Hair as a Biomarker for Manganese Exposure Among Welders

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Abstract

Hair as a Biomarker for Manganese Exposure Among Welders

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Quantifying exposure to manganese (Mn) in airborne welding fume, and the resultant dose, presents many challenges. Common biomarkers such as Mn in blood or Mn in urine have not proven to be practical even in studies where positive associations were observed. Hair Mn (MnH) is another potential biomarker. It is easy to obtain and grows slowly, so it has the advantage over blood and urine of being less influenced by short term variability in Mn exposure levels. The objective of this research was to determine whether hair can be used as a biomarker for welders' exposure to manganese. This body of work investigates three aspects of using hair as a biomarker: (1) whether airborne Mn (MnA) is associated with bulk hair Mn ($MnH_{(B)}$), (2)

whether $MnH_{(B)}$ is associated with Mn determined by laser ablation of individual hair strands ($MnH_{(La)}$), and (3) whether $MnH_{(La)}$ can be used as an indicator of MnA exposure. $MnH_{(B)}$ samples (1 cm length) were collected from each of 47 welding school students and personal air sampling was conducted to determine their individual MnA exposures. A moderate association between bulk MnH ($MnH_{(B)}$) and MnA was found. The investigation of whether Mn measured by laser ablation inductively coupled mass spectrometry (LA-ICP-MS) of individual hair strands ($MnH_{(La)}$) is associated with $MnH_{(B)}$, and could be used to improve the limited time resolution provided by $MnH_{(B)}$, led to the development of a new calibration standard for this work. Hair strands with known Mn concentrations are not available, so a series of calibration standards consisting of gelatin samples spiked with known concentrations of Mn (MnG) were developed. The MnG calibration results were compared to Mn concentrations determined via acid digestion of bulk hair samples followed by inductively coupled mass spectrometry (ICP-MS), and indicated an association between the MnG calibration standards developed for this study and $MnH_{(B)}$. The final investigation found no association between MnA and temporally-resolved $MnH_{(La)}$. Further steps toward the achievement of this objective are discussed.

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Chapter 1. BACKGROUND

1.1 MANGANESE

1.1.1 *Description*

Mn is a naturally occurring transition metal, which is most commonly used in manufacturing to harden steel, as a pigment, in welding rods, and in dry cell batteries. In consumer products, Mn occurs naturally in food, and is found in nutritional supplements, fertilizers, paints, and cosmetics. It is also used as an imaging contrast agent (mangafodipir trisodium; MnDPDP) during magnetic resonance imaging to detect lesions in body organs, and has replaced lead as a fuel additive in the form of methylcyclopentadienyl manganese tricarbonyl (MMT or MCMT) to improve the octane rating of gasoline (Agency for Toxic Substances and Disease Registry 2012).

Mn is found in air, soil, water and food. In the natural environment Mn is present in limited forms of inorganic salts, such as manganese chloride ($MnCl_2$), manganese sulfate ($MnSO_4$), manganese acetate (MnOAc), manganese phosphate ($MnPO_4$), manganese dioxide (MnO_2), manganese tetroxide (Mn_3O_4), and manganese carbonate ($MnCO_3$). The fuel additive MMT is a ubiquitous organic source for Mn. However, MMT breaks down very rapidly to an inorganic form when exposed to sunlight and thus MMT levels are expressed as Mn levels (Agency for Toxic Substances and Disease Registry 2012). Ambient Mn levels due to MMT are estimated to be approximately 0.5 to 15 ng/m^3 in remote areas, < 10 to 30 ng/m^3 in non-polluted urban and rural areas, and 10–70 ng/m^3 in large urban centers (excluding point sources) (Pfeifer et al. 2004). Importantly, MnO_2 is present in occupational environments where welding occurs.

Health effect assessments focus on inorganic manganese in the oxidation states Mn(III), Mn(IV), and most importantly Mn(II), because these forms of Mn are relevant to human health and can lead to manganism (Agency for Toxic Substances and Disease Registry 2012). Mn(II) is the important form for humans, because in low doses it is an essential nutrient, yet in higher doses it is a neurotoxicant (Aschner et al. 2005).

1.1.2 *Health effects*

Mn functions as a co-factor in enzymes involved in detoxification of reactive oxygen species (ROS), including superoxide dismutase 2 (SOD2) which is found in mitochondria (United States Environmental Protection Agency 2003). The provisionally estimated safe and adequate daily dietary intake (ESADDI) of Mn is 2 to 5 mg/day (National Research Council (NRC) (U.S) Subcommittee on the Tenth Edition of the RDAs 1992; Greger 1998). NRC uses a range because a minimum amount of Mn is required for a human health. Mn intake study results are not consistent (Greger 1998). Chronic aerosol exposure of >5 mg/m^3 inhaled Mn may induce manganism, a Parkinson-like neurodegenerative disease (Aschner M. et al. 2005). Workers with manganism show behavioral changes and slow, clumsy movements. Children are more sensitive to Mn exposures because their nervous systems are continuing to develop. Learning disabilities, and in severe cases, difficulties with speaking and walking, have been observed in children with elevated exposure to Mn (Bouchard et al. 2007). The mechanisms for manganism and the underlying presumed Mn deposition in the brain are not yet fully understood, partially because of the challenges associated with quantifying exposure to airborne particles containing Mn and the resultant dose (Dorman et al. 2002; Takeda 2003; Yokel 2006).

1.1.3 *Mn exposures*

Mn fume is released into air during welding and inhaled. It is absorbed primarily through the lung, and to a lesser extent, through the gastrointestinal tract. Once absorbed, Mn may pass the blood-brain barrier and deposit in the brain and other tissues (Aschner et al. 2007).

Welding fume exposure varies widely depending on the type of welding, the specific welding filler metal, and the base metal composition. Mn exposure from welding fume has been shown to vary from less than 10 $\mu g/m^3$ in gas tungsten arc welding (GTAW) to more than 585 $\mu g/m^3$ in flux core arc welding (FCAW) (Pesch et al. 2012). Exposure levels are also affected by the welding environment, the use of ventilation and the use of respiratory protective equipment (Flynn and Susi 2009; Liu 2010a; Hobson et al. 2011). For example, welders may work in open environments such as construction sites, or in small confined spaces with poor ventilation, such as in shipbuilding. The variability of welding fume constituents adds to variability in exposure, making accurate estimation of welders' exposures challenging. The current standard practice for measuring welding fume exposures requires welders to wear personal sampling pumps with specific filter cassettes for their work shift. Biomarkers that reflect exposure over time could be an attractive alternative to the traditional exposure monitoring approach.

1.2 BIOMARKERS OF EXPOSURE

Typical biomarkers for Mn include its concentration in blood (or blood components) and in urine - fluids which are relatively easily accessible and commonly collected. Studies have examined relationships between ambient airborne concentrations of Mn and the level of Mn in whole blood, red blood cells, plasma, serum, and urine of exposed workers (Roels et al. 1987; Järvisalo et al. 1992; Roels et al. 1992; Apostoli et al. 2000; Myers et al. 2003). Some of these

studies have found associations between concentrations of airborne particles containing Mn and Mn levels in blood, urine, or both when grouping subjects into exposure categories. However, no associations were found on an individual subject level.

A recent meta-analysis showed that these associations are observed only at higher exposure levels, above about 10 $\mu g/m^3$ (Baker et al. 2014). Even in studies with positive associations, blood and urine have not proven to be practical biomarkers due to the narrow range of Mn concentrations in these biological matrices compared to wide variations in airborne Mn (Smith et al. 2007). At an individual level, dietary intake may also mask associations between inhalation exposure and Mn in blood or urine because on average, the daily dietary intake of Mn is greater than the daily airborne intake (Greger 1998; Agency for Toxic Substances and Disease Registry 2012).

An important advantage of using hair as a biomarker is that its slow growth rate makes it less influenced by short term variations in Mn exposure levels. Hair growth rates are estimated to range from 0.35 mm per day (approximately 1 cm per month) (Tobin 2005a) to about 0.5 mm per day (approximately 1.5 cm per month) (Chamberlain and Dawber 2003). Thus, Mn levels in hair (MnH) levels may be more representative of an integrated average exposure.

Most methods for the analysis of Mn and other metals in hair involve acid-digestion of a hair bundle followed by determination of the metal concentration in the resulting solution using instruments such as ICP-MS. The usefulness of this approach is limited by the large mass of hair required, the relatively long exposure time being assessed (about one month, corresponding to ~1cm of hair), and the difficulty of consistently cutting a bundle of hair strands to the same length. The Environmental Health Laboratory in the Department of Environmental & Occupational Health Sciences at the University of Washington (UW-DEOHS-EHL) requires at least 10 mg of hair for ICP-MS analysis. This mass corresponds to approximately 20 hair strands of 1 cm length, which

is the upper limit that subjects are willing to provide because it creates a visible "hole" in the overall appearance of the hair. To detect Mn in hair that represents a time frame of less than 1 month, hair samples shorter than 1 cm in length would be needed, requiring an increase in the number of strands collected. While more hair strands could be obtained from a human skull, scissors do not provide enough accuracy when cutting hair much shorter than 1 cm. As the length of hair samples decreases, the impact of the cutting error increases. A 2 mm variation in the length of hair samples translates to a 20% error on 1 cm long hair and to a 40% error on 0.5 cm long hair. Inaccuracies in cutting a specific bundle of hair may result in the hair strands of a sample reflecting different periods of exposure. Therefore, acid digestion and ICP-MS limits the minimum exposure duration that can be measured to approximately 1 month.

1.3 STUDIES OF HAIR AS BIOMARKER OF EXPOSURE

Bulk MnH ($MnH_{(B)}$) levels have been used previously as a biomarker for exposure in environmental studies. For example, a comparison study of exposures to low and high levels of Mn in water in rural Quebec, where no other source of Mn was known, showed $MnH_{(B)}$ levels to be significantly higher and IQ levels significantly lower in children who consumed water with the higher Mn concentrations (Bouchard et al. 2007).

In occupational settings, $MnH_{(B)}$ was used as a biomarker for air exposures in studies of manufacturing workers in dry cell battery facilities (Bader et al. 1999). Workplace MnA concentrations ranged from 1 to about 800 $\mu g/m^3$ at three different job sites, with averages of 4, 40, and 400 $\mu g/m^3$. In this study, $MnH_{(B)}$ was found to be associated with MnA levels when subjects were grouped into categories of exposure, but $MnH_{(B)}$ was not found to be associated with MnA on an individual basis.

There are a few studies reporting a relationship between ambient Mn exposure among welders and their $MnH_{(B)}$ (Xie et al. 1995; Zhang et al. 1996; Ramakrishna et al. 1996; Lin 2002; Huang and Cao 2003). These studies (Table 1-1) show higher $MnH_{(B)}$ in welders compared to unexposed controls, but they did not include quantitative assessment of Mn exposure levels. In two of these studies, no association was observed between years welding and MnH (Zhang et al. 1996; Huang and Cao 2003). In contrast, two of the papers demonstrated a relationship with years of exposure as a welder. This finding suggests a relationship with cumulative exposure, but not with exposure occurring during the time period represented by the length of the hair sample (Xie et al. 1995; Lin 2002). These papers did not provide consistent evidence of a quantitative relationship between airborne exposure and levels of Mn in hair.

1.4 LASER ABLATION INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) is a new analytical technique that allows analysis of metals in small defined locations of tissues or other solids. And thus it can be used to trace along a single hair strand. A laser beam moves along a hair shaft and aerosolizes hair material. Aerosols are transported via a stream of carrier gas into the ICP-MS, analyzed, and signal intensity is measured for each point where the hair surface was ablated by the laser. With an ablation interval of as low as 5 μm and an estimated hair growth rate of 350 μm per day, LA-ICP-MS could theoretically allow retrospective exposure measurements with a time resolution of 20 minutes, provided that sufficient amounts of Mn are present in the 5 μm interval being ablated (Stadlbauer et al. 2005). Alternately, the minimum time resolution may be substantially greater than 20 min, because the burn area required to detect a target element may need to be larger than the minimum burning interval. For example, a 4 hour time resolution

was estimated for using LA-ICP-MS to measure mercury in hair following a low exposure. A burn area of 50 μm, far exceeding the minimum burn interval of 5 μm, was found to be required (Legrand et al. 2004).

In addition, uptake of Mn into the body and subsequent incorporation of Mn into the hair could be slower than short term changes in exposure.

