants represents variability in the data, and the total sum of squares can be found by summing the two values in our ANOVA. The percent of total variability in the data that is due to between-participant variability depends on the metal. We see for zinc that 85% of the total variability is due to between-group variation in zinc concentrations.

Between-Hair Variation

In order to describe and summarize variation between and hairs, we ran a one-way repeated measurement ANOVA with the R package "rstatix" in Rstudio (Kassambara, 2023b; Rstudio Team, 2020). A regular one-way ANOVA cannot be used for observations that are dependent, or related, but a repeated-measures ANOVA is designed to look at these related measures within a subject ("Repeated Measures ANOVA in R," n.d.). Because we have five hairs from each participant, each of these hairs are dependent, or related, to one another. The statistics from this ANOVA compare the variability in metal concentrations between hairs from each participant and the P value reports whether there are significant differences across hairs

accounting for hairs related to each participant (T-Test, Chi-Square, ANOVA, Regression, Correlation..., n.d.).

The repeated-measures ANOVA has a few requirements of the input data. The data cannot have extreme outliers, must be normally distributed, and must have sphericity (or have sphericity corrections in the ANOVA output) ("Repeated Measures ANOVA in R," n.d.). We ran a Shapiro-Wilkes test in Rstudio using the package "rstatix" to assess normal distribution of hair concentrations for each participant (Rstudio Team, 2020; Kassambara, 2023b; "Repeated Measures ANOVA in R," n.d.). We found that one out of 21 participants had a non-normal distribution of hair concentrations for zinc and mercury, and two out of 21 participants had a non-normal distribution of hair concentrations for lead. We went ahead with the repeated-measures ANOVA because very few of our participants didn't have a normal distribution of hair concentrations. Our data also had a small number of extreme outliers, so we ran this repeated-measures ANOVA twice for each element, with retained extreme outliers, and with removal of extreme outliers. There was no significant change in the outcome of our ANOVA, but it did decrease the number of observations per participant, so we decided to leave in the outliers for our reported results. Sphericity is another important characteristic of the data in a repeated-measures ANOVA, and it occurs when the variance of differences between all combinations of hairs for a participant are equal (Understanding Sphericity - An Introduction to, Testing for, and Interpreting Sphericity | Laerd Statistics, n.d.). We applied the Greenhouse-Geisser correction to our data when running this ANOVA, as the data did not have sphericity (Kassambara, 2023b).

Table 7 Between Hairs, within Participants ANOVA

Type	DF	Variability (Sum Squares)	Critical F Value	F Statistic	P Value	Element
Between Hairs	1.63	222	3.28	0.125	0.842	Zn
Residual	32.62	35652				
Between Hairs	2.0	0.099	3.23	1.746	0.188	Hg
Residual	39.98	1.137				
Between Hairs	1.1	0.112	4.30	0.674	0.434	Pb
Residual	22.02	3.333				

The results from our repeated measures ANOVA show that there was no significant difference across hairs from the same participant (P value is greater than 0.05). This means that the concentrations of zinc, lead and mercury were similar across all hairs. This furthers our confidence that analysis of five hairs is an appropriate sample size for describing a subject's zinc, lead, or mercury concentration because we see consistent concentrations across five hairs, with little variability.

Chapter 4: Discussion

Aim 1:

Determine the feasibility of measuring zinc hair concentrations using LA-ICP-MS. We hypothesize that zinc supplementation will give adequate peaks in zinc hair concentrations that can be detected by LA-ICP-MS.

These results do not show the obvious peaks in zinc concentrations that we hypothesized we would see from zinc supplementation. Although there are publications on LA-ICP-MS hair

analysis, there are few examples of LA-ICP-MS analysis of zinc concentrations along the hair (Noël et al., 2015; Christensen & LaBine, 2023). As far as we are aware, there are no studies that positively correlated zinc uptake into hair with dietary supplement zinc intake. Studies that utilized zinc supplementation with doses similar to what we gave participants did see plasma zinc fluctuations correlated with zinc intake, but the relationship between zinc hair concentrations and plasma zinc concentrations is not well characterized. Siepmann et al. (2005) had an average plasma zinc concentration increase of about 400 µg/dL after a single dose of 20 mg of zinc gluconate (~ 50% increase in plasma concentrations). Henderson et al. (1996) found a single dose of 50 mg of zinc as zinc acetate had an average increase of blood plasma ~ 150 µ/dL (150% increase from baseline). If plasma zinc levels are proportional to hair zinc uptake, we would expect a 50-100% increase in hair zinc concentrations, based on literature. From our average hair concentration, that would be a 70-140 ppm increase in zinc hair concentrations for about 88 micrometers of hair—a fluctuation our analysis is able to detect. However, fluctuations with similar parameters are seen all along the hairs analyzed, suggesting that the background concentration and variability of zinc in hair may mask fluctuations in the plasma concentration of zinc due to single ingested doses. The lack of identifiable zinc peaks correlated to ingested zinc supplements emphasizes the need for a model of zinc uptake. Smith et al. (2014) successfully modelled cortisol uptake from blood into hair, and was even able to use hair concentrations to predict blood and saliva concentrations. Similar research for metals, particularly zinc, would allow for better study design with future supplementation and hair growth studies.

Some participants noted nausea and gastrointestinal upset from the supplements, which suggests that using higher doses of zinc supplementation in future studies would not be appropriate. Studies have also shown that zinc absorption is a saturable process, and we chose a

dose that was on the upper end of concentrations with increased absorption, so higher concentrations are unlikely to see much greater increases in plasma zinc concentrations (Henderson et al., 1996; Hambidge et al., 2005). Given this unsuccessful attempt to utilize this metal to measure hair growth rates, future work would likely need to repeat a similar study design with a different metal supplement.

However, we can see intrapersonal (i.e. between-hair from the same participant) variation in zinc concentrations as well as interpersonal variation (between participants). This supports the notion that individualized biomonitoring for metals is feasible and important, as individuals show different baseline levels of zinc (and other metals) in their hair.

Aim 2:

Determine hair growth rate using the time-resolved zinc concentrations measured by LA-ICP-MS. The dates of zinc supplementation, integrated with the distance between peaks on the hair shaft, can calculate an average growth rate of hair.

Unfortunately, we were unable to detect peaks of zinc concentrations along the hair shafts that we can attribute to zinc supplements taken by participants. Because of this, we are unable to draw any conclusions about hair growth rates.

Aim 3:

Examine components of variability (between-hair, within-subject, and between-subject) for multiple metals in human hair samples.

Characterizing Variability Between and Within Participants

Our Results from Aim 3 showed significant variability in concentrations of zinc, lead, and mercury between participants, with much lower variability in hair concentrations of metals

within a participant, compared to between participants. This data supports our method's ability to characterize an individual's metal concentrations for zinc, our primary element of interest, as well as lead and mercury. Unfortunately, we were unable to characterize concentrations of arsenic, manganese, and cadmium in the hairs of participants due to low detection frequencies for these analytes.

Comparisons between our detected average concentrations of zinc, lead, mercury, arsenic, manganese, and cadmium and literature values for these metals in hair, saw good agreement. Table 5 shows that the average concentrations that we determined for our 21 participants are within the published range of concentrations detected with liquid-introduction ICP-MS, or very near this published range (for lead) (Rodushkin & Axelsson, 2000). Liquid introduction ICP-MS is most commonly used for trace metal analysis in hair, so agreement between our laser ablation method and this method suggests that LA-ICP-MS is an adequate analysis method for detection of trace metals in hair (Stadlbauer et al., 2005).

Appropriate Number of Hairs for Analysis

We can also assess the suitability of analyzing five hairs per participant and the effect of sample size on the precision of the measured metal concentrations in the participants' hair. In Table 8, 95% confidence intervals around the average hair concentrations of zinc, lead, and mercury are shown. The width of these intervals is calculated with our sample size of 5 hairs, as well as with one, ten, and twenty hairs.

Table 8 Effect of Sample Size on Accuracy

Element	Sample Size	Avg. Concentration (ppm)	Average 95% Confidence Interval	CI expressed as % of the mean
Zn	1	142	±27	±19%
	5		±12	±9%
	10		±9	±6%
	20		±6	±4%
Pb	1	0.191	±0.129	±67%
	5		±0.058	±30%
	10		±0.041	±21%
	20		±0.029	±15%
Hg	1	0.665	±0.150	±23%
	5		±0.067	±10%
	10		±0.048	±7%
	20		±0.034	±5%

With a sample size of five, our confidence intervals around our mean are less than 10% of our average hair concentration for zinc, from which we conclude that our sample size is appropriate for these metals. However, lead and mercury have larger confidence intervals relative to there average concentrations at a sample size of five. For a 95% confidence interval that is close to 10% of the average concentration, lead and mercury analysis would need a sample size of about twenty hairs.

Although greater sample size would be beneficial for narrowing the confidence intervals around the mean and thus reducing misclassification of exposure, it introduces increases in cost and time of analysis. If twenty hairs were analyzed for each participant, the cost of analysis would likely become infeasible for large studies with many participants. Weighing both the feasibility and the accuracy of results, we conclude that five hairs is an acceptable sample size for zinc analysis, and five hairs may be appropriate for lead and mercury analysis considering the time and cost of analysis for a sample size that would significantly decrease the confidence interval for this metal. In all cases, analyzing only one hair gave a large confidence interval, with

the margin of error making up 20-67% of the average concentration, was insufficient. Future work with zinc, mercury, and lead, should use at least five hairs in analysis.