Quantifying the results of LA-ICP-MS hair analysis is challenging because well-characterized, matrix-matched calibration standards are not available (Stadlbauer et al. 2005; Legrand et al. 2007), i.e., hair strands with standard known concentrations of Mn do not exist. Several procedural proxies are needed to calibrate a LA-ICP-MS system. A proxy standard with a chemical composition similar to hair is needed for calibrating the ICP-MS. Mixtures of manganese in gelatin can be used for this purpose.

A second proxy standard, with a physical structure that is similar to the biological sample material (Limbeck et al. 2015), i.e., hair, is needed for calibrating the laser ablation and to evaluate the stability of the LA-ICP-MS system. Hair powder can be used for this standard. It must be ground, homogenized, and compressed with a binding agent because the laser ablation process can break up a mechanically weak pellet (Borisov et al. 2001).

Finally, an internal standard inside the biological material (hair) is required to compensate for instabilities in the laser ablation. The ubiquitous sulfur isotope ^{34}S in both the hair strand and the compressed hair powder can serve as the internal standard to compensate for laser beam variations during individual hair strand ablations.

Accurately measuring the time of Mn exposure when analyzing hair strands using LA-ICP-MS is complicated by the difficulty in finding a temporal reference point in a hair strand. Individual hair strands have variable growth rates that differ by sample locations on the scalp, time, and

subject (Tobin 2005a). Ingestion of a specific, identifiable element could be used to create time points in the MnH profile by creating peaks in the profile that can be linked to the time of ingestion. Markers of time that are not related to the Mn exposure [such as selenium (Se)] are preferred because high exposures to Mn may mask the controlled ingestion Mn peak intended to mark a point in time.

Additional issues that arise from using LA-ICP-MS include finding appropriate methods to attach a hair strand in the laser ablation cell, as well as potential surface contamination on a hair strand (Stadlbauer et al. 2005). A hair strand must be fixed to a surface to allow accurate scanning along the shaft. Most commonly, hair has been stapled, taped, or glued to a surface. Glues are preferred over staples and tape because they provide more secure fixation for the small diameter of the hair (~70 μm). However, glues must be assessed for contamination (Stadlbauer et al. 2005).

1.5 HAIR SURFACE CONTAMINATION

A major problem with the use of hair as a biomarker is the issue of direct contamination from the environment, which may be enhanced by electrical charge and residual oil on the hair (Eastman et al. 2013). Personal exposure can influence the extent of external hair contamination. In addition, subjects may contaminate hair by using shampoos that contain metals such as selenium or zinc (e.g. Selsun Blue and other deep cleansing products). Effective washing to remove external contamination is critical to the use of hair as a biomarker.

Different research groups have used different hair washing procedures. Water-based detergent or acetone have been used often, but only a few methods have been thoroughly tested (Eastman et al. 2013; Wołowiec et al. 2013). One commonly used method is a detergent-water mix such as water, Triton X-100, and EDTA (ethylenediaminetetraacetic acid). This approach may

leave a detergent residue, requiring thorough rinsing. Surface tension is another problem related to washing hair with water-based detergents because additional rinsing steps are needed to completely remove the residual detergent. In contrast, acetone has a lower surface tension, does not leave a residue on hair surfaces and, evaporates quickly at room temperature, reducing drying time. Further, detergents may release Mn from the welding fume particles attached to the hair surface, whereas this is unlikely to occur when acetone is used as the solvent.

Triton X-100 (0.5%) sonication plus 1 N nitric acid sonication (TN) was shown with LA-ICP-MS to be an effective cleaning method to remove exogenous metal contamination on hair in preparation for LA-ICP-MS analysis (Eastman et al. 2013). Although this method has not been confirmed by other research groups, the authors provided substantial data and a solid experimental setup supporting their method for assessing residual Mn contamination on the surface of hair. Hair from 10 subjects was contaminated with two methods (dust, Mn solution), analyzed using two methods (hair bundle: ICP-MS, individual hair strands: LA-ICP-MS), and visually inspected for morphological changes following hair washing. The authors noted that hair contamination levels differed from subject to subject and between contamination methods. However, washing hair with TN eliminated those differences. In addition, when measuring Mn contamination as a ratio of Mn to the stable sulfur isotope ^{34}S, $Mn/^{34}S$ ratios were essentially identical for TN washed, unwashed and un-treated hair for all depths of measurement. No morphological changes were observed during the visual inspection of hair strands with an electron microscope (Eastman et al. 2013). These results indicated that TN is an effective method to remove Mn contamination from the outside of hair.

1.6 FACTORS AFFECTING THE MEASUREMENT MN IN HAIR

In addition to the accurate measurements of an individual hair strand along it's hair shaft, it is important to understand the differences in MnH levels between hair strands, locations on the skull, between subjects, and possibly between different hair colors. For example, darker hair seems to contain more trace metals than lighter colored hair. This effect appears to be different for different metals (Sturaro et al. 1994). Peak positions, peak areas, and peak width must be obtained from the MnH profile so that hair growth rates, time between peaks, the minimum measurable time of exposure, intake dose to hair level ratios, and the smallest Mn exposure detectable in hair can be calculated.

Other factors affecting the measurement of Mn in hair are the variabilities of both MnH and ^{34}S profiles in hair measured by LA-ICP-MS; in particular the stability of ^{34}S must be confirmed (Stadlbauer et al. 2005; Legrand et al. 2007).. Quantifying MnH levels requires the use of an internal standard that is measured in parallel to MnH, in order to correct for ablation efficiency changes of the LA unit (Stadlbauer et al. 2005; Legrand et al. 2007). ^{34}S, a very stable isotope in hair, has been used as an internal hair standard by others (Legrand et al. 2007).

The variability of all of the above mentioned factors must be assessed in order to determine the number of hair strands, scalp locations, and subjects to be sampled to get a reliable measure of exposure.

1.7 TABLES

Table 1-1 Mn in hair welding studies

Author	Group	N	Mean ± SD (µg/g)*	Range (µg/g)*	P-value*
(Huang and Cao 2003)	Welder	807	2.015 ± 1.647	0.53 - 11.22	< 0.01
	Control (electrician)	110	1.648 ± 1.151	0.47 - 8.75	
(Zhang et al. 1996)	Electric Welder with manganism	216	8.439 ± 5.673	-	< 0.01
	Control ("healthy people")	47	2.423 ± 0.848	-	
(Lin 2002)	Welder	265	6.16 ± 1.61	1.9 - 12.9	< 0.01
	Control	163	4.43 ± 1.18	1.2 - 7.6	
(Ramakrishna et al. 1996)	Welder	22	6.07	4.10 - 10.9	Difference not tested
	Control ("mostly students")	21	3.7	3.46 - 4.48	
(Xie et al. 1995)	Welders "working"	55	0.2639 ± 0.2234 **(µmol/g)**	-	< 0.005
	Welders "after work"	55	0.1684 ± 0.0761 **(µmol/g)**	-	

Note:
(*) Numbers exactly as reported in publications

1.8 OBJECTIVE

Occupational inhalation exposures to Mn are often chronic and vary substantially from day to day (Flynn and Susi 2009; Hobson et al. 2011; Pesch et al. 2012). Estimating long-term exposures requires intensive effort to obtain continuous measurements on-site. Persons exposed to Mn have to wear sampling equipment at their work-site throughout the exposure periods. Welders, for example, have a very heterogeneous work environment and perform a job that requires a

substantial amount of effort to characterize their exposures. Ongoing personal environmental monitoring is essentially unrealistic.

The objective of the research described in this dissertation was to assess the use of hair as a biomarker of welders' exposure to airborne manganese.

1.9 SPECIFIC AIMS

The specific aims are to assess the association between MnA and Mn levels from digested bulk hair ($MnH_{(B)}$), $MnH_{(B)}$ and $MnH_{(La)}$, and MnA and LA-ICP-MS-derived counts of Mn in individual hair strands $MnH_{(La)}$.

1.9.1 *Aim 1: To assess the feasibility of using Mn in hair to measure Mn exposure*

To characterize the association between Mn exposure and $MnH_{(B)}$ among a cohort of apprentice welders. Total Mn in a hair sample was compared to an appropriate time window of the individual's exposure history in order to quantify the association between Mn exposure and Mn expression levels in hair.

1.9.2 *Aim 2: To demonstrate proof of concept for feasibility of measuring Mn in hair with Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS).*

To develop a calibration procedure to quantify $MnH_{(La)}$ measurements in 1 cm hair strands using 1 cm $MnH_{(B)}$ and Mn in gelatin (MnG). To calculate the limit of detection for the method.

1.9.3 *Aim 3: To demonstrate the application of LA-ICP-MS for measurement of time resolved MnH in welders exposed to Mn*

To measure $MnH_{(La)}$ in individual hair strands from those subjects of the cohort that have high contrast in Mn exposures. The analysis with LA-ICP-MS of individual hair strands was used to provide a MnH profile that was linked to the MnA exposure profile of the individual worker.

Chapter 2. HAIR MANGANESE AS AN EXPOSURE BIOMARKER AMONG WELDERS

2.1 ABSTRACT

Quantifying exposure and dose to manganese (Mn) containing airborne particles in welding fume presents many challenges. Common biological markers such as Mn in blood or Mn in urine have not proven to be practical biomarkers even in studies where positive associations were observed. However, hair Mn (MnH) as a biomarker has the advantage over blood and urine that it is less influenced by short term variability of Mn exposure levels because of its slow growth rate. The objective of this study was to determine whether hair can be used as a biomarker for welders exposed to manganese.

Hair samples (1 cm) were collected from 47 welding school students and individual air Mn (MnA) exposures were measured for each subject. MnA levels for all days were estimated with a linear mixed model using welding type as a predictor. A 30-day time-weighted average MnA (MnA_{30d}) exposure level was calculated for each hair sample. The association between MnH and MnA_{30d} levels was then assessed.

A linear relationship was observed between log-transformed MnA_{30d} and log-transformed MnH. Doubling MnA_{30d} exposure levels yields a 20% (95% confidence interval: 11% – 29%) increase in MnH. The association was similar for hair washed following two different wash procedures designed to remove external contamination.

Hair shows promise as a biomarker for inhaled Mn exposure given the presence of a significant linear association between MnH and MnA_{30d} levels.

2.2 INTRODUCTION

Manganese (Mn) is a naturally occurring transition metal (Agency for Toxic Substances and Disease Registry 2012). It is most commonly used to harden steel, as a pigment, in welding rods, and in dry cell batteries. In low doses it is an essential nutrient and functions as a co-factor in enzymes that are important in detoxification of reactive oxygen species (United States Environmental Protection Agency 2003). Above the estimated safe and adequate daily dietary intake of Mn of 2-5 mg/day (Greger 1998), inhaled Mn may induce a Parkinson-like neurodegenerative disease called manganism (Aschner and Aschner 2005). The mechanisms for this disease are not yet fully understood, partly due to challenges associated with quantifying exposure and dose to Mn-containing airborne particles from sources such as welding fume.

During welding, fume containing Mn is released into the air, inhaled, and may be absorbed mostly through the lung and to a lesser degree through the gastrointestinal tract. Once absorbed, Mn may pass through the blood and deposit in the brain and other tissues (Aschner et al. 2007).

Welding fume exposure varies widely due to the type of welding, the specific welding filler metal, and the base metal composition. For instance, Mn exposure from welding fume may vary from < 10 $\mu g/m^3$ in gas tungsten arc welding (GTAW) to > 580 $\mu g/m^3$ in flux core arc welding (FCAW) (Pesch et al. 2012). In addition exposure levels may vary widely due to the welding environment, use of ventilation and use of respiratory protective equipment (Flynn and Susi 2009; Liu 2010a; Hobson et al. 2011). For instance, welders may work in open environments such as construction sites, or in small confined spaces with poor ventilation, especially in ship-building. The variability of welding fume constituents and exposure makes accurate estimation of welders' exposures challenging. Consequently, biomarkers that reflect exposure integrated over time and

multiple uptake routes are an attractive alternative to traditional exposure monitoring using personal air samples.

Typical biomarkers for Mn include its concentration in blood (or blood components) and in urine, fluids which are relatively easily accessible and commonly collected. Studies have examined relationships between ambient airborne concentrations of Mn and the level of Mn in whole blood, red blood cells, plasma, serum, and urine of exposed workers (Roels et al. 1987; Järvisalo et al. 1992; Roels et al. 1992; Apostoli et al. 2000; Myers et al. 2003). Some of these studies found associations between airborne particles containing Mn and Mn in blood or urine (or both) at the group level, but not at the individual subject level. A recent meta-analysis showed these associations are in general only observed at higher exposure levels above ~ 10 $\mu g/m^3$ (Baker et al. 2014). However, even in studies with positive associations, blood and urine have not proven to be practical biomarkers due to little variability in biomarker concentrations over a wide range of air exposures (Smith et al. 2007). In addition, associations between Mn in blood or urine and inhaled Mn at an individual level, may be masked by dietary intake, because the daily dietary intake of Mn is on average higher than the daily airborne Mn intake of welders (Greger 1998; Agency for Toxic Substances and Disease Registry 2012).