Potential Future Work

Although this experiment was unable to validate the use of zinc supplements and LA-ICP-MS for measuring hair growth rate, future work could use many aspects of this study design, with a different metal supplement. One of zinc's shortcomings is its naturally high background concentration in our bodies and our hair (Rodushkin & Axelsson, 2000). There is also a relatively narrow range between everyday consumption of zinc and the upper tolerable limit or LOAEL (*Office of Dietary Supplements—Zinc*, 2022).

This experiment could be repeated in the future with a low-toxicity metal with much lower background levels in our bodies, as well as a greater range of doses between normal intake and its LOAEL. Other potential metals for a controlled-exposure study include selenium and molybdenum. A summary of the intake and upper limits of these metals is shown below, with data specific to adults.

Table 9 Alternative Metals for Future Study

Metal	Average Daily Intake (ADI)*	Upper Tolerable Limit (UL)*	Avg. Hair Conc. (unexposed)**
Selenium	108 – 116 µg/day	400 µg/day	0.830 µg/g
Molybdenum	109 µg/day (women) 76 µg/day (men)	2,000 µg/day	0.042 µg/g

*ADI and UL's are described by the *Office of Dietary Supplements - Molybdenum (2021)* and *Office of Dietary Supplements – Selenium (2024)*.
**Average Hair concentrations published by Rodushkin & Axelsson (2000)

Based on these results in table 10, molybdenum (Mo) has the largest range between average intake by U.S. adults and the upper tolerable limit (*Office of Dietary Supplements* -

Molybdenum, 2021). That gives us a greater range of doses to administer participants while staying below a level that may produce adverse effects. The low concentration in an unexposed population also suggests that there may be low background concentrations in the hair, which may make visualizing peaks due to supplementation easier (Rodushkin & Axelsson, 2000). However, it should be noted that Molybdenum levels are usually measured in the urine, because it is rapidly excreted (Martinez-Morata et al., 2023). This may limit the deposition of Mo into the hair, and may account for the low concentrations measured by Rodushkin and Axelsson, (2000).

Selenium is another potential metal for future study. Selenium has an upper tolerable limit about four times that of daily intake levels (see Table 9) (Martinez-Morata et al., 2023). Selenium also has a plasma half-life on the scale of days, which increases the length of time a high-selenium exposure may be present in the blood and deposited in the hair (Martinez-Morata et al., 2023). Lastly, Martinez-Morata et al. (2023) cites that selenium in the blood is highly correlated with selenium in toenails. This suggests that the long half-life and variation in plasma selenium levels would also be reflected in hair samples, which are also keratin based like toenails (Tobin, 2005). It should be noted that Selenium is another anti-dandruff shampoo ingredient, and deposition of selenium onto hair by shampoo may play a role in hair selenium levels (Martinez-Morata et al., 2023).

A recent publication by Christensen & LaBine (2023) used LA-ICP-MS to detect metals including Molybdenum and Selenium in single hairs from human subjects. They had low detection of Molybdenum in their hair samples (detection frequency above LOD of 2% for cuticle concentrations near the scalp) (Christensen & LaBine, 2023). For selenium, Christensen & LaBine (2023) had much better detection frequency above their LOD (92%) in the cuticle of the hair, near the scalp. However, Christensen & LaBine (2023) did not report the limits of

detection for each element in this paper. This study also analyzed zinc and the other metals in our study. Christensen & LaBine (2023) reported a detection frequency in the hair cuticle, near the scalp of 11% for cadmium, 100% detection frequency for zinc, and 92% detection frequency for mercury (Christensen & LaBine, 2023). These detection frequencies of cadmium, zinc, and mercury are similar to our results (5%, 100%, and 90% respectively), which supports their findings on molybdenum and selenium detection frequencies are applicable to our methods and future work. However, it should be noted that they reported good detection frequency of manganese (84%) and we had much poorer detection with 5% of participants (Christensen & LaBine, 2023). This work by Christensen & LaBine (2023) would suggest much higher detection of selenium than molybdenum, and thus may support use of selenium supplements in future work.

Relevance

The conclusions we can draw from this study include confirmation of the performance of our methods analyzing zinc and some trace metals, and improvements to our method for future attempts to measure hair growth rates. The confidence intervals for our detection of zinc and mercury show acceptable margin of error with five hairs analyzed per participant, and suggest ten hairs may provide even better confidence in detection of lead from participants. We also found collision cell gas did not hinder the performance of our calibration curves and detection of metals in hair. Lastly, we determined that a washout period of at least thirty seconds helps prevent carryover of one ablated sample into another. All of these conclusions further refine the method by which hairs are analyzed using LA-ICP-MS – an approach that is still developing and remains relatively novel.

More generally, we successfully detected environmentally-relevant concentrations of metals in hair, whereas many LA-ICP-MS studies used occupationally or medically-exposed populations for analysis. Because there are many individuals who are exposed to heavy metals through their drinking water, diet, or general environment, validating this technology for detection of these lower concentrations and fluctuations in hair expands the applicability of this method to a more general population.

Limitations

Some participants noted nausea from the supplements, but never indicated it was severe enough to quit participation. One participant reported vomiting after their second dose, but did not drop out of the study. This was a known risk of zinc supplements, with further review of literature showing nausea as a reported side effect in doses 20 mg and higher (Siepmann et al. 2005; Henderson, 2013). These observations do suggest that using higher doses of Zn supplementation in future studies would not be appropriate.

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Appendices

Appendix A: Calibration curves

In order to convert from the instrument's output unit of "Counts per Second", a measure of intensity, to element concentration, we utilize a calibration curve. We prepared a series of gelatin standards, spiked with a known amount of heavy metals, ranging from 0.05 ppm to 1000 ppm in concentration. By plotting the known concentration of these standards versus the output signal from the ICP-MS, we can create a conversion equation to use on the hair samples, for which we won't have a known concentration in the hair.

The average signal (in counts per second) is found for each standard after trimming the time series, removing outliers and smoothing, as described previously in the methods section, and a linear regression model is run either in Excel or in R (for weighted regression curves). The resulting calibration curve equations are summarized in the table below for a suite of metals, including zinc.

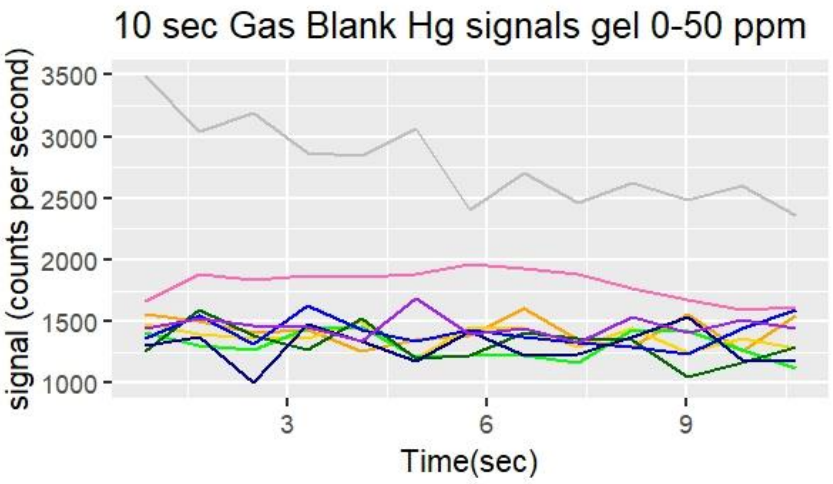
In method development experiments we ran calibration curves using gels instead of hair samples, so that we had a known concentration in the gels, and could compare between experiments and see how different methods affected the instrument's detection of certain concentrations.

The range of calibration gel concentrations varied based on the concentrations relevant to the hair samples' concentrations and we eliminated gels where the ICP-MS signal was not clearly above the zero gel. If the adjusted average gel signal was below that of the blank "zero" gel, thus a negative number, it was not included in the calibration curve. The 1/x weighting applied to some calibration curves also could not be done with a 0 ppm concentration for the blank gel, so these calibration curves did not included 0 ppm in their range (dividing by zero gave an error) (R

Core Team, 2023). These scenarios contributed to some of the differences between the calibration curves for our metals of interest.

Appendix B: Adding a Washout period

Each ablation run on the laser has a 10 second warm up time, during which the ICP-MS is collecting data, but the laser has not yet begun ablating material. This warm up time collects what is called "gas blank" data, or data that should give only background signal from the Helium and Argon gases. We noticed that this gas blank data had trends with certain metals showing a carry-over of signal from one run to the next run. For example, mercury (Hg202) can be seen with higher gas blank signals before running the 50 and 25 ppm gels, compared to the 0-10 ppm warm up periods. The gelatin standards were one after the other from lowest to highest in concentration.