Hair as a moiety for biomarkers has the advantage over blood and urine, because it is less influenced by short term variability of Mn exposure levels because of its slow growth rate. Estimates for hair growth rate range from 0.35 mm per day (~ 1 cm per month) (Tobin 2005a) to ~ 0.5 mm per day (~ 2 cm per month) (Chamberlain and Dawber 2003). Thus, hair levels of a contaminant may be more representative of an integrated average exposure.

However, a major problem with the use of hair is its direct contamination from the external environment, which may be enhanced by electrical charge and residual oil on the hair (Eastman et al. 2013). In addition, individual exposure can influence the extent of external hair contamination. Detergents used to remove external contamination may also release Mn from welding fume particles attached to the hair surface. Different research groups have used different hair washing procedures commonly using either a water-based detergent (i.e. mixed with Triton X-100) or acetone for washing, but only a few methods have been thoroughly tested (Eastman et al. 2013; Wołowiec et al. 2013) and an accepted standard method has not been identified (Kempson and Henry 2010; Wołowiec et al. 2013).

The objective of this project was to determine the relationship between quantitatively assessed exposure to Mn in welding fume, and Mn levels in scalp hair.

2.3 METHODS

The samples for this study were derived from a longitudinal inception cohort study of welding trainees; details have been presented elsewhere (Baker et al. 2014). Briefly, we recruited 53 students enrolled in a welding training program between April, 2011 and June 2013. Most students enter the program without prior welding experience and then progress through a 5-quarter training schedule. They learn and practice different welding techniques typically in the order: oxyacetylene, shielded metal arc welding (SMAW), FCAW encompassing both dual shield (DS) and inner shield (IS), gas metal arc welding (GMAW), and GTAW. We recruited students into the study during the first week of their program or at study inception, and followed them through the end of their enrollment or until the end of the study. Participants were asked to provide blood and urine samples on Monday and Friday of the first and last week of each school quarter and were

monitored for exposure to welding fume on the same days. At the end of each sampling day, students completed a daily questionnaire to assess their workplace and personal characteristics such as smoking habits, respirator usage, and welding duration. At the beginning of the first quarter, and at the end of each quarter including the first quarter, subjects were asked to give a scalp hair sample. All study protocols were reviewed and approved by the University of Washington Institutional Review Board and study subjects provided written informed consent.

Forty-seven subjects provided a total of 154 hair samples in ~ 3-month intervals. Six subjects did not submit hair samples, because they were bald, or for personal reasons. A hair sample bundle was cut from the occipital region of the head with ceramic scissors as close as possible to the scalp. The cut hair bundles were attached with tape inside a new Ziploc bag for storage and transport to the laboratory. In the laboratory hair bundles were cut into first and second centimeter segments from the proximal end. The 1 cm hair samples were electrically discharged (Mettler Ionizer Antistatic System Model 11238-354) and weighed with an analytical balance (Mettler Toledo model AG285; limit of quantification: 0.1 mg). Only the first centimeter of the samples was used for this analysis.

The first set of 56 hair samples was washed with a solution of 0.5 ml 1% Triton X-100 mixed with 930 mg EDTA in 50 ml ultrapure water. Hair samples were vortexed for 30 min and rinsed with ultra-distilled water until hair samples demonstrated no residual detergent, and were dried for 24 h at room temperature (Triton X-100, Vortex, Water; TVW procedure). When washing hair with TVW we noticed that due to static effects occasionally hair strands were not suspended into the washing solution and some hair stands were lost, even though we increased the time to electrically discharge the hair and test tubes. Thus further testing of alternative washing procedures was indicated.

In order to identify a washing procedure with less static charge effects and adequately efficient washing, we evaluated four washing procedures to test the effectiveness of detergent (acetone and Triton X-100), mechanical agitation (vortex and sonication), and rinse solvent (ultrapure water, acetone, or water followed by acetone). Details of the experiment and results are provided in the Supplementary Data.

Briefly, a sample of human hair was obtained from a local barbershop, and collected into two bundles. One bundle was put aside for an 'uncontaminated' hair, and the other was exposed to high concentrations of SMAW welding fume in the study training facility by mechanically rotating the sample in the welding plume for ~ 10 min. The contaminated bundle was subsequently divided and cut into samples for analysis, and weighed on an analytical balance (Mettler Toledo, Model AG285; limit of quantification: 0.1 mg). Five contaminated samples were used for the positive control, and triplicate samples of contaminated and uncontaminated hair were prepared for each of four wash procedures.

Washed hair samples were dried in a vacuum oven, then digested with nitric acid using open vessel microwave assisted digestion (Puchyr et al. 1998), and analyzed following EPA method 6020a Rev.1, using an Agilent 7500-CE ICP-MS (inductively coupled plasma-mass spectrometry) operated in He collision mode to eliminate polyatomic interferences (United States Environmental Protection Agency 2007). Details of the quality control measures for the analytic method are provided in the Supplementary Data.

We used ordinary least squares regression with robust standard errors to determine whether the washed contaminated hair was significantly cleaner than the unwashed contaminated hair and analysis of variance was used to test whether the four washing procedures differed from each other in residual Mn content. Washing contaminated hair produced on average 80% lower MnH levels

than unwashed contaminated hair ($P < 0.01$). There was no significant difference between the MnH levels associated with the four washing procedures ($P = 0.239$), and there were substantially fewer problems with static charge when washing hair with acetone. Thus the more efficient acetone wash procedure was adopted for subsequent samples (n=98).

Subsequent to the results of the hair washing evaluation study, the second set of 98 hair samples was washed and rinsed with acetone (Fischer scientific: Optima A929-4 ultragrade and Alfa Aesar Acetone HPLC Grade 99.5%). Hair samples were covered with 25 ml of acetone, shaken for 15 s, and sonicated for 30 min. Acetone was removed and the procedure was repeated. Samples were dried in a vacuum-drying oven at ~ 80 °C for ~ 1 h. All dried hair samples were prepared for the analysis and analyzed as described above.

Throughout the study welding students wore personal air sampling pumps during their work day on Monday and Friday of the first and last week of each school quarter. For each student on average 3.7 ± 1.6 air samples were taken per quarter and 10.7 ± 5.5 throughout the study, respectively. Total particulate matter was collected on 37 mm mixed cellulose ester (MCE) filters in a closed face filter cassette connected to a personal sampling pump and worn outside of the welding helmet, which is not expected to introduce an overall bias (Harris et al. 2005). Pumps were pre- and post-calibrated to ~ 2 l per minute. At the end of each sampling day, samples were transported to the Environmental Health Laboratory at the University of Washington for analysis. At least two field blank MCE filters were collected on each sampling day.

Filters were analyzed for Mn by using ICP-MS based on a modified EPA 6020a Rev.1 procedure using an Agilent 7500-CE ICP-MS operated in He collision mode to eliminate polyatomic interferences (United States Environmental Protection Agency 2007). Filters and deposited fume were digested with 10 ml of a 1:1 mixture of concentrated nitric acid and deionized

water, using open vessel microwave assisted digestion (MarsXpress, CEM Corp., Matthews, NC, USA). Quality control samples including field blank and spike filters were included with each batch of field samples. Assay accuracy and precision based on the spike recovery samples were 103 ± 6%. Reporting limits for Mn ranged from 0.01 to 0.02 µg depending on analysis-batch-specific field blanks, and were based on three times the standard deviation of the blanks, which were treated the same as the samples in the field. Values below the reporting limit were replaced with the analytical-batch-specific reporting limit divided by square root of two (Hornung and Reed 1990). MnA concentrations were calculated using the mass of Mn determined and divided by the volume of air sampled. The resulting air concentrations were standardized to an 8-h time-weighted average.

Mn air concentrations were log-transformed to normalize their distribution and used in a linear mixed model to estimate daily Mn exposure levels by welding type (fixed effect), adjusted for individual subject (random effect) (Stata Version 11, xtmixed, College Park, TX, USA). The model estimates were then used to predict daily individual and welding-type-specific Mn exposures as the maximum likelihood estimate of the arithmetic mean, using the within subject variance. The estimated exposure level was then assigned for each subject-day depending on the subjects' welding activity, attendance, and duration of welding activity, as reported by the welding school and the individual. For each hair sample a 30 day time-weighted average Mn exposure was calculated for the 30 days prior to the sample collection date using the individual's daily estimated exposures.

Each subject was classified as a consistent respirator user or non-user based on whether they self-reported respirator use for more than 90% of welding days. Although crude and stringent,

this classification was designed to address the lack of systematic respiratory protection fit testing, and the inconsistent reporting of respiratory protective equipment observed at the location.

MnH and predicted MnA_{30d} concentrations were log-transformed to normalize their distributions and used in a multivariate linear mixed model to estimate the effects of predictors on log MnH levels. In addition to the log-transformed MnA_{30d}, which was forced into all models, age, body weight, gender, race, ethnicity, smoking status, pack-years, self-reported drinking status (yes/no), respirator user (yes/no), time in welding program, and washing procedure were tested for contributions to the model using the Akaike information criterion (AIC). We also evaluated the interaction between log-transformed MnA_{30d} and washing procedure to determine whether differences in the washing procedure modified the effect of exposure. The model also estimated the between and within subject variance components. Analysis was conducted using the R3.1.1 (32-bit) platform with the lme-function from the nlme-package (version: nlme_3.1-117). The resulting coefficients and 95% confidence intervals were exponentiated to the base 2 in order to determine the percentage increase of MnH levels for doubling the exposure (MnA_{30d}).

2.4 RESULTS

The demographic and exposure characteristics of the welder trainees who provided hair samples are presented in Table 2-1. The forty-seven trainee welders had a mean age of 26.7 ± 9.1 years, ranging between 18 and 56 years. Seventy-five percent were white, 13% black, and 4% each were Asian, American Indian, and other or mixed race. The group offering hair samples was similar to the entire apprentice cohort (n=53) except for race, because fewer hair samples were collected from black subjects. Approximately 43% of subjects who provided a hair sample never smoked and 34% were current smokers.

Over the ~2-year study duration, we collected a total of 600 personal MnA and 154 hair samples. Table 3-1 Sample overview shows MnA exposure concentrations by type of welding. The highest mean concentration and variability was found in FCAW-DS (40.7 $\mu g/m^3$) followed by SMAW (34.7 $\mu g/m^3$). Lower levels were found among oxyacetylene (5.2 $\mu g/m^3$), and GTAW (5.5 $\mu g/m^3$).

Table 2-1 shows a summary of the predicted MnA_{30d} air concentrations and the observed Mn in hair for the 47 subjects who provided 154 hair samples. The average hair sample mass was 12.1 ± 7.3 mg (range: 1.4 to 47.6 mg). Those with samples washed with acetone only had slightly higher MnA concentrations, though the distributions were widely overlapping. MnH levels were more widely separated, with the acetone wash samples demonstrating a higher average concentration of Mn, and a wider distribution of concentrations.

Figure 2-1 shows measured MnH levels as a function of predicted MnA_{30d} air concentrations. A simple linear regression line and its 95% confidence interval are overlaid. MnH levels increase with increasing MnA_{30d} levels.

Table 2-3 shows the results of the mixed model analysis. The only variables included in the final model were the log transformed Mn_{30d} exposure and the wash method. Thirty-day MnA levels were significantly associated with an increase in MnH, yielding a 20% [95% confidence interval (CI): 11 – 29%) increase in MnH with doubling of the MnA_{30d} exposure levels. More of the remaining variance is within individuals (77.1%) than between subjects (22.9%). When washing procedure was not included in the model, the effect estimate increased to a 25% (95% CI: 16 – 36%) increase in MnH for each doubling of MnA_{30d} levels.

Although the results of our hair washing method evaluation substudy suggested that differences between the washing procedures should not influence MnH, the acetone washing

procedure did yield higher estimated MnH levels than detergent washing in the model. However, the association between exposure and MnH was unaffected by the different wash procedure, as evidenced by a small and non-statistically significant interaction of the washing procedure on the exposure related increase. The interaction produces a doubling effect of 7.2% (95% CI: - 7.9% to 24.8%, P= 0.362).