Legend: Gel Standards

Yellow – 0 ppm

Gold – 0.05 ppm

Green – 0.1 ppm

Dark green - 0.5 ppm

Blue – 1.0 ppm

Dark blue – 5.0 ppm

Purple – 10 ppm

Pink – 25 ppm

Grey – 50 ppm

Figure 6 Gas blank data for Mercury - Without Washout time

We decided to add a 30 sec washout period to help "flush" residual material from the sample line before beginning the next ablation run. An experiment was done prior to our final analysis in which a second calibration curve was run with 30 seconds of data collection after each gel:

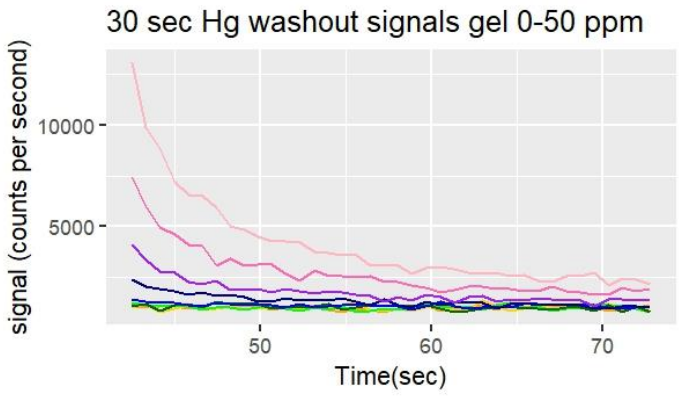


Figure 7 Mercury Signals During Washout Period

From these graphs you can see that mercury takes about 30 seconds for the signals to come back down to baseline from an ablation, as can be seen from the time series of the washout period. Zinc did not have a similar problem, but because we were measuring multiple elements during our analysis we decided it was best practice to include the washout period. This 30 second washout period has also been employed in other LA-ICP-MS publications (Steely et al., 2007).

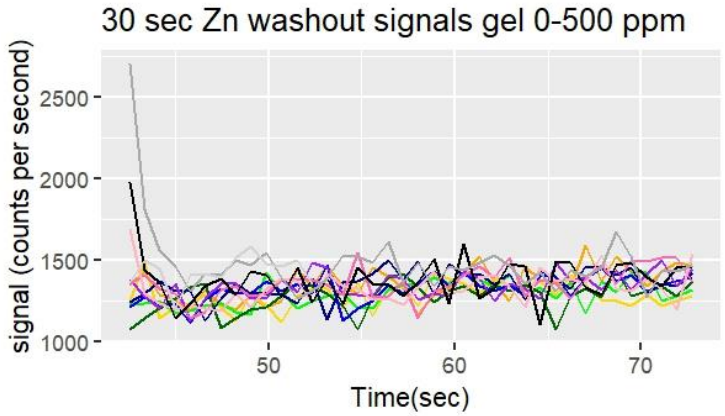


Figure 8: Zinc Signal During Washout Period

Appendix C: Collision Cell Gas Experiments:

The masses we are interested in, namely Zn66, have polyatomic interference with sulfur oxides – a species that is likely to form with high-sulfur content matrices, like hair (May & Weidmeyer, n.d.; Gellein et al., 2008). A common practice for removing polyatomic interferences in an ICP-MS is to utilize a collision cell (Wilschefski & Baxter, 2019). A collision cell bombards the sample stream with a gas to break up polyatomic species, and this gas can be either reactive or inert (Wilschefski & Baxter, 2019). We chose to experiment with an inert gas, to avoid the complication of chemical reactions, while still physically breaking up any molecular clusters – like sulfur oxides (McCurdy et al., 2006). These experiments were run with the help of the Environmental Health Lab (EHL) team in the Department of Environmental and Occupational Health Sciences, University of Washington.

A major downside to collision cell gas is that it lowers the overall signal intensity for elements being detected, which lowers the instrument's sensitivity (Neubauer, 2010). For elements that are relatively scarce in hair, like gadolinium, collision cell gas may decrease the sensitivity of the instrument to levels found in the hair.

Zinc, on the other hand, is a relatively abundant metal in hair, compared to other metals (Gellein et al., 2008). Because of this, collision cell gas may offer the benefit of reducing polyatomic species, while not conferring lower sensitivity. To test this hypothesis, we experimented with our gel standards, running a full calibration curve at three flow rates of helium cell gas: 0 mL/min, 2 mL/min, and 4.3 mL/min. Our highest flow range was recommended by EHL as it is the flow rate for liquid-introduction ICP-MS analysis.

We decided to use helium cell gas because it is inert, and would not introduce chemical reactions into our sample stream. Agilent Technologies published an experiment detailing their

own tests showing successful removal of polyatomics, including SO_2 polyatomic species from the Zn66 mass peak (McCurdy et al., 2006). Helium gas was also used by the EHL team for their analysis, so they were most familiar with the use and functions of helium as a collision cell gas.

Hydrogen gas was experimented with by the EHL team prior to the LA-ICP-MS experiments. The EHL team saw reduced instrument signal ("counts") using hydrogen gas for the collision cell, and with the limited number of counts provided by laser ablation, decided helium would be a better option.

Due to limitations of the collision cell's gas introduction system, xenon and oxygen collision cell gases were not tested.

Table 10 Experimental Parameters for Collision Cell Gas Experiments

	No Gas	Low He	High He
Laser parameters	60% energy, 100 m/s velocity, 30 µm spot size.	60% energy, 100 m/s velocity, 30 µm spot size.	60% energy, 100 m/s velocity, 30 µm spot size.
ICP-MS Acq. Time	0.817 sec	0.817 sec	0.817 sec
Collision Cell Gas (He)	0.00 mL/min	2.0 mL/min	4.3 mL/min

The average zinc signal acquired for each gel (0 – 1000 ppm), at each flow rate of cell gas, is summarized below. The linear regression model was used to calculate a concentration based on the line of best fit, and the % error between the calculated concentration and the known standard concentration was found.

Table 11 Calibration Curves from Collision Cell Gas Experiments

Zinc Calibration Curves									
Collision Gas - Flow (mL/min)									
0.0				2.0			4.3		
Gel Standard	Signal	Calc. Conc.	% Error	Signal	Calc. Conc.	% Error	Signal	Calc. Conc.	% Error
0	6780	11.8	N/A	1170	20.7	N/A	15.77	18.8	N/A
0.05	4780	9.30	18502%	331	8.88	17660 %	5.77	7.91	15711 %
0.1	4530	8.99	8892%	351	9.16	9055%	5.26	7.35	7250%
0.5	5280	9.92	1884%	379	9.56	1811%	6.28	8.46	1592%
1	8980	14.5	1354%	519	11.5	1053%	8.72	11.1	1010%
5	7000	12.1	141%	535	11.8	135%	7.05	9.30	86%
10	12100	18.4	84%	987	18.1	81%	16.2	19.2	92%
25	22500	31.4	26%	1930	31.5	26%	23.7	27.4	9%
50	45800	60.6	21%	4000	60.7	21%	52.3	58.4	17%
100	91200	117	17%	8030	118	18%	105	115	15%
357	192000	243	32%	17500	251	30%	234	255	29%
500	355000	447	11%	29800	425	15%	416	453	9%
1000	851000	1060	6%	75600	1070	7%	974	1060	6%
Slope	y = 801.47x - 2675.2			y = 70.81x - 297.38			y = 0.9223x - 1.5221		
R^2	$R^2 = 0.9805$			$R^2 = 0.9786$			$R^2 = 0.9843$		
Range	0.05-1000			0.05-1000			0.05-1000		

The average signal from each gel standard is shown, as well as the linear regression equation created using the average signals and known concentration of the standards. These Zinc calibration curves were not processed with the final analysis' 1/x weighting, sliding mean, or adjustments for gas blank signal. The 2 mL/min (low-flow) Helium run did not reduce the performance of the calibration curve, as the table shows comparable percent error and R squared values between the no gas, and 2 mL/min curves. The 2 mL/min flow rate of Helium collision cell gas also did not reduce the zinc signal to a concerningly low level. The 4.3 mL/min flow rate shows drastically lowered signal from the ICP-MS. Because the zinc signal was strong enough to avoid sensitivity issues with a flow rate of 2 mL/min, we decided to use collision cell gas at 2 mL/min to address our concerns with polyatomic interferences by sulfur-oxides.

Appendix D: Acquisition Time Experiments:

The last parameter that needed to be changed was the time the ICP-MS spent analyzing for each element. A quadrupole ICP-MS cannot simultaneously detect all of the elements selected for analysis. Instead, the detector cycles through all of these elements, detecting one at a time. The dwell time is the time spent on each element, and the acquisition time is the total amount of time the ICP-MS takes to cycle through all of the elements of interest. The sampling time is the total time it takes the instrument to cycle through all of the elements it is programmed it to detect.

Our initial experiments used a much larger acquisition time, with a focus on longer dwell times to optimize instrument sensitivity. However, our zinc literature review suggests that a lower acquisition time may be necessary to acquire data points within a small hair portion with elevated zinc from our supplementations.