The association between MnA_{30d} and MnH did not change with the inclusion of the variables age, body weight, gender, race, ethnicity, smoking status, pack-years, self-reported drinking status, respirator user, and time in welding program. The *P*-values of these variables were not significant ($P > 0.05$) and the inclusion of these variables did not substantially change the AIC.

2.5 DISCUSSION

In this study, welders with higher air exposure to Mn in welding fume had higher Mn levels expressed in scalp hair. These relationships remain significant and consistent, despite using two different washing procedures to prepare the samples. These relationships indicate that MnH may be a useful biomarker for Mn exposure, even at the relatively low exposure levels observed in this study.

The MnA concentrations of our study (Table 2-2) are substantially lower than MnA concentrations found in other studies. For example, an average SMAW MnA concentration of 160 ± 190 μg/m^3 was reported by (Hobson et al. 2011) and 543 ± 1530 μg/m^3 by (Liu et al. 2011) while our average was 34.7 ± 32.2 μg/m^3. Thus, the levels observed in this setting are useful for evaluating hair as biomarker of Mn exposure in moderately well controlled, or short duration occupational exposures.

The MnA levels observed in this study exceed the recently lowered current and very stringent American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV) of 0.02 mg/m^3 for respirable Mn 52% of the time. The ACGIH TLV of 0.1 mg/m^3 for inhalable Mn was exceeded 4% of the time. None of the samples exceeded either the National Institute for Occupational Safety and Health (NIOSH) Recommended Exposure Limit (REL) of 1.0 mg/m^3 or the prevailing Occupational Safety and Health Administration (OSHA) regulatory standard permissible exposure limit (PEL) of 5 mg/m^3 as a ceiling value.

MnH levels have been used previously as a biomarker for exposure in environmental and occupational studies. MnH was used as a biomarker of exposure in a comparison study of low and high Mn water level exposures in rural Quebec where no other source of Mn was known. MnH levels were significantly higher in children who consumed water with high Mn levels (Bouchard et al. 2007). Because the source of Mn was drinking water, the hair samples were not washed in this study, although the potential for external contamination from bathing in the water cannot be ruled out.

We investigated Mn levels in drinking water in our study area, because MnH levels could be influenced by Mn in water (Bouchard et al. 2007). In the study region Mn in drinking water is monitored and removed with green sand filters by the local water utility company. Manganese levels are measured daily and are estimated to be on average 0.08 ± 0.026 mg/l pre- and 0.01 ± 0.0 mg/l post treatment (personal communication Thomas Malphrus, City of Renton). These treated water levels are sufficiently low to have little impact on the observed levels in our study. Further, in order to have influenced our results, they would have to have been associated with duration in the welding program.

MnH in children's hair was also used as biomarker of Mn exposure downwind of a ferro-manganese alloy production plant. Hair samples were prepared similarly to our study and washed with a Triton X-100 solution. The authors reported an increase of MnH with time of mother's exposure before child birth and a decrease of MnH with increasing distance to plant (Menezes-Filho et al. 2011).

MnH has also been used in occupational settings as a biomarker for air exposures. MnH was associated with MnA levels on a group level, but not on an individual level in studies with manufacturing workers in dry cell battery facilities (Bader et al. 1999). Workplace MnA concentrations ranged from 1 to ~800 $\mu g/m^3$ in three different job sites with averages of 4, 40, and 400 $\mu g/m^3$, and hair levels of 4.6 ± 5.8, 5.2 ± 4.5, and 8.2 ± 6.7 µg/g, respectively.

There are a few studies available reporting a relationship between Mn exposure among welders and Mn in hair (Xie et al. 1995; Zhang et al. 1996; Ramakrishna et al. 1996; Lin 2002; Huang and Cao 2003). However, each of these studies has important limitations. All of these studies show higher MnH among welders in comparison to unexposed controls, but they lack a quantitative assessment of subject-specific Mn exposure levels. In only one of these five papers the washing procedure was described (Ramakrishna et al. 1996). They followed the procedure of the International Atomic Energy Agency (IAEA) (International Atomic Energy Agency 1985), which is an acetone, a detergent, water and acetone rinse procedure. The lack of exposure assessment and details of the hair washing procedures used makes it difficult to evaluate the findings.

Additionally, two of the papers demonstrate a relationship with years of exposure as a welder, which suggests a relationship with cumulative exposure, rather than exposure occurring during the time period expressed in the length of the hair sample (Xie et al. 1995; Lin 2002). Given

the growth of hair of ~1-2 cm per month, years of welding would only be associated with MnH levels if it reflects a long-term reservoir of Mn in other body tissues. Consequently, these papers do not provide compelling evidence of a quantitative relationship between exposure and MnH.

In contrast to the previous discussed welding studies, a significant strength of our study was the large number of individual air exposure measurements, and our ability to model these data to estimate individual 30-day MnA subject-specific exposures, the time window directly relevant to the hair samples collected. To the best of our knowledge, no other Mn biomarker study has had such a rich exposure dataset with which to quantitatively estimate air exposures over an etiologically appropriate time scale. Furthermore, matching the segment of hair to the integration period of exposure as we have done is an important component of biomarker evaluation, but heretofore has frequently been overlooked.

The bioavailability of the manganese in airborne exposures is of potential consequence to this analysis, and is related to the particle size distribution (PSD) of the metal fume. We measured the PSD of fume in the training facility using a 10 stage Micro-Ofrice Uniform Deposit Impactor. The mass median aerodynamic diameters (MMADs) ranged from 0.88 to 1.25 μm, depending on welding type. Except for GTAW, the average GSD ranged from 3.5 to 4. The average GSD for GTAW was 6.21 (Warner 2014). While the MMADs are somewhat higher than those observed in other studies (Taube 2013), they indicate primarily alveolar deposition, and potential uptake through both pulmonary and gastrointestinal routes.

In this study we found the majority of variability in MnH to be within subjects as opposed to between subjects, indicating substantial remaining variability in the MnH measurement, that was unaccounted for by either individual differences in expression of Mn in hair, or by our estimated exposure levels. Uncertainty in both the quantification of MnH due to imprecision in

cutting the first cm from the scalp, transfer of the weighed sample of hair to the digestion tube, and residual external contamination of the sample could contribute to this error. In addition, our estimated 30 day air exposure for each subject contains error due to inter-individual variability in exposure for a specified welding type, and the individual ascertainment of welding duration, effects of respirator use, etc. These errors necessarily contribute to the residual error in our model despite our large exposure sample size and individually assessed welding parameters.

A challenging element of our study was the hair washing procedure. While the importance of hair washing has been previously discussed (Eastman et al. 2013), its importance is often overlooked in hair biomarker studies. Due to issues with static electrical charge when washing our initial hair samples we conducted a sub-study to evaluated four washing procedures. Ultimately two wash procedures were used with the hair samples in this analysis. We first used a Triton X-100 procedure and then changed to an acetone based wash procedure because the two approaches did not show any statistical difference in the sub-study. However, our final results indicated that hair washed with the Triton X-100 procedure had MnH levels that were consistently lower than samples washed with acetone, potentially due to more complete washing. Nevertheless, the relationship between air exposure and hair levels on the log scale was not significantly modified by the use of the two methods.

Various hair washing procedures have been proposed since the early attempts of measuring chemicals in hair as an exposure biomarker (Bate 1965). The need for standardization of the procedures has also been recognized (Bencko 1995). Although the IAEA proposed a standard hair washing procedure in 1985 (International Atomic Energy Agency 1985) it has still not been widely adopted. Very little consensus exists on which hair washing procedures should be used (Kempson and Henry 2010). Very thorough work on wash procedures for using hair as a biomarker for Mn

was also done earlier (Salmela et al. 1981). However, that analysis was limited to bulk hair samples and had higher limits of detection than are available today. Most recently, as an alternative analytical approach, laser-ablation ICP-MS has also been proposed to verify the effectiveness of a washing procedure (Eastman et al. 2013).

We quantified four different washing procedures on purposely contaminated hair (see the Supplementary Data). The four washing procedures produced on average 80% lower MnH levels than what was measured in unwashed contaminated hair. However, we likely experienced residual contamination, because the Mn content in contaminated hair after washing (0.8 ± 0.5 μg/g) was higher than the Mn content of washed not contaminated hair (0.6 ± 0.7 μg/g).

Given the apparent difference in the MnH between the two wash procedures, it is important to consider if the observed relationship between MnA and MnH could have been due to residual contamination. Given the prior evidence of Mn accumulation in the hair from environmental sources including water supplies, the demonstration of good washing efficiency in our washing study (80%), the use of two wash procedures with different types of solvents, and an essentially parallel association of MnA and MnH in the log-log space, we think it unlikely that residual external contamination could explain the observed results.

However, the possibility that proportional washing efficiency and residual contamination left on the surface of the hair cannot be fully ruled out in this study. Thus additional work on efficient hair washing techniques is needed before a fully validated and quantitative biomarker for Mn exposure in hair can be developed.

2.6 CONCLUSION

At relatively low occupational levels of exposure to welding fume, on a log-log scale MnH was linearly related to exposure, and thus may prove useful as an exposure biomarker in similar settings. We demonstrated this relationship using a time integrated quantitative estimate of exposure over the previous 30 day period, associated with the first centimeter of hair proximal to the scalp – the hair nominally grown over this same period of time. Removal of surface contamination of the hair is clearly an important component of the procedure for use of hair as a biomarker for airborne environmental contaminants, and requires additional development before this technique can be widely accepted as a quantitative exposure biomarker.

2.7 TABLES

Table 2-1: Characteristics of welders who contributed hair samples

Characteristic		K (%)	N	mean ± SD (range)
Age (years)				26.7 ± 9.1 (18.0 - 56.4)
Body weight (kg)				83.8 ± 19.2 (56.7- 156.5)
Male		43 (91.5)		
Race	White	35 (74.5)		
	Black	6 (12.8)		
	Asian	2 (4.3)		
	American Indian	2 (4.3)		
	Other	2 (4.3)		
Ethnicity	Non-hispanic	43 (91.5)		
	Pack years			2.8 ± 6.0 (0-36)
Smoker	Never	20 (42.6)		
	Current	16 (34.0)		
	Past	11 (23.4)		
Drinks alcohol		31 (66.0)		
Time welding/day (min)		47	527	312.6 ± 52.9 (66-405)
Respirator user		11 (23.4)		
MnA_{30d} ($\mu g/m^3$)		47	154	13.1 ± 10.2 (0.2 - 44.7)
	Acetone wash only	33	98	13.8 ± 10.2 (0.2 - 44.7)
	Triton-X wash only	27	56	11.9 ± 10.1 (0.2 - 32.8)
Manganese concentration in hair (μg/g)		47	154	3.9 ± 7.2 (0.1 - 51.5)
	Acetone wash only	33	98	5.2 ± 8.6 (0.1 - 51.5)
	Triton-X wash only	27	56	1.7 ± 2.1 (0.1 - 10.6)

Notes: K = number of subjects, N = number of samples

Table 2-2: 8-hour TWA measured air Mn concentration by welding type (µg/m^3)

Welding type	N	AM	GM	GSD
All	600	29.1	16.5	3.4
Flux Core Arc Welding - Dual Shield (FCAW-DS)	75	40.7	25.5	3.6
Flux Core Arc Welding - Inner Shield (FCAW-IS)	32	34.5	23.6	3.0
Gas Metal Arc Welding (GMAW)	62	28.6	21.0	2.3
Oxyacetylene (Oxy)	80	5.2	4.2	2.0
Shielded Metal Arc Welding (SMAW)	315	34.7	22.8	3.0
Gas Tungsten Arc Welding (GTAW)	36	5.5	4.0	2.3

Notes: N = number of samples, AM = arithmetic mean, GM = geometric mean, GSD = geometric standard deviation

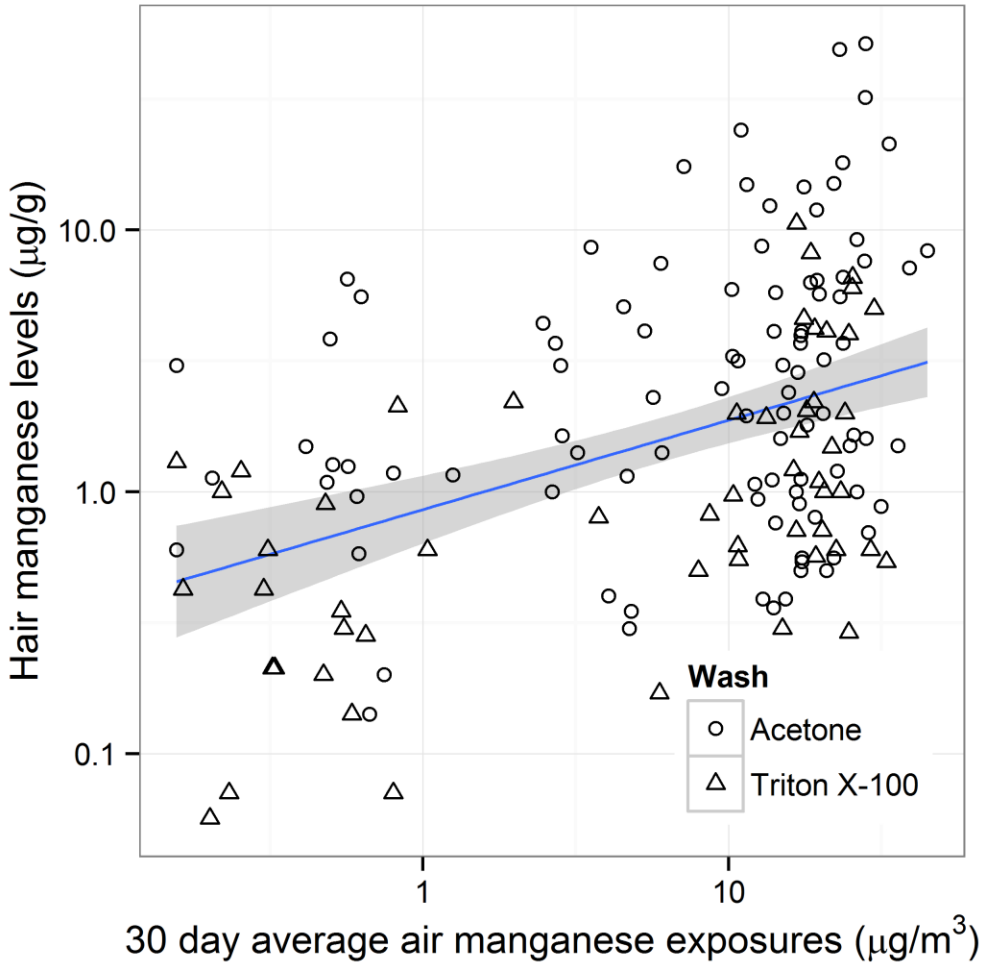
Table 2-3: Determinants of MnH levels (ln-ln)

Fixed effects	**ln MnH estimate (95% CI)**	**SE**	**p-value**
Intercept	0.35 (-0.72 - -0.01)	0.18	0.055
ln MnA_{30d}	0.26 (0.16 - 0.37)	0.05	<0.001
Wash Procedure[a]	-1.06 (-1.47 - -0.65)	0.21	<0.001
Variance components			
	ln MnH		**%**
Within subject variance	1.027		77.1
Between-subject variance	0.305		22.9
Total variance	1.332		

Notes: [a] 0: acetone washed, 1: Triton X-100 washed

2.8 FIGURES

Figure 2-1: Relationship between 30-Day average Air Mn (MnA_{30d}) Exposure and Hair Mn (MnH) level



Chapter 3. CALIBRATION OF LASER ABLATION INDUCTIVELY COUPLED PLASMA SPECTROMETRY FOR QUANTIFICATION OF MANGANESE IN HUMAN HAIR

3.1 ABSTRACT

Measuring exposure to Mn-containing particles in airborne welding fume presents many challenges. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) seems a promising method for measuring Mn levels in hair strands. However, hair strands with known Mn concentrations are not available for use as calibration standards. The objective of this study was to calibrate LA-ICP-MS response to Mn in individual 1cm hair strands ($MnH_{(La)}$) with a series of gelatin samples that had been spiked with known concentrations of Mn (MnG), and to calibrate the LA-ICP-MS response to Mn in the individual 1cm hair strands to Mn concentrations determined via acid digestion of bulk hair ($MnH_{(B)}$) samples followed by ICP-MS. ^{34}S was used to normalize the ^{55}Mn measurements. One cm bulk hair samples were collected from 47 welding school students and analyzed. Fifty-three hair strands from 22 bulk hair samples with concentrations of 0.3 to 51.5 µg/g and ten Mn-gelatin mixtures (0-250 ppm) were analyzed with LA-ICP-MS for ^{55}Mn and ^{34}S. The association between ln-transformed Mn levels in gelatin and in bulk hair, and ln-transformed Mn/S ratios in individual hair strands were assessed with a mixed model using the ln-transformed Mn concentration as predictor.

Mn/S ratios were found to be significantly associated with the standard Mn concentrations, yielding an 8.0% [95% confidence interval (CI): 6.0–11.0%] increase in Mn/S with a 10% increase

in the MnG concentration. The Mn/S intercept was not significantly different for the gelatin or bulk hair calibration series (p=0.101) and the interaction term for the calibration matrix (gelatin vs. bulk hair) was not significant (p=0.968). This result indicates that the calibration slopes are equivalent for both the gelatin calibration and the calibration based on digestion of the bulk hair samples. Gelatin shows promise as a proxy calibration standard for measuring Mn levels in hair strands.

3.2 INTRODUCTION

Manganese (Mn) is a naturally occurring transition metal that is commonly used to harden steel, and as a component of welding rods and dry cell batteries (Agency for Toxic Substances and Disease Registry 2012). Below the estimated safe and adequate daily dietary intake of Mn of 2–5 mg day^{-1}, it is an essential nutrient (Greger 1998). It functions as an enzymatic cofactor in the detoxification of reactive oxygen species (United States Environmental Protection Agency 2003). At higher levels, inhaled Mn may induce manganism – a Parkinson's-like neurological disorder (Aschner and Aschner 2005). The pathogenesis of manganism is not fully understood, in part because of difficulties measuring exposure to airborne Mn and the resultant dose.

Typical Mn biomarkers such as the concentration of Mn in blood (or blood components) or in urine are relatively easily measured; however multiple repeat measurements are needed to understand temporal variations in exposures. Several studies have examined relationships between ambient airborne concentrations of Mn (MnA) and the level of Mn in whole blood, red blood cells, plasma, serum, and urine of exposed workers (Roels et al. 1987; Järvisalo et al. 1992; Roels et al. 1992; Apostoli et al. 2000; Myers et al. 2003). Only some of these studies found associations between Mn in blood or urine and MnA at the group level, and no association was found at the

individual subject level. Even in studies with positive associations, blood and urine have not proven to be practical biomarkers because only limited variability was found in in the biomarker concentrations over a wide range of MnA exposure concentrations (Smith et al. 2007).

Hair as a biomarker of exposure has an important advantage over blood or urine in that it can preserve the temporally varying signature of past exposures as it grows. Several studies have reported associations between integrated Mn levels from digested bulk hair and occupational exposure concentrations (Grund et al. 1980; Bergert et al. 1982; Gorban et al. 1992; Ramakrishna et al. 1996; Reiss et al. 2015).

A minimum length of 1 cm of bulk hair was typically analyzed in these studies, because the analytical limits of detection dictated a certain minimum mass of hair and because handling of multiple hair strands of less than 1 cm length is mechanically very difficult. Reported hair growth rates ranged from about 1 cm per month (Tobin 2005a) to about 1.5 cm per month (Chamberlain and Dawber 2003). Accordingly, 1 cm bulk hair may represent integrated exposures over only the past month. To the best of our knowledge, the associations between MnA and Mn in hair for time intervals shorter than 30 days and for hair strands shorter than 1 cm length have not been investigated.

Laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been used in several studies to estimate the quantities of multiple elements in micrometer segments of single hair strands. A laser beam is directed to move along the shaft of a hair strand where a small amount of the hair is ablated. The ablated aerosols are directed into the ICP-MS for analysis. A concentration profile is created as the laser moves along the hair shaft. This profile shows quantities of the material of interest deposited in the hair at specific points in time, allowing a time resolution of substantially less than one month. Most previous studies using LA-ICP-MS on hair

focused on mercury (Rodushkin and Axelsson 2000a; Rodushkin and Axelsson 2000b; Rodushkin and Axelsson 2003; Legrand et al. 2004; Stadlbauer et al. 2005; Legrand et al. 2007), but one study measured Mn hair levels using LA-ICP-MS in children exposed to ferroalloy Mn emissions and evaluated procedures for washing hair strands to remove external contamination from air and water. (Eastman et al. 2013). In the Eastman study, hair strands were soaked in a $MnCl_2$ solution to contaminate them with Mn. The quantity of Mn absorbed into the hair was found to be proportional to the Mn concentration in the solution, after sufficient time in solution. Several washing procedures were found not to remove absorbed Mn.

Calibration standards for LA-ICP-MS are often unavailable for biological materials (Russo et al. 1998) and proxy calibration approaches, with a matrix similar to the sample material, are used. For example, droplets of calibration solution dried onto filter paper were used for brain tissue sections (Bonta et al. 2016; Shariatgorji et al. 2016); spiked rat brain tissue encapsulated into a sol-gelatin matrix was used for other studies (Sela et al. 2011).

Matrix-matched calibration standards i.e, hair strands with known Mn concentrations, are not easily obtainable. It has been suggested that Mn concentrations in hair could be created using the contamination procedure described (Eastman et al. 2013) to evaluate hair washing procedures (Donald Smith, Microbiology and Environmental Toxicology Department, University of California, Santa Cruz, California 95064, United States; personal communication). However, in the contamination study only two Mn concentrations were used, differences in absorption rates between hair samples were observed, and the stability of the Mn concentrations in hair strands over time is unclear. The process remains difficult, requires more testing, and has not been validated for calibration purposes.

Gelatin spiked with a range of Mn concentrations, was used in this study in place of contaminating individual hair strands. Gelatin is somewhat similar to hair in composition. The relatively constant sulfur concentrations of keratin proteins in hair have been suggested as a suitable internal standard for LA-ICP-MS of hair, in order to compensate for variability in the ablation process, and temporal drift in the ICP-MS response (Eastman et al. 2013; Limbeck et al. 2015). Gelatin also contains sulfur due to the protein collagen (Baernstein 1932). Sulfur 34 (^{34}S) has been used as a chemical reference in isotopic analysis of archaeological bone collagen (Nehlich and Richards 2009).

In contrast to LA-ICP-MS analysis of single hair shafts, analysis of bulk hair samples is typically accomplished by acid digestion followed by use of ICP-MS. Calibration of the Mn response in this case is relatively simple. The dilute acid matrix that is introduced into the ICP-MS can be readily spiked with Mn-containing solutions to create a calibration series.

The objective of the current study was to compare two approaches to calibrating the LA-ICP-MS response to Mn in individual 1cm hair strands. In the first approach, a calibration equation for the LA-ICP-MS was based on analysis of a series of gelatin samples that had been spiked with known concentrations of Mn. In the second approach, measurements of Mn concentrations in hair strands from the LA-ICP-MS method were compared to Mn concentrations determined via acid digestion of bulk hair samples followed by ICP-MS.

3.3 METHODS AND MATERIALS

3.3.1 *Study design*

The hair samples used in this study were obtained from welding trainees who participated in a longitudinal inception cohort study between April, 2011 and June, 2013. Details of the study

are described elsewhere (Baker et al. 2014). Typically, these welding trainees progress through a 5-quarter training schedule without prior exposure to welding. They learn 6 welding techniques with a range of Mn exposure potential: oxyacetylene; shielded metal arc welding (SMAW); both flux core arc welding (FCAW) types, i.e., dual shield (DS) and inner shield (IS); gas metal arc welding (GMAW); and gas tungsten arc welding (GTAW). Previous work on this study demonstrated a range of Mn in air exposure values from 0.18 – 185.64 $\mu g/m^3$ and a relationship between MnA and MnH using bulk hair analysis (Reiss et al. 2015). All study protocols were reviewed and approved by the University of Washington Institutional Review Board and study subjects provided written informed consent.

3.3.2 *Sample preparation*

Forty-seven students provided a total of 154 hair samples in approximately three-month intervals. Hair samples were cut from the occipital region of the head, as close as possible to the scalp, with ceramic scissors; taped inside a new Ziploc bag; and transported to the laboratory. In the laboratory, the first centimeter segment from the proximal end of the hair bundles was cut and weighed with an analytical balance (Mettler Toledo model AG285; limit of quantification: 0.1 mg). The 1 cm segments were washed with either Triton-X100 (N=56) or acetone (N=99) and rinsed with ultra-distilled water. Details on the hair washing have been reported previously (Reiss et al. 2015). Washed hair samples were dried in a vacuum oven, and 3 individual hair strands ("triplicate hair strands") from each hair sample were put aside for LA-ICP-MS. The remaining bulk hair sample was digested with nitric acid using open vessel microwave assisted digestion (Puchyr et al. 1998), and analyzed following EPA method 6020a Rev.1, using an Agilent 7500-CE ICP-MS operated in He collision mode to eliminate polyatomic interferences (United States Environmental Protection Agency 2007). Details of the analytic method are provided in the

appendix (Reiss et al. 2015). For the current study, a subset of 22 hair samples was selected from the 154 samples available from the cohort study. Samples were selected to cover the full concentration range of the results from the digestion analysis (0.3-51.5 µg/g). From each of the 22 samples, three hair strands were selected for ablation.