Table 12 Calibration Curves from Acquisition Time Experiments

Zinc Calibration Curve							
Acq. Time:	0.179 sec				0.817 sec		
Conc.	Signal	Calc. Conc.	% Error	Conc.	Signal	Calc. Conc.	% Error
0	278	5.85	#DIV/0!	0	805	16.33	#DIV/0!
0.05	213	4.87	9649%	0.05	328	9.60	19100%
0.1	213	4.87	4772%	0.1	351	9.92	9822%
0.5	323	6.54	1208%	0.5	379	10.32	1964%
1	337	6.74	574%	1	338	9.75	875%
5	531	9.67	93%	5	535	12.5	150%
10	909	15.4	54%	10	987	18.9	89%
25	1900	30.3	21%	25	1930	32.2	29%
50	3820	59.5	19%	50	4000	61.4	23%
100	6870	106	6%	100	8030	118	18%
357	17900	273	24%	357	17500	252	29%
500	32600	496	1%	500	29800	425	15%
1000	68035.53	1031.07	3%	1000	75600	1070	7%
Equation	y = 66.1x - 109			Equation	y = 70.9x - 352		
R squared	$R^2 = 0.992$			R squared	$R^2 = 0.979$		

Both calibration curves were run with the same laser parameters and 2 mL/min of collision cell He gas. This table shows comparable performance of the calibration curve with a

lowered acquisition time. These average signal values were not processed with any adjustment for gas blank signal or baseline gelatin signal, and were not smoothed before averaging. These steps were only added to the final processing of the calibration curves used on the hair samples.

These results gave us confidence that we could acquire data from the hairs at a resolution that would elucidate an elevated zinc peak, if one existed, for the single dose supplementation events.

The lowered acquisition time meant we needed lower dwell times for the detection of each element. Because different elements are more or less abundant in the hair, and produce lower or higher counts on the ICP-MS, we wanted to keep the dwell times highest for elements that would have the lowest counts at the concentrations likely to be found in the hair.

We averaged the instrument background signal and the hair signal for elements in our preliminary analysis of hairs, and created a “signal-to-noise” ratio. A low ratio indicated that it would need a larger acquisition time to acquire as much data in the shorter time frame as possible to keep the hair ablation signal above the background signal of the instrument. The signals were averaged from all five hairs analyzed for 5 participants in a preliminary analysis of samples. These signal to noise ratios helped inform the detection time of each element that you can see below.

Table 13 Signal to Noise Ratios of Elements for Analysis

The Average Element Signal-to-Noise Ratios	
Zn66	94.6
As75	1.22
Cd111	2.05
Hg202	3.27
Pb208	25.4
Mn55	1.63
S34	17.1

Table 14 ICP-MS Integration Times for Each Element - Final Analysis

Isotope	**Integration time (sec)**	**Tune Mode**
^{34}S	0.0100	Gas
^{32}S	0.0050	Gas
^{55}Mn	0.0300	Gas
^{66}Zn	0.0050	Gas
^{75}As	0.0400	Gas
^{111}Cd	0.0300	Gas
^{202}Hg	0.0200	Gas
^{208}Pb	0.0200	Gas
Total Sampling Period	**0.179 sec**	

Appendix E: Normalization of the Zinc Signal

Sulfur was analyzed for potential normalization of the zinc signal, and for use as a qualitative check on the laser's line scan success. Sulfur is very abundant in the hair's keratin and a significant dip or decrease in sulfur signal can indicate the laser has migrated off the hair sample during ablation. Once the acquisition parameters were decided upon, we tested the performance of our calibration curve at the lower sampling time.

Sulfur normalization is a process that uses the hair's endogenous sulfur as an internal standard by which other elements' concentrations are adjusted. The zinc signal being interpreted becomes a ratio of zinc to sulfur, which in theory addresses the issue presented with Participant 7's hairs.

However, the use of sulfur normalization is not always used in LA-ICP-MS research and there are examples of researchers decided for and against this data processing step. Pozebon et al. (2008) analyzed hair using LA-ICP-MS for detection of platinum from cancer drugs, and used sulfur normalization in his analysis. Legrand et al. (2007) also described their use of ^{34}S for normalization of their mercury signal in hair analysis using LA-ICP-MS.

But other publications, like the study done by Duncan et al. (2021) on gadolinium in hair, did not find that sulfur normalization decreased variability. Duncan et al. (2021) processed hairs using sulfur normalization and a rolling average function. Their goal was to decrease variation not due to real fluctuation of gadolinium in the hairs, and they found that the rolling average smoothing (done in R) was more effective than normalizing to sulfur based on the coefficient of variation (CV) measured for each technique (Duncan et al., 2021).

In our study, we did have one sample which may have benefitted from sulfur normalization:

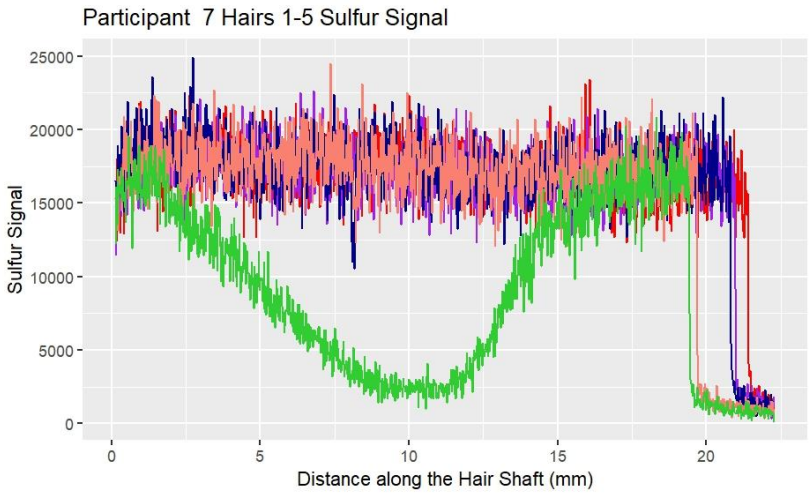


Figure 9 Participant 7 Sulfur Signal

This graph shows the raw sulfur signal from the ICP-MS instrument for five hairs (differently colored lines represent different hairs) from Participant 7 in our study. The green line's sulfur signal distinctly drops below the rest of the hairs, before recovering close to its original signal strength. This drop is most likely due to a decrease in the laser's ablation of the hair. This has been observed to occur from either an out-of-focus laser, a laser that deviates from the hair, or from a transient decrease in the laser's energy.

The zinc signal from the ICP-MS is shown below. You can see a similar drop in signal to what is observed with this participant's sulfur signal.

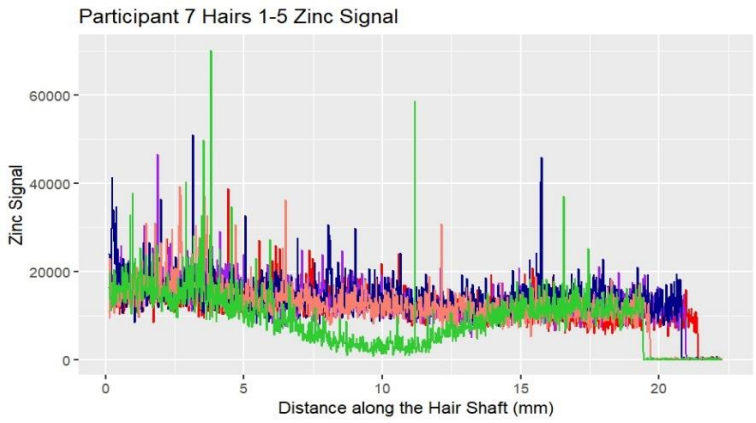


Figure 10 Participant 7 Zinc Signal along Hairs

If the zinc signal is graphed without accounting for the overall decrease in signal, this may look like a characteristic of the hair: a portion of the hair with much lower zinc concentration. However, because the sulfur signal also decreases in this way, we interpret this zinc signal fluctuation as an instrument mistake

However, among 105 hairs, only one seemed to have a zinc signal that would potentially benefit from this normalization. Because the majority of our hair signals were not affected by large decreases likely due to ablation problems, we decided normalization would not be helpful. Our conclusion echoes that of Duncan et al. (2021).