Double-sided adhesive tape was glued onto the entire surface of petrographic glass slides (Beta Diamond Products, Inc., Yorba Linda, California, catalog number PS2746) and single-sided tape was placed upside down on the double-sided tape. Individual hair strands were glued onto the adhesive top. No Mn signal above the ICP-MS background was detected in the tape or the glass slides. A maximum of six glass slides were placed in the ablation chamber. The laser ablation path was programmed using the laser control software to define the start, end, and directional changes of the burn path. After each burn path was defined, the Z-value (focus point) of the laser was fine-tuned and the burn path was re-adjusted as needed. The length of the ablation path was also recorded.

A calibration series was prepared by adding Mn (0.05-250 µg/g) to gelatin (Sigma, from bovine skin, Type B, Bioreagent, Product # G9391-100G Lot 041M0052V). Powdered gelatin (200 mg) was added to plastic weigh boats (3 x 3 inch hexagonal) and dissolved in 4 ml boiling deionized water. Each separate gelatin solution was spiked with different quantities of Mn (prepared from a certified Mn standard solution, Fluka Analytical, catalog number 77036, Sigma-Aldrich, St. Louis, Missouri). The spiked gelatin solutions were mixed with plastic pipets and dried in an incubator at 37˚C. Dried gelatin standards were cut using ceramic scissors and small pieces (5 x 5 mm) were mounted on glass slides using double-sided tape. Each of the dried gelatin standards was ablated twice.

An additional matrix-matched reference sample was prepared from finely ground hair powder compressed into a pellet in order to evaluate the stability of the LA-ICP-MS analytical system. The pellet was prepared by mixing 12 g hair with 3 g copolywax binder, followed by grinding in a SPEX Freezer Mill 6870 for 15 min. The ground mixture was pressed into a 13 mm pellet.

Both the ^{34}S and the ^{32}S isotopes of sulfur have previously been recommended for use as internal standards in LA-ICP-MS analyses of hair because S is homogeneously distributed within and between hair strands (Rodushkin and Axelsson 2003; Legrand et al. 2004). The *m/z* 34 isotope of sulfur (^{34}S) was used in this study as an internal standard to compensate for drift in the MS response and to correct for variation in the amount of material ablated. The ^{34}S isotope was used to avoid potential isobaric interferences from O_2 (*m/z* 32) in the quadrupole mass spectrometer.

3.3.3 *Conditions for laser ablation-ICP-MS analysis*

Hair samples were analyzed using a NWR213 laser ablation system (ESI, Portland OR), connected to an Agilent 7500 CE ICP-MS. The ICP-MS was equipped with a collision cell using He, which removes polyatomic interferences. Data acquisition from the ICP-MS was triggered by the laser ablation software (New Wave Research Laser Ablation System Version 4.1.0 Build 29 Copyright 2014 New Wave Research). ICP-MS data acquisition was initiated 30 sec before the ablation started in order to establish the background MS signal in the absence of ablated material. Data acquisition continued for 4 minutes, which was estimated to be longer than the time required to ablate the individual hair strands.

The Nd:YAG laser operated at a wavelength of 213 nm. Measurements were made with a laser energy output of 60% (11.79 J/cm^2), using a spot size of 30 µm, firing at a rate of 20 Hz. The sample stage was programmed to move at a rate of 100 µm per second.

The effluent stream from the laser ablation system (He, 800 L/minute) was introduced directly into the torch of the ICP-MS. Make-up gas, Ar (0.5 mL/min), was T-ed into the line immediately upstream of the torch. Elements of interest were quantified with He mode using the collision cell. The ICP-MS was set to measure the following isotopes (dwell time), in order: ^{34}S (0.1 ms), ^{53}Cr (0.4 ms), ^{55}Mn (0.4 ms), ^{56}Fe (0.1 ms), ^{57}Fe (0.1 ms), ^{60}Ni (0.1 ms), ^{63}Cu (0.1 ms), ^{66}Zn (0.1 ms), ^{208}Pb (0.1 ms). One data point was collected every 1.52 seconds.

Mass spectrometer signal intensities were exported to .csv files and then further processed using the R 3.2 software package, as described in detail in the online supplementary information. The mean and standard deviation for ^{34}S and ^{55}Mn were calculated. Additionally, the mean and standard deviation of the ratio of ^{55}Mn to ^{34}S (Mn/S) were determined.

The normality of the Mn and Mn/S ratios in the hair samples was examined with qq-plots and histograms, and determined to be log normal. Mn/S ratios for the hair samples were ln-transformed. Mn/S ratios for the gelatin samples were also ln-transformed in order to compare them to Mn levels in the same numerical space.

Several corrections were applied to the LA-ICP-MS data in order to compensate for Mn background levels in the gelatin and variations in instrument response over time.

Mn contamination was observed to be present in gelatin blanks. To estimate this background contamination, the linear response of Mn/S ratio for the gelatin blanks and calibrations series was visually inspected; the plot showed a linear response for gelatin calibrations standards above 1 ppm of added Mn. The Mn/S ratios for concentrations below 1 ppm were averaged to determine the background Mn/S signal (1.01). This average was subtracted from all Mn/S ratios in in order to compensate for Mn background concentration in the gelatin.

Analysis of the compressed hair pellet demonstrated that the Mn and S signals, and the Mn/S ratios, varied from day to day (daily CV: 0.19 ± 0.02). Because the hair samples and the gelatin calibration standards were analyzed on different days, the daily Mn/S ratio measured in the compressed hair pellet was used to adjust the Mn/S data from the hair and gelatin samples to correct for this between-day drift in instrument response.

3.3.4 *Statistical analysis*

A multivariate linear mixed model was used to estimate the association between the known Mn concentration from digestion of the bulk hair samples and the average Mn/S ratio from each hair strand. The same model was used to estimate the association between the known manganese standard concentrations in gelatin (MnG) and the Mn/S ratio from each ablated MnG calibration standard. An interaction term was included in the model to evaluate whether the calibration function differed for the MnG compared to the Mn measured from the bulk hair digests.

A random effects model was used to assess the between and within sample variance components for the hair strands only. Ln-$MnH_{(La)}$ was entered as a random slope and sample id as a random intercept.

The limit of detection (LOD) of the Mn/S ratio for measuring Mn in hair and gelatin with LA-ICP-MS was estimated using a regression technique, because no true blank samples were available for both matrices. The LOD can be estimated from $C_{LOD} = \frac{3 * s_{yx}}{\beta_1}$, where β_1 is the slope of the mixed model regression and s_{yx} is the standard error on the prediction $s_{yx} = \sqrt{\frac{\sum_{n=1}^{i}(y_i - \hat{y}_i)^2}{n-2}}$ (Miller et al. 1998), y_i are the measured values of the LA-ICP-MS, and $\hat{y}_i$ the predicted LA-ICP-MS values respectively. s_{yx} is also called root mean squared error (RMSE). The LOD in ln-transformed data was calculated as follows:

$\ln(C_{LOD}) = \ln\left(\frac{3*s_{yx}}{\beta_1}\right) = \ln(3) + \ln(s_{yx}) - \ln(\beta_1)$. The fixed effects of the mixed model were used to calculate the LOD. Random effects errors were not included in the assessment of the RMSE.

3.4 RESULTS

In order to assess the within- and between-day variability in the LA-ICP-MS system, the compressed hair pellet was analyzed 22 times on eight days across a 100 day period (Figure 3-1).

Data are expressed as absolute counts-per-second (cps) for ^{55}Mn and ^{34}S, as well as the Mn/S ratio, calculated on a point-by-point basis. The raw Mn measurements exhibit substantial within-day and between-day variability, with mean values ranging from ~4000 cps on July 28, to ~8000 cps for one sample on July 24. In comparison, the Mn/S ratio was found to be more reproducible both within-day, and between days – with the notable exception of the November 11 data. Between the July and November analyses, the ICP-MS instrument underwent major service including repairing an air leak in the makeup gas and replacing the torch ground, lens base and skimmer cone. Evidently when the instrument was re-tuned after this servicing, the sensitivity for Mn relative to sulfur was higher. In summary, as shown in Figure 3-1, use of ^{34}S as an internal standard helped to correct for within- and between-day variations in the response of the ICP-MS system, except when the instrument underwent major maintenance and was re-tuned. Therefore, the ^{55}Mn signal was normalized to the ^{34}S signal for all subsequent analyses. Because the Mn/S response in November when the gelatin samples were analyzed was 2.4 times higher than in July when the hair strands were analyzed, the average Mn/S response of an ablated hair strand was multiplied by 2.4 to allow comparison of Mn/S responses of ablated hair strands to the response in gelatin. Table 3-2 Calibration relationship between ln-transformed Mn/S from LA-ICP-MS, and

ln-transformed Mn levels in bulk hair and gelatin standards presents the results of the analyzed hair and gelatin samples. Of the selected 66 individual hair strands (triplicate strands from 22 unique hair samples), a total of 53 hair strands (an average 2.4 ± 0.8 hair strands per hair sample) were analyzed with LA-ICP-MS. Thirteen (13) individual hair strands were not analyzed because the hair strand did not remain attached to the glue of the tape. This situation occurred most frequently when the hair was curled.

The average Mn concentration of the bulk hair was 12 ± 15 μg/g, ranging from 0.3 to 51.5 μg/g. The average count of ablated Mn in the hair strands was 8700 ± 11000 counts per second (cps) and of ablated ^{34}S was 66000 ± 11000 cps. The coefficients of variation (CV) for Mn in the bulk hair was 1.3. The average ^{55}Mn CV for within individual hair strands was 0.33 ± 0.21 ranging from 0.11 to 1.4, where 1.4 is substantially higher than the other CVs. The average ^{34}S CV for within individual hair strands was 0.13 ± 0.02 ranging from 0.09 to 0.2. The average Mn/S CV for within individual hair strands was 0.36 ± 0.19 ranging from 0.14 to 1.4. The low CV for ^{34}S supports the hypothesis that sulfur concentrations are relatively constant in hair samples (both within and between subjects) and hence ^{34}S can be used as an internal standard.

Figure 3-2 shows the measured Mn/S values as a function of MnG. The fixed-effect mixed-model regression line and the 95% CI for the fixed mean estimate are overlaid. Mn/S levels are shown to increase with increasing MnG levels. Table 3-2 shows the results of the mixed model regression describing the association between ln-transformed Mn/S ratios from the single hair strands and the ln-transformed known Mn concentrations in the gelatin calibration standards and the bulk hair digests. Mn/S ratios were significantly associated with the standard Mn concentrations, yielding an 8.0% [95% confidence interval (CI): 6.0–11.0%] increase in Mn/S with a 10% increase in the MnG concentration. The association between Mn/S and Mn concentration

was unaffected by the different matrix. The Mn/S intercept was not significantly different for the gelatin or bulk hair calibration series (p=0.101) and the interaction term for the calibration matrix (gelatin vs. bulk hair) was not significant (p=0.968), which indicates that the calibration slopes are equivalent for both the gelatin calibration and the calibration based on digestion of the bulk hair samples. Based on the replicate hair samples analyzed, the average coefficient of variation for the LA-ICP-MS analyses was 0.28 ± 0.26 with a range of 0.053 to 1.0. The RMSE error was 1.2 ppm and the LOD was estimated to be 0.53 ppm.

Table 3-3 Variance components for 1 cm hair strands shows that more of the remaining variance is between samples (88.0%) than within samples (12.0%).

3.5 DISCUSSION

Mn/S ratios measured with LA-ICP-MS on 1 cm hair strands and gelatin flakes were positively associated with the Mn concentration in the hair and the gelatin. The association between Mn/S response ratio and Mn concentration was not found to be significantly different for the MnG calibration series and Mn concentrations determined independently in the bulk hair samples by acid digestion and ICP-MS. This finding indicates that calibration standards prepared by spiked Mn in gelatin may be used to directly calibrate measurements of Mn in hair by LA-ICP-MS. Although the current study focused exclusively on Mn, we anticipate that the approach would also be effective for measurement of other metals in hair by LA-ICP-MS. An LOD of 0.53 ppm was calculated, which excludes only approximately 23% of the hair samples collected in the apprentice study in which MnA concentrations were low compared to MnA values reported from other studies of welders. In a study of measuring $^{202}Hg/^{34}S$ in hair strands with LA-ICP-MS, a CV of 0.02 to 0.06 for ^{34}S was reported for five hair strands (Legrand et al. 2004; Legrand et al. 2007).

All our ^{34}S CVs for individual hair strands were higher and ranged from 0.09 to 0.19. However, our ^{34}S CV of 0.13 across all hair strands (Table 3-1) was below the range of ^{34}S CVs reported for pelleted hair standard reference materials (0.16 to 0.49) (Legrand et al. 2007). Although our relative precision is lower than the Legrand studies, it is still within the same range.

Our data suggest that LA-ICP-MS is suitable for measuring Mn levels in welders exposed to airborne Mn, given that an association between air and hair levels has previously been demonstrated (Reiss et al. 2015).The low variance within a sample and the relative low CV indicate that only a few hair stands may be needed to characterize a welder's past Mn exposure. At any given time, approximately 10% of scalp hair follicles are in the telogen i.e., resting, phase where is no growth. The telogen phase is estimated to last from to 1-3 months (Tobin 2005b). For this reason, analyzing a minimum of three hair strands is recommended in order to minimize the influence of harvesting hair strands that were not in the growth phase during the exposure period.

Although this analysis strongly suggests that gelatin may be a suitable matrix to prepare calibration standards for LA-ICP-MS analyses of hair, one issue may warrant further investigation. The non-significant offset of -0.83 between ln-transformed Mn/S ratios determined in the ablated hair strands using the bulk hair digested calibration values compared to the gelatin standard could represent a small bias when measuring Mn concentrations in hair by LA-ICP-MS. One possibility is that the sulphur content of hair may differ from that of gelatin. The Mn signal was normalized to sulphur (to account for variation in instrument response): if the sulphur content in gelatin is lower than in hair, a higher Mn/S ratio would be found in a gelatin standard than in a hair sample containing the same concentration of Mn.

In this analysis that used a subset of the full apprentice study, approximately 6.4% of the bulk hair samples had Mn levels lower than the calculated LOD of 0.53 ppm. Although those

numbers are small, lowering the LOD would be beneficial because the fraction of samples below the LOD of 0.53 ppm may increase with lower environmental exposures compared to the occupational exposures of this study. A simple approach would be to limit the number of analyzed elements to ^{55}Mn and ^{34}S, and to reduce dwell times in order to measure both elements over a shorter time period as the laser moves. A slower traveling speed of the laser maybe beneficial too, because measurements of both elements would be closer in time in comparison to the measurement cycle of the ICP-MS. As more time is spent measuring fewer elements, the sensitivity is expected to improve.

3.6 CONCLUSIONS

The results of this study indicate that gelatin shows strong promise as a proxy calibration standard for measuring Mn levels in hair strands because the slopes for both the gelatin calibration and the calibration based on digestion of the bulk hair samples are equivalent. The offset between these slopes needs further investigation with a larger sample size to determine its statistical significance.

3.7 FUNDING

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3.8 TABLES

Table 3-1 Sample overview

Sample Type		N	AM	ASD	GM	GSD	CV
Hair strand 1cm	^{55}Mn (cps)	53	8700	11000	5000	2.7	0.33*
	^{34}S (cps)	53	66000	11000	65000	1.2	0.13*
	^{55}Mn/^{34}S$	53	0.14	0.21	0.078	2.8	0.36*
Bulk hair digested	^{55}Mn (μg/g)	22	12	15	5.4	3.9	1.3**

Notes:
N = number of samples, AM = arithmetic mean, ASD = arithmetic standard deviation, GM = geometric mean, GSD = geometric standard deviation,
CV = coefficient of variation (ASD/AM)
*: average CV of within individual hair strand CVs
**: CV = ASD/AM
$: Mn/S on a shot-by-shot measurement

Table 3-2 Calibration relationship between ln-transformed Mn/S from LA-ICP-MS, and ln-transformed Mn levels in bulk hair and gelatin standards

Parameters	estimate (95% LCI, 95% UCI)	SE	p-value
Intercept	-3.0 (-3.9, -2.1)	0.44	<0.001
Mn	0.85 (0.6, 1.1))	0.12	<0.001
Matrix [A]	-0.83 (-1.8, 0.18)	0.48	0.1
Interaction: Mn * matrix	0.0058 (-0.29, 0.30)	0.14	0.97

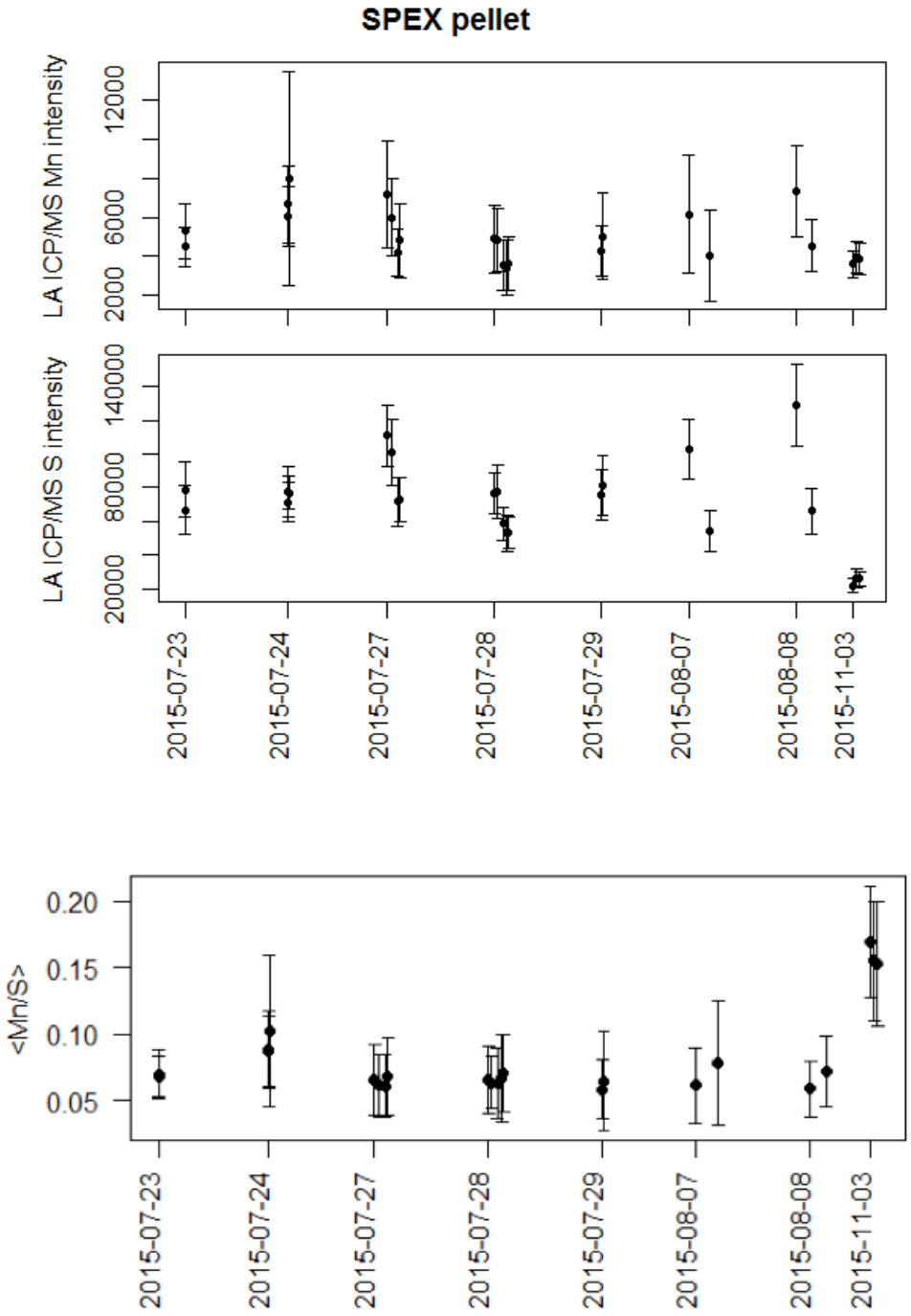
Note:
A: 0: gelatin 1: hair

Table 3-3 Variance components for 1 cm hair strands

Variance component	**Variance ln Mn/S**	**Value%**
Within hair	0.0647	12.0
Between-hair	0.475	88.0
Total	0.540	100

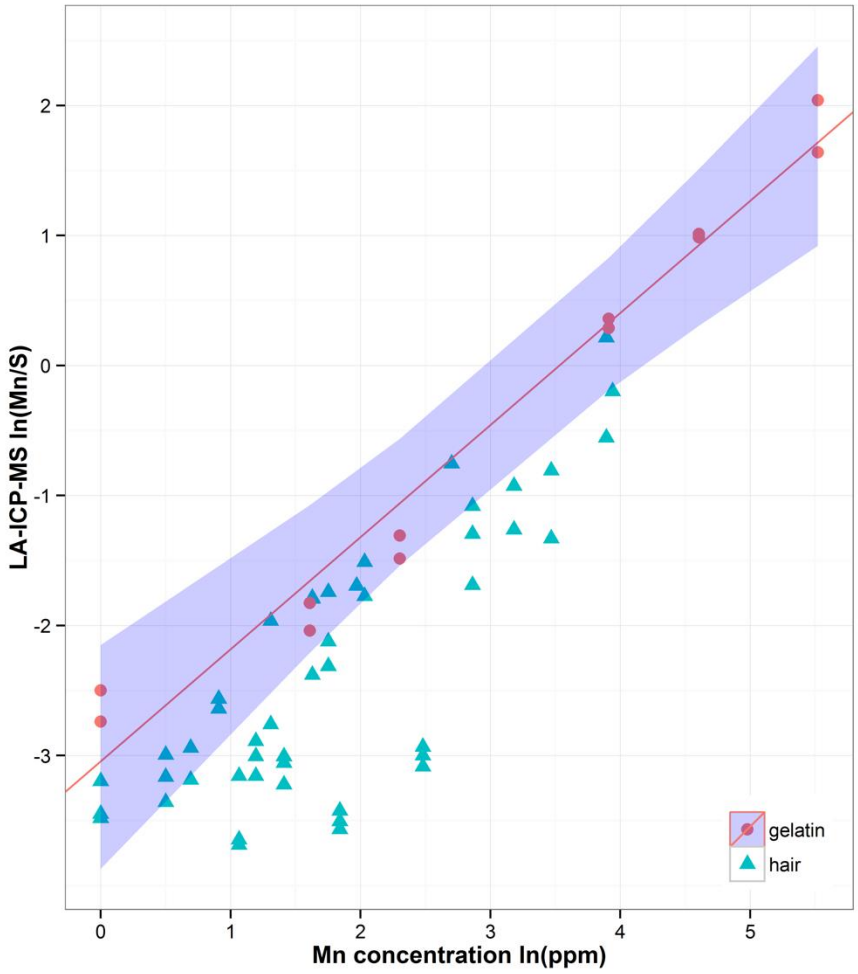
3.1 FIGURES

Figure 3-1 Within- and between-day variation in Mn measurements on ground hair pellet



Note: Black circles represent the mean value for each analysis; vertical lines represent one standard deviation for the time series of measurements acquired during each analysis.

Figure 3-2 Relationship of Mn levels (hair, gelatin) and Mn/S. Linear mixed model fixed effects regression line and 95% CI shown for MnG matrix



Chapter 4. ASSOCIATION BETWEEN TIME-RESOLVED MEASUREMENTS OF MANGANESE IN HUMAN HAIR AND DAILY OCCUPATIONAL EXPOSURE TO WELDING FUME.

4.1 ABSTRACT

Assessing exposure to manganese (Mn) in welding fume, and the resultant dose, presents many challenges. Ongoing personal air sampling to determine welders' widely varying exposures is impractical. One recent study has shown a moderate association between the welders' individual exposures to airborne Mn and Mn detected in bulk samples of their hair (Reiss et al. 2015). Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) analyses of Mn in individual hair strands has been shown to be highly correlated with those bulk hair measurements, suggesting that LA-ICP-MS-derived counts of Mn in individual hair strands ($MnH_{(La)}$) may be associated with airborne Mn exposures (MnA) (Chapter 1Chapter 3). The objective of this study was to assess a temporal association between LA-ICP-MS measurements of Mn in individual hair strands with Mn concentration profiles in air.