Appendix F: Concentrations in Hairs – All Participants

The plots of hair concentrations are generated with ggplot2, in R using R Studio (Wickham H., 2016; R Core Team, 2023; Rstudio Team, 2020),

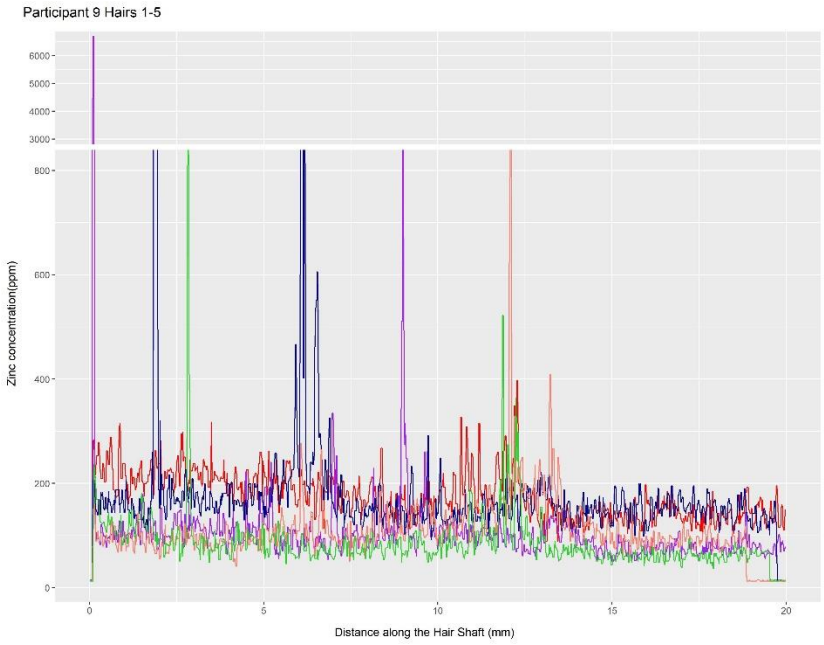


Figure 11 Participant 9 Truncated Axis Hairs 1-5

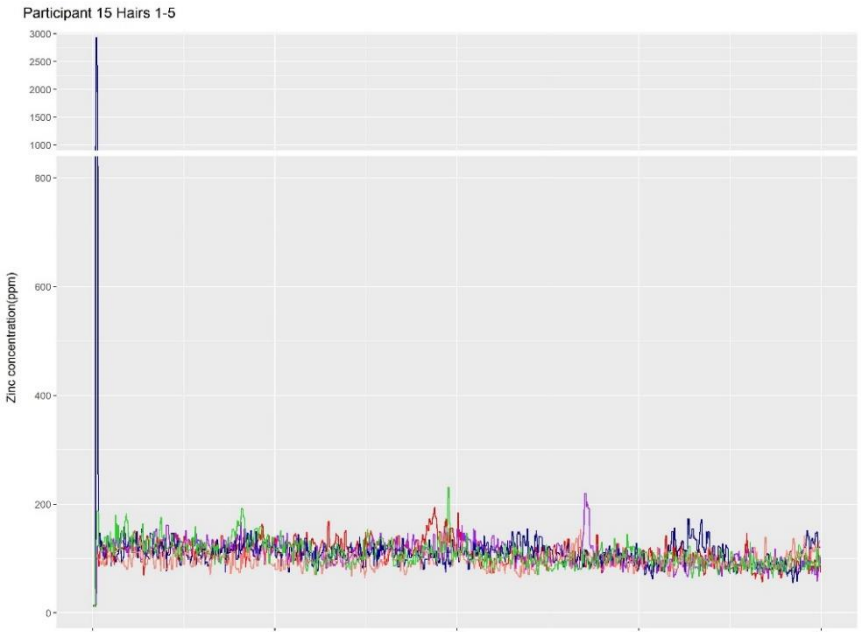


Figure 12 Participant 15 Truncated Axis Hairs 1-5

Below are the concentrations of all six metals along the hairs from each participant. Each series of 21 graphs shows a different metal's concentrations. The different hairs are overlayed and graphed for each participant.

Figure 13 Arsenic Concentration Along Participant Hairs

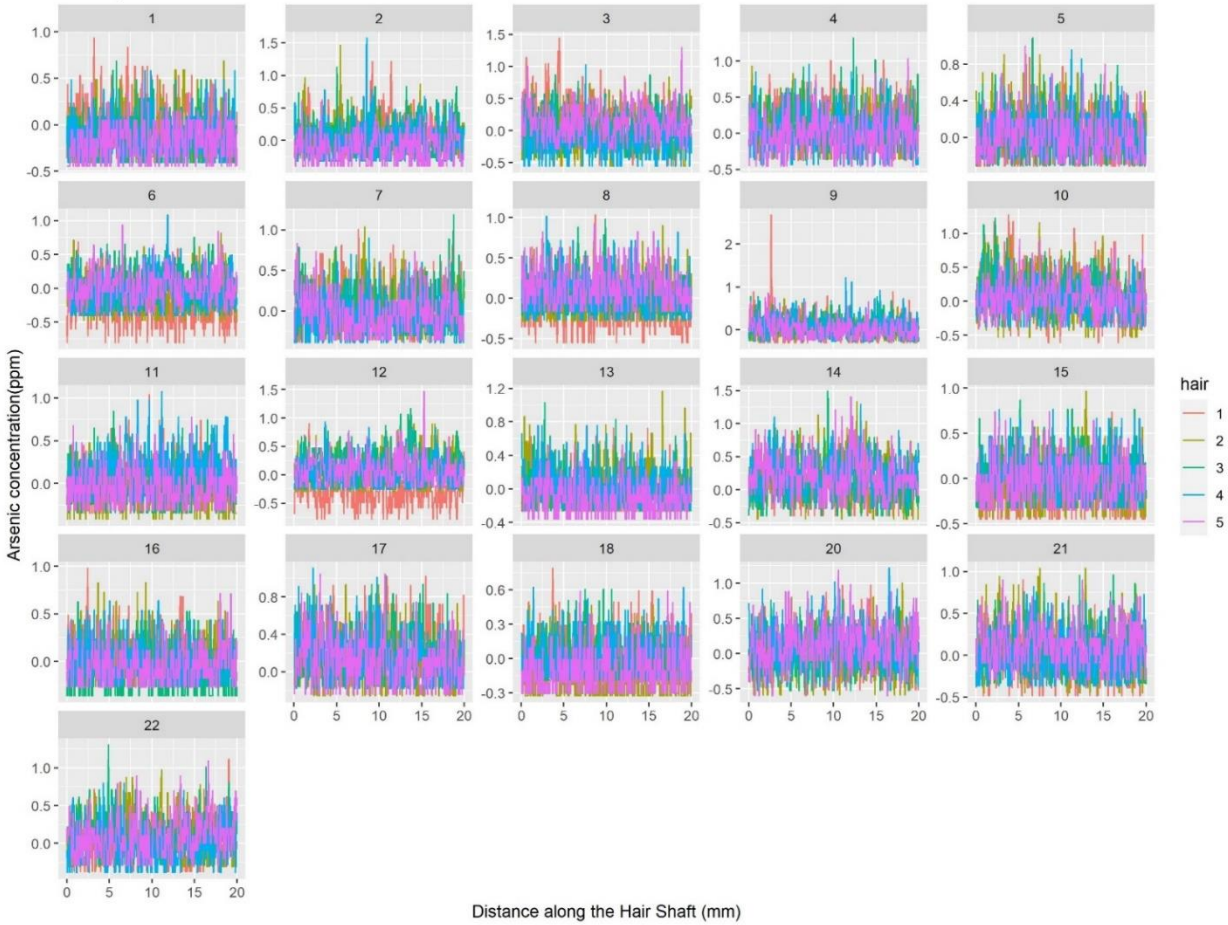


Figure 14 Cadmium Concentration Along Participant Hairs

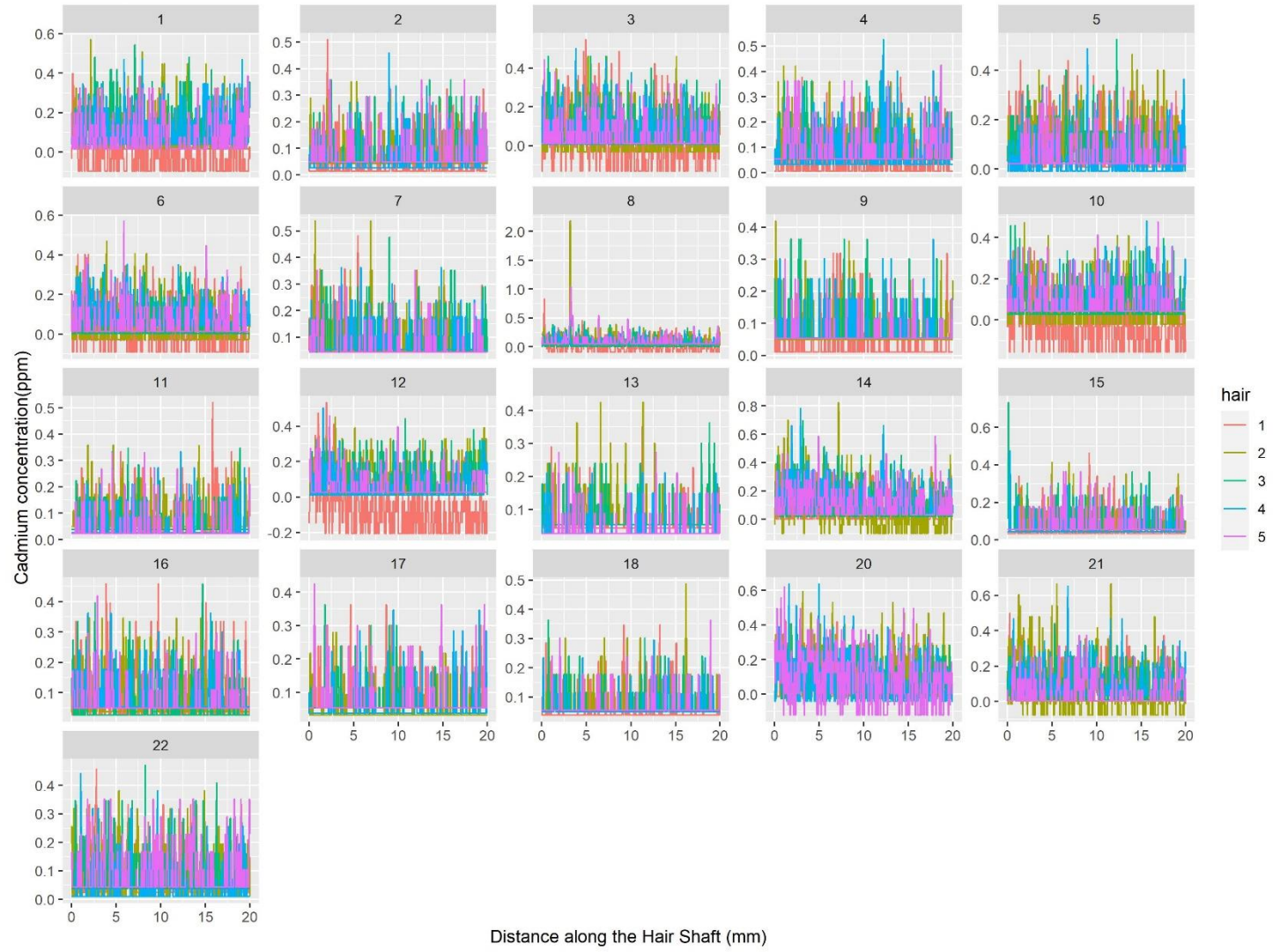
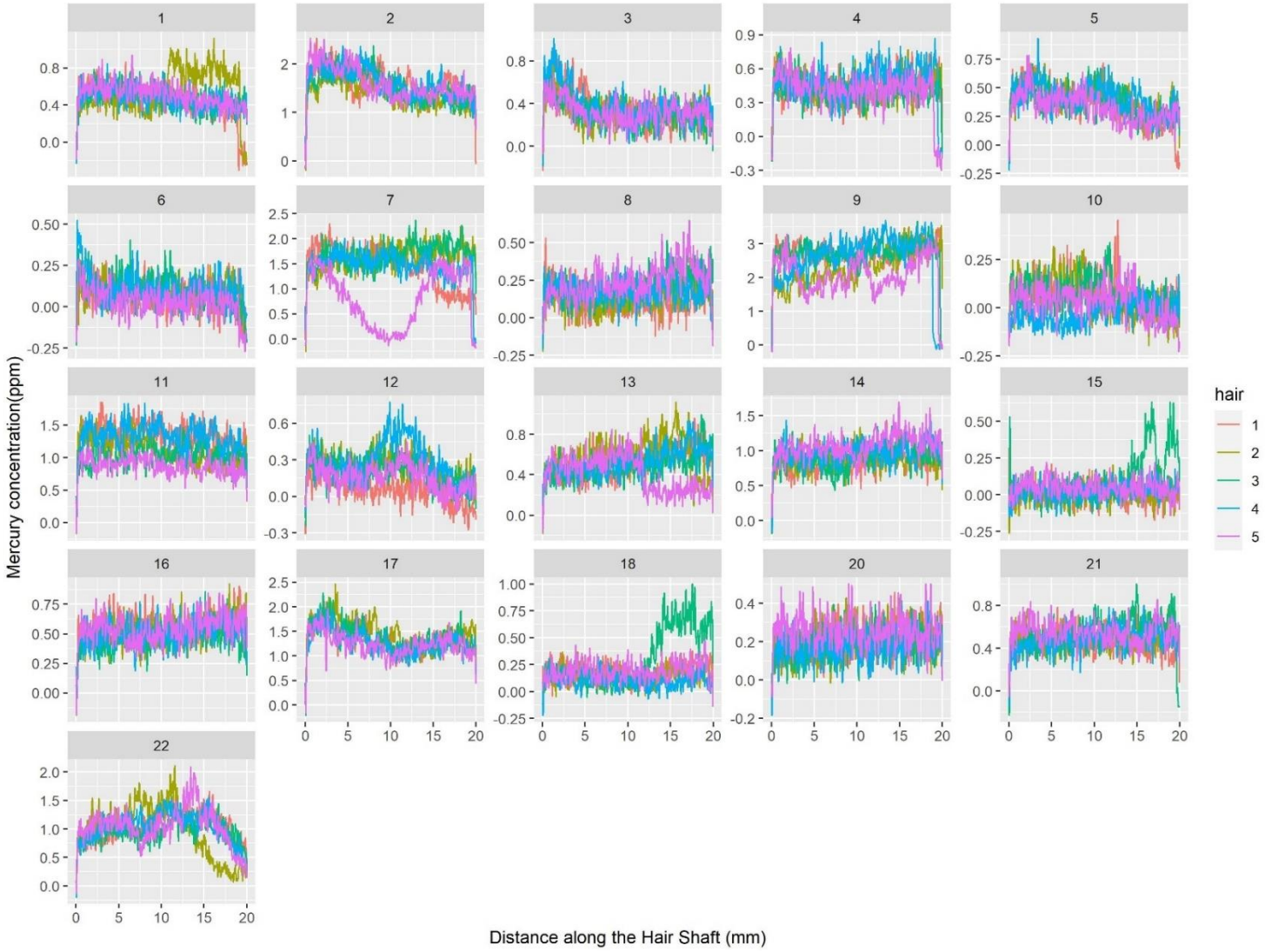


Figure 15 Lead Concentrations Along Participant Hairs

Figure 16 Manganese Concentrations Along Participant Hairs

Figure 17 Mercury Concentration Along Participant Hairs



Appendix G: Visuals of Variation – Between and Among Participants

For the elements with more than half the participants' concentrations above our limit of detection, we characterized the variability within and between participants. Below are the box plots for Mercury and Lead showing such variation.

Figure 18 Mercury Concentrations Between and Within Participants

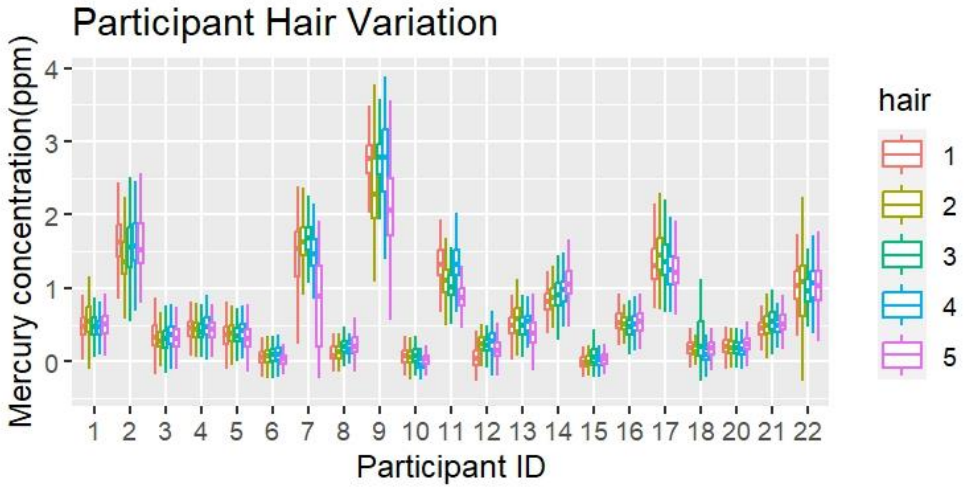
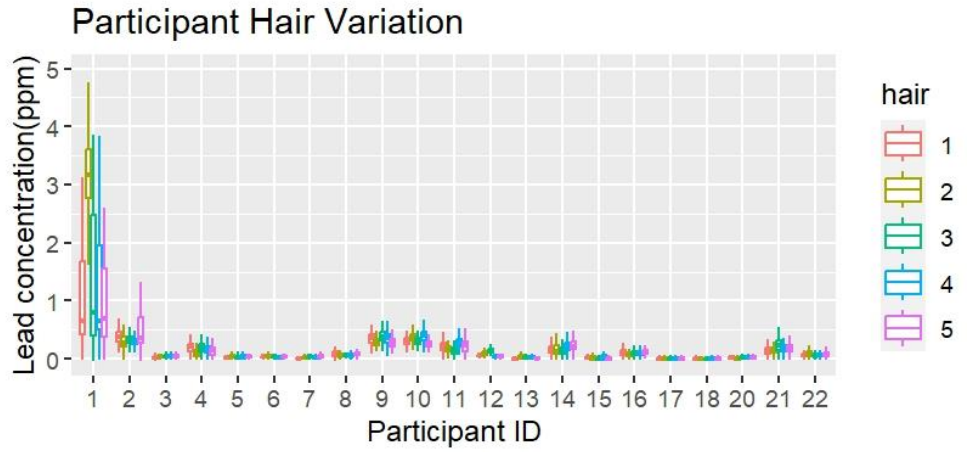


Figure 19 Lead Concentrations Between and Within Participants



To visualize the number of hairs below our limit of detection, the average concentration for each metal is tabulated below for each participant. Here, values are shown in red that are below the limit of detection, but are kept in the table to illustrate the variation and estimated concentrations across participants.

Table 15 Metal Concentrations by Participant for All Metals of Interest

Average Concentrations (ppm)		Participant ID										
Element	LOD	1	2	3	4	5	6	7	8	9	10	11
Zinc	5	247	159	146	132	129	142	164	142	132	120	103
Lead	0.05	1.53	0.418	0.046	0.162	0.036	0.038	0.031	0.073	0.336	0.324	0.212
Mercury	0.05	0.493	1.56	0.340	0.436	0.371	0.078	1.41	0.176	2.49	0.044	1.14
Manganese	1.0	0.923	0.704	1.76	0.670	0.695	0.707	0.860	0.868	0.743	0.731	0.726
Arsenic	0.05	-0.0992	0.0268	0.0367	0.0679	0.0456	-0.0674	0.0245	0.0486	0.0635	0.0771	0.0336
Cadmium	0.1	0.0918	0.0726	0.0713	0.0897	0.0769	0.0616	0.0828	0.0782	0.0724	0.0702	0.0623

Average Concentrations (ppm)	Participant ID									
Element	12	13	14	15	16	17	18	20	21	22
Zinc	95.7	87.4	275	108	150	118	114	149	123	152
Lead	0.0768	0.0257	0.196	0.0194	0.110	0.0106	0.0066	0.0224	0.181	0.0741
Mercury	0.193	0.504	0.932	0.0362	0.521	1.339	0.187	0.204	0.503	1.02
Manganese	0.727	0.743	0.717	0.669	0.748	0.710	0.659	0.704	0.741	0.730
Arsenic	0.0409	0.0008	0.164	-0.0121	-0.0122	0.166	-0.0355	0.0351	0.0603	0.0608
Cadmium	0.0508	0.0595	0.117	0.0780	0.0815	0.0688	0.0695	0.0941	0.0902	0.0787

We also calculated the average concentrations for all elements (Zinc, Lead, Mercury, Arsenic, Cadmium, and Manganese) across all hairs for each participant. These results are shown below in Table 20 -25.

Table 16 Zinc Hair Concentrations

Average Zinc Hairs (ppm)	Participant ID										
	1	2	3	4	5	6	7	8	9	10	11
Hair1	220	183	121	150	132	134	164	141	175	129	103
Hair2	268	154	142	120	135	151	177	130	114	112	101
Hair3	237	162	149	118	117	149	184	145	177	121	92.9
Hair4	262	151	159	144	139	126	164	144	109	110	117
Hair5	246	143	161	130	123	152	129	151	85	128	98.5
Average	247	159	146	132	129	142	164	142	132	120	103

Average Zinc in Hairs (ppm)	Participant ID									
	12	13	14	15	16	17	18	20	21	22
Hair 1	99.0	80.7	177	110	162	121	113	157	119	149
Hair 2	99.8	100	275	108	145	129	116	141	129	151
Hair 3	103	79.1	258	115	152	105	109	145	107	141
Hair 4	92.3	97.0	302	97.0	154	109	110	145	118	143
Hair 5	83.1	79.4	359	109	136	125	121	155	139	172
Average	95.7	87.4	275	108	150	118	114	149	123	151

Table 17 Hair Concentrations of Lead

Average Lead (ppm) Across Hairs	Participant ID										
Hairs	1	2	3	4	5	6	7	8	9	10	11
Hair1	1.01	0.403	0.0430	0.203	0.0263	0.0344	0.0171	0.0935	0.365	0.305	0.218
Hair2	3.11	0.328	0.0340	0.104	0.0545	0.0484	0.0264	0.0642	0.286	0.372	0.164
Hair3	1.40	0.326	0.0483	0.196	0.0305	0.0382	0.0345	0.0560	0.386	0.302	0.148
Hair4	1.18	0.291	0.0513	0.165	0.0367	0.0277	0.0273	0.0644	0.335	0.383	0.290
Hair5	0.955	0.744	0.0554	0.140	0.0326	0.0399	0.0501	0.0859	0.307	0.259	0.240
Average Concentrations (ppm)	1.53	0.418	0.0464	0.162	0.0361	0.0377	0.0311	0.0728	0.336	0.324	0.212

Average Lead (ppm) Across Hairs	Participant ID									
Hairs	12	13	14	15	16	17	18	20	21	22
Hair1	0.0583	0.0105	0.166	0.0223	0.128	0.0137	0.00916	0.0281	0.141	0.0676
Hair2	0.0946	0.0577	0.170	0.0182	0.0790	0.0153	0.00287	0.00759	0.147	0.0950
Hair3	0.139	0.0213	0.148	0.0130	0.109	0.0106	0.00399	0.0252	0.243	0.0657
Hair4	0.0460	0.0225	0.252	0.0347	0.104	0.00614	0.0121	0.0213	0.189	0.0609
Hair5	0.0459	0.0165	0.244	0.00885	0.128	0.00712	0.00508	0.0297	0.186	0.0815
Average Concentrations (ppm)	0.0768	0.0257	0.196	0.0194	0.110	0.0106	0.00664	0.0224	0.181	0.0741

Table 18 Mercury Hair Concentrations

Average Mercury (ppm) Across Hairs	Participant ID										
Hairs	1	2	3	4	5	6	7	8	9	10	11
Hair1	0.454	1.64	0.356	0.450	0.365	0.0628	1.45	0.114	2.75	0.0704	1.34
Hair2	0.549	1.40	0.311	0.433	0.385	0.0890	1.63	0.137	2.33	0.0640	1.12
Hair3	0.484	1.54	0.323	0.406	0.365	0.0924	1.68	0.215	2.73	0.0780	1.03
Hair4	0.470	1.61	0.389	0.485	0.412	0.115	1.47	0.178	2.59	-0.0269	1.33
Hair5	0.510	1.61	0.322	0.408	0.326	0.0327	0.809	0.239	2.06	0.0325	0.875
Average Concentrations (ppm)	0.493	1.56	0.340	0.436	0.371	0.0783	1.41	0.176	2.49	0.0436	1.14

Average Mercury (ppm) Across Hairs	Participant ID									
Hairs	12	13	14	15	16	17	18	20	21	22
Hair1	0.0560	0.508	0.828	-0.00390	0.557	1.34	0.178	0.207	0.452	1.04
Hair2	0.249	0.583	0.882	0.00713	0.520	1.47	0.153	0.200	0.495	0.987
Hair3	0.214	0.490	0.907	0.103	0.472	1.41	0.306	0.187	0.542	0.970
Hair4	0.286	0.531	0.969	0.0268	0.508	1.25	0.106	0.174	0.481	1.04
Hair5	0.162	0.410	1.1	0.0485	0.546	1.23	0.191	0.255	0.545	1.04
Average Concentrations (ppm)	0.193	0.504	0.932	0.0362	0.521	1.34	0.187	0.204	0.503	1.02

Table 19 Manganese Hair Concentrations

Average Manganese (ppm) Across Hairs	Participant ID										
Hairs	1	2	3	4	5	6	7	8	9	10	11
Hair1	0.904	0.710	1.51	0.700	0.666	0.712	0.849	0.876	0.769	0.687	0.739
Hair2	0.943	0.682	1.67	0.655	0.724	0.690	0.881	0.839	0.765	0.729	0.734
Hair3	0.886	0.718	1.85	0.635	0.658	0.708	0.895	0.881	0.687	0.724	0.725
Hair4	0.963	0.738	1.87	0.659	0.710	0.704	0.844	0.860	0.760	0.830	0.730
Hair5	0.918	0.673	1.87	0.702	0.716	0.722	0.831	0.883	0.735	0.683	0.703
Average Concentrations (ppm)	0.923	0.704	1.76	0.670	0.695	0.707	0.860	0.868	0.743	0.731	0.726

Average Manganese (ppm) Across Hairs	Participant ID									
Hairs	12	13	14	15	16	17	18	20	21	22
Hair1	0.733	0.704	0.680	0.691	0.811	0.710	0.681	0.684	0.706	0.738
Hair2	0.747	0.840	0.695	0.688	0.709	0.701	0.664	0.706	0.730	0.742
Hair3	0.768	0.708	0.748	0.638	0.752	0.718	0.671	0.728	0.756	0.699
Hair4	0.711	0.712	0.715	0.675	0.728	0.720	0.621	0.680	0.743	0.765
Hair5	0.678	0.752	0.746	0.653	0.737	0.703	0.659	0.721	0.768	0.706
Average Concentrations (ppm)	0.727	0.743	0.717	0.669	0.748	0.710	0.659	0.704	0.741	0.730

Table 20 Concentrations of Arsenic

Average Arsenic (ppm) Across Hairs	Participant ID										
Hairs	1	2	3	4	5	6	7	8	9	10	11
Hair1	-0.00667	0.0906	0.131	0.136	0.0568	-0.270	0.0752	-0.0890	0.0587	0.151	0.0435
Hair2	-0.0685	0.0518	0.00288	0.0777	0.0779	-0.0918	0.0485	0.0264	0.0372	0.0544	-0.0347
Hair3	-0.140	0.0774	0.0707	0.0993	0.0110	0.0346	0.0822	0.110	0.0981	0.139	0.00502
Hair4	-0.102	0.00823	-0.143	0.0349	0.0725	-0.0393	-0.0489	0.0394	0.104	-0.00561	0.182
Hair5	-0.179	-0.0941	0.122	-0.00862	0.0100	0.0290	-0.0346	0.156	0.0197	0.0466	-0.0277
Average Concentrations (ppm)	-0.0992	0.0268	0.0367	0.0679	0.0456	-0.0674	0.0245	0.0486	0.0635	0.0771	0.0336

Average Arsenic (ppm) Across Hairs	Participant ID									
Hairs	12	13	14	15	16	17	18	20	21	22
Hair1	-0.288	-0.00782	0.0758	-0.122	0.0680	0.214	0.0331	0.0243	-0.0219	0.0415
Hair2	0.132	0.0824	0.160	-0.0421	0.00492	0.118	-0.130	-0.0604	0.104	0.0562
Hair3	0.217	-0.0154	0.134	0.0694	-0.0981	0.193	0.00349	-0.0251	0.0830	0.117
Hair4	0.0548	0.0494	0.232	0.0476	0.000616	0.169	0.0224	0.120	0.0127	0.00580
Hair5	0.0886	-0.104	0.217	-0.0132	-0.0364	0.135	-0.107	0.117	0.124	0.0840
Average Concentrations (ppm)	0.0409	0.000834	0.164	-0.0121	-0.0122	0.166	-0.0355	0.0351	0.0603	0.0608

Table 21 Concentrations of Cadmium

Average Cadmium (ppm) Across Hairs	Participant ID										
Hairs	1	2	3	4	5	6	7	8	9	10	11
Hair1	0.00767	0.0600	0.0436	0.0690	0.103	0.0398	0.0896	0.0397	0.0513	-0.0114	0.0690
Hair2	0.116	0.0773	0.0703	0.0956	0.0984	0.0488	0.0861	0.0946	0.0749	0.0794	0.0826
Hair3	0.130	0.0824	0.105	0.0927	0.0774	0.0573	0.0870	0.0705	0.0841	0.0881	0.0628
Hair4	0.118	0.0585	0.0689	0.0918	0.0444	0.0939	0.0841	0.0781	0.0847	0.0968	0.0513
Hair5	0.0877	0.0848	0.0685	0.0993	0.0612	0.0680	0.0673	0.108	0.0669	0.0981	0.0457
Average Concentrations (ppm)	0.0918	0.0726	0.0713	0.0897	0.0769	0.0616	0.0828	0.0782	0.0724	0.0702	0.0623

Average Cadmium (ppm) Across Hairs	Participant ID									
Hairs	12	13	14	15	16	17	18	20	21	22
Hair1	-0.0636	0.0634	0.0767	0.0696	0.0928	0.0827	0.0625	0.0695	0.104	0.0783
Hair2	0.108	0.0755	0.104	0.0737	0.0838	0.0536	0.0752	0.1373	0.0982	0.0679
Hair3	0.0856	0.0733	0.132	0.0924	0.0632	0.0714	0.0747	0.0738	0.0831	0.0952
Hair4	0.0615	0.0419	0.145	0.0676	0.0919	0.0598	0.0663	0.0948	0.0965	0.0535
Hair5	0.0624	0.0435	0.125	0.0866	0.0760	0.0766	0.0689	0.0953	0.0690	0.0983
Average Concentrations (ppm)	0.0508	0.0595	0.117	0.0780	0.0815	0.0688	0.0695	0.0941	0.0902	0.0787

Appendix H: Data Distribution and Normality

Below are the Quantile-Quantile plots generated using ggplot2 and ggpubr packages in R (Wickham, 2016; Kassambara, 2023a). These plots visualize all 105 hairs' zinc, lead, and mercury concentrations and an ideal normal distribution (solid black line) with a 95% confidence level shaded in grey. Data points that fall outside of the grey shaded region are considered outside a normal distribution.

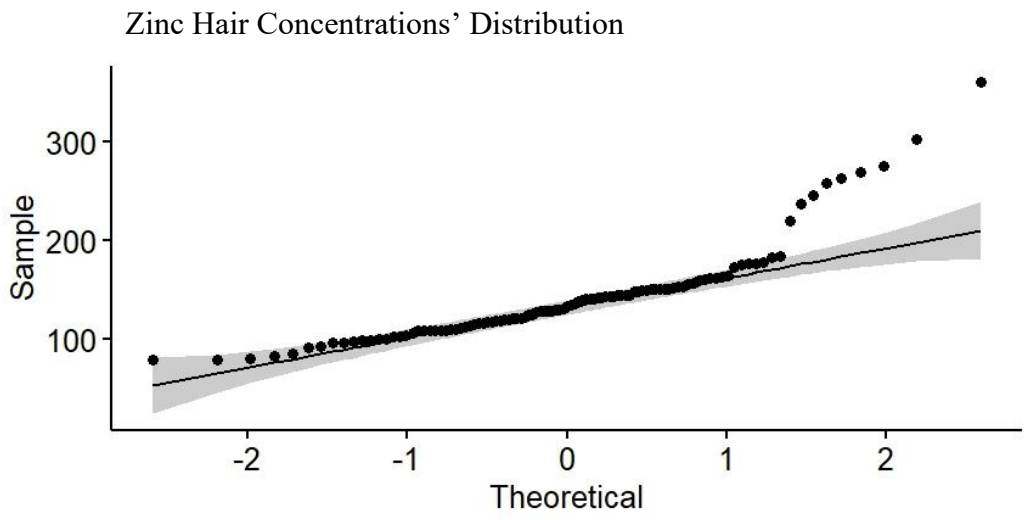


Figure 20 Zinc Data Distribution

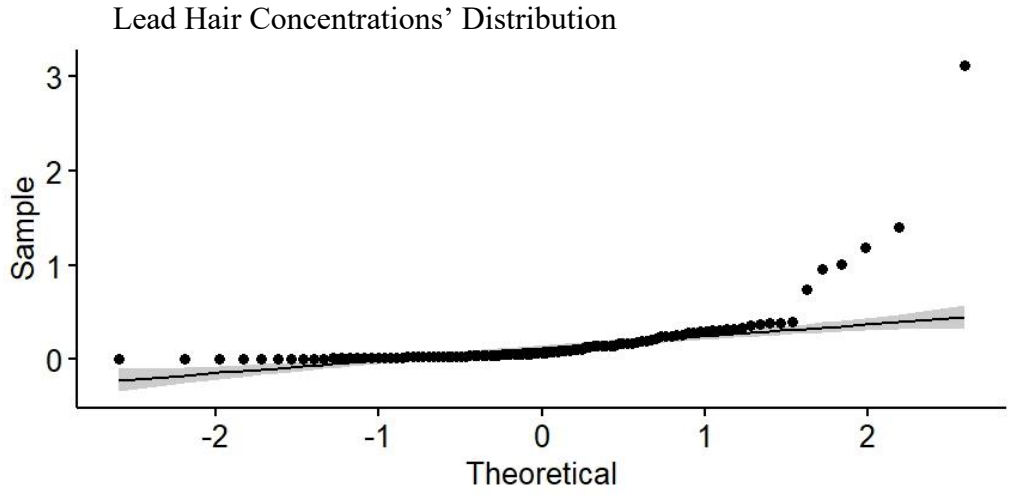


Figure 21 Lead Data Distribution

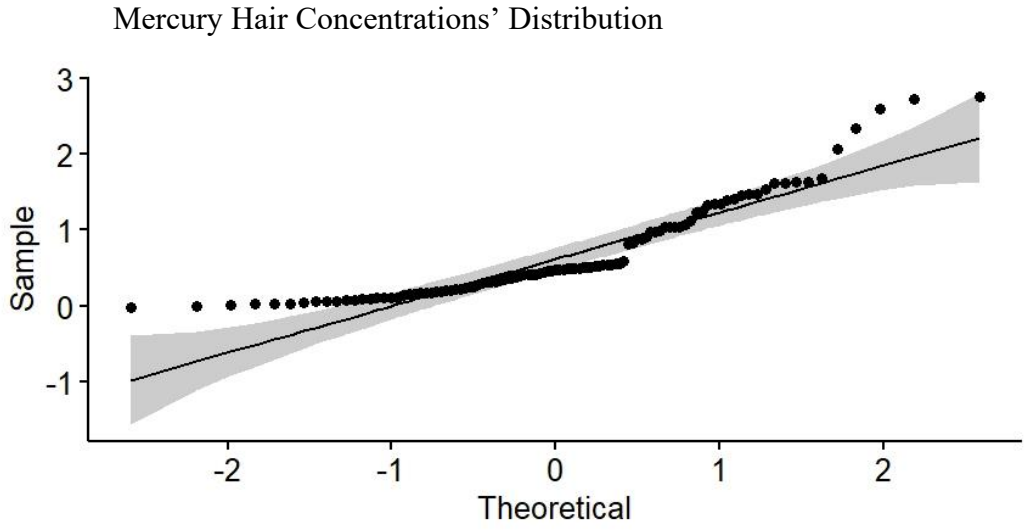


Figure 22 Mercury Data Distribution