$MnH_{(La)}$ was measured with LA-ICP-MS along the shafts of nine hair strands (2.7 – 12 cm). Each strand was from a different test subject. $MnH_{(La)}$ measurements were spatially-resolved and translated into a time series. These $MnH_{(La)}$ time-resolved measurements were linked to estimated MnA via the hair sampling data. The cross-correlation between $MnH_{(La)}$ and MnA (cc) was calculated for each hair strand. In addition, $MnH_{(La)}$ values were randomized ($MnH_{(LaR)}$) 10,000 times in a simulation and the cross-correlation between MnA and $MnH_{(LaR)}$ (ccr) were

calculated. The p-value was calculated for the chance of observing the measured cross-correlation randomly in the distribution of the 10,000 ccr values. A significant negative significant cross-correlation was observed between the nine $MnH_{(La)}$ samples and the MnA exposure data (mean ccr: -0.184 ± 0.167; average p-value of 0.875 ± 0.267). The association between natural-log (ln) transformed $MnH_{(La)}$ and ln-transformed MnA was also assessed with a mixed model using the ln-transformed MnA concentration as a predictor for $MnH_{(La)}$. MnA levels were found to be significantly negatively associated ($p < 0.001$) with doubling MnA concentrations, yielding an 4.0% [95% confidence interval (CI): 3.5 to 4.5%] decrease in $MnH_{(LA)}$. These results are in strong contrast with previous findings where a moderate association was found between MnA and $MnH_{(B)}$ and a strong correlation was found between $MnH_{(B)}$ and $MnH_{(La)}$. The indications are that the relationship between $MnH_{(La)}$ and MnA maybe too weak to be assessed with the presented methods and that the concentration of Mn in hair may change with time. Given that associations between MnH and MnA have been found previously - including in this dissertation, greater sample size, hair samples of subjects exposed to much higher MnA, and different statistical tools may be needed in order to assess the relationship between MnA and $MnH_{(La)}$.

Keywords: Manganese, hair, laser ablation ICP-MS, biomarker of exposure

4.2 INTRODUCTION

LA-ICP-MS has been used in several studies to estimate the quantities of multiple elements in micrometer-long segments of single hair strands, because hair has been found to incorporate metals from xenobiotic exposures (Tobin 2005c). A laser beam is directed along a hair strand, ablating a small amount of the hair as it progresses. The ablated aerosols are directed into the ICP-

MS for analysis. A $MnH_{(LA)}$ profile is created as the laser moves along the hair shaft. This profile shows quantities of the material of interest deposited in the hair at specific points in time, providing a time resolution of substantially less than one month.

Most previous LA-ICP-MS hair studies have focused on mercury (Rodushkin and Axelsson 2000a; Rodushkin and Axelsson 2000b; Rodushkin and Axelsson 2003; Legrand et al. 2004; Stadlbauer et al. 2005; Legrand et al. 2007). One study, however, used LA-ICP-MS to measure Mn in the hair of children exposed to airborne ferroalloy Mn emissions; this study also evaluated procedures for washing the hair strands to remove external contamination from air and water (Eastman et al. 2013).

Most of these studies focused on short hair segments with few exposure reference points. The association between highly spatially-resolved $MnH_{(La)}$ measurements over longer hair strands and an exposure time-series had not been a not been conducted previously. The moderate association between Mn levels determined from digested bulk hair ($MnH_{(B)}$) and MnA exposures (Reiss et al. 2015) and the strong association between $MnH_{(B)}$ with $MnH_{(La)}$ (Reiss et al. in progress) suggests that $MnH_{(La)}$ could also be associated with MnA exposures. MnA exposures are an important concern in professions where exposures are high, such as welding. Individual long hair strands may have the potential for use in evaluating individual welder's past MnA exposures and their Mn dose.

Several studies have reported associations between integrated Mn levels from digested bulk hair ($MnH_{(B)}$) and exposures to Mn in ambient air. In occupational studies, associations between $MnH_{(B)}$ samples and MnA levels have been observed in dry-cell battery facilities (Bader et al. 1999) and in several welding studies (Grund et al. 1980; Bergert et al. 1982; Gorban et al. 1992; Ramakrishna et al. 1996; Reiss et al. 2013). In children exposed to high Mn levels in drinking

water (Bouchard et al. 2007) and children exposed to ferro-manganese dust (Menezes-Filho et al. 2011), $MnH_{(B)}$ was found to be associated with the exposure levels. In most of these studies, bulk hair samples were a minimum of 1 cm long because analytical limits of detection require a certain mass of hair and because handling multiple hair strands of less than 1 cm length is mechanically very difficult. Reported hair growth rates range from 0.35 mm per day (~1 cm per 28 days) (Tobin 2005a) to ~0.5 mm per day (~1 cm per 20 days) (Chamberlain and Dawber 2003). Accordingly, 1 cm bulk hair may represent integrated exposures over only the past month. To the best of our knowledge, associations between airborne exposure and $MnH_{(B)}$ for time intervals shorter than 30 days and for hair strands shorter than 1 cm length have not been investigated.

Inhaled Mn from welding fume is absorbed mainly through the lung, with a smaller fraction absorbed through the gastrointestinal tract. Mn reaches the brain and other tissues via the blood stream (Aschner et al. 2007). Welding fume exposure varies widely due to the type of welding, the specific welding filler metal, the base metal composition, the welding environment, the use of ventilation, the use of respiratory protective equipment, and the individual welder's behavior (positioning with respect to the welding plume, etc.) (Flynn and Susi 2009; Liu 2010b; Hobson et al. 2011). Reported Mn exposures from welding fume range from <10 μg m^{-3} [gas tungsten arc welding (GTAW)] to >580 μg m^{-3} [flux core arc welding (FCAW)] (Pesch et al. 2012). Welders' work settings also influence their exposures. For example, the open environments of construction sites typically result in different exposures than the small, poorly ventilated work spaces associated with shipbuilding. Accurate estimation of welders' exposures is challenging because of the variability of welding fume constituents and exposure durations.

Biomarkers that preserve variations in exposures over time are an attractive alternative to traditional exposure monitoring using personal air sampling. Hair has a potential advantage over

other exposure biomarkers such as blood in that it may preserve the temporally varying signature of past exposures as it grows.

LA-ICP-MS provides a tool to assess Mn levels in individual hair strands at measurement intervals of micrometers, potentially permitting retrospective evaluation of exposures to Mn with a finer temporal resolution than has previously been achieved.

The objective of this project was to determine the association between daily time-resolved exposure to Mn in welding fume and highly spatially-resolved Mn levels in individual hair strands.

4.3 METHODS AND MATERIALS

4.3.1 *Study design*

Samples for this study are a subset of samples collected from a longitudinal inception cohort study of welding trainees. The general overview of the cohort study design, details, and other statistical analysis has been presented in several prior publications (Baker et al. 2014; Baker et al. 2015a; Baker et al. 2015b; Reiss et al. 2015; Baker et al. 2015c). A brief summary of the study follows: From April 2011 to June 2013, 56 students from a Washington State welding training program were enrolled in the study cohort during the first week of a program quarter. The recruited students reported not having prior occupational exposure to Mn related to welding activities. The students provided hair, blood, and urine samples, and were monitored for exposures to airborne Mn at specific intervals as they progressed through the program. All study protocols were reviewed and approved by the University of Washington Institutional Review Board, and the study subjects provided written informed consent.

In the five-quarter academic welding training program, students learned five welding processes: oxyacetylene, shielded metal arc welding (SMAW), flux core arc welding dual shield (FCAW-DS), flux core arc welding inner shield (FCAW-IS), gas metal arc welding (GMAW), and gas tungsten arc welding (GTAW). Students also conducted other metalworking tasks such as cutting and grinding. Students attended the program from Monday through Friday, 8 AM to 2 PM, except during academic breaks and holidays. Students alternated welding activities with classroom instruction throughout the training day. The time spent on specific welding activities varied between test subjects, depending on their individual progress. Students worked in welding booths with adjustable exhaust ventilation hoods. There was no formal respiratory protection training or fit testing. Twenty-five (25%) of the students reported wearing respiratory protective equipment.

Air samples were collected on the Monday and Friday of the first and last week of each school quarter (4 days per quarter in total). On average, 10.7 ± 5.5 air samples per participant were collected throughout the study (average of 3.7 ± 1.6 air samples per quarter). Scalp hair samples were collected on a Monday or Friday at the beginning of the first quarter and at the end of each quarter. At the end of each sampling day, students completed a questionnaire to provide information on their work activities that day, including time spent welding, and on personal characteristics such as smoking habits and respirator usage.

4.3.2 *Air samples*

Air samples for particulate matter were collected with personal air sampling pumps (SKC AirChek XR4000, Eighty Four, PA, USA) equipped with closed-face polystyrene cassettes, each containing a pre-weighed 37-mm 0.8-µm pore mixed cellulose ester filter (MCE). The cassette was attached on the student's collar outside the welding garments and helmet. The pumps were calibrated to a flowrate of 2.0 L/min.

Filters were analyzed gravimetrically, pre- and post-sampling, for total particulate mass using an XS3DU analytical microbalance (Mettler Toledo, Columbus, OH) and then digested in 10 mL of a 1:1 mixture of concentrated nitric acid and deionized water using open vessel microwave assisted digestion (MarsXpress, CEM Corp., Matthews, NC, USA). The digestate was analyzed for Mn by a modified EPA 6020a Rev.1 procedure (United States Environmental Protection Agency 2007), using an Agilent 7500 CE ICP-MS (Agilent Corp, Santa Clara, CA) in He collision mode to eliminate polyatomic interferences. Analytical results for Mn mass identified as "below the reporting limit" were replaced with the analytical-batch-specific reporting limit divided by square root of two (Hornung and Reed 1990). MnA concentrations were calculated using the mass of Mn determined divided by the volume of air sampled and standardized to an 8-h time-weighted average. The maximum LOD for MnA was estimated to be 0.13 μg/m^3, based on three times the standard deviation of the field blanks.

MnA data were ln-transformed, because MnA data were found to be approximately lognormal distributed. Daily ln-transformed Mn concentrations were estimated in a linear mixed model using welding type (fixed effect) and attendance records. Individual subjects were entered as a random effect to adjust for within-subject variance. (Stata Version 11, xtmixed, College Park, TX, USA). A total of 19000 subject-specific daily exposure estimates were created.
Weekends, holidays, and days before subjects entered the welding program which are considered non-welding exposure days were set to the MnA LOD divided by the square root of two $\left(\frac{0.13\ \frac{\mu g}{m^3}}{\sqrt{2}} = 0.09\ \frac{\mu g}{m^3}\right)$ (Hornung and Reed 1990).

4.3.3 *Hair samples*

Forty-seven students provided hair samples at approximately three-month intervals, for a total of 154 samples. Hair samples were cut from the occipital region of the head, as close as possible to the scalp, using ceramic scissors. The hair samples were taped inside a new Ziploc bag, the bag was closed, and the samples were transported to the laboratory. The tape had been analyzed in advance and found to contain no detectable Mn.

A subset of hair samples from the inception cohort was selected for the current analysis. In order to increase the likelihood of detecting MnA exposure-related changes within a single strand, hair strands longer than 3 cm were selected from subjects who had large differences between MnA values when they moved from one welding activity to the next. A single hair strand was selected (from all collected hair samples) for analysis from each of nine subjects whose MnA concentration over the entire study period ranged across all subjects from 6.3±1.5 to 45.5±11.0 μg/m^3. Two of the nine subjects who's hair was used in this study reported to be respirator users.

4.3.4 *Sample preparation*

The individual hair strands were washed with Triton-X100 and acetone, and rinsed with ultra-distilled water between and after washes (Reiss et al. 2015). Washed hair samples were dried in a vacuum oven at 80°C for about 1 hr.

The hair strands were glued with double-sided adhesive tape (ScotchTM Double Sided Tape) onto petrographic glass slides (Beta Diamond Products, Inc., Yorba Linda, California; catalog number PS2746). No Mn signal above the ICP-MS background was detected in the tape or the glass slides. The ablation chamber accommodated a maximum of six glass slides simultaneously. The laser ablation path was programmed using the laser control software to define

the start (proximal end of hair strand), end (distal end of the hair strand), and directional changes of the burn path. After each burn path was defined, the Z-value (focus point) of the laser was fine-adjusted and the burn path was re-adjusted if needed. The length of the ablation path was also recorded.

4.3.5 *Analytical methods*

The hair samples were analyzed using an NWR213 laser ablation system (ESI, Portland OR), connected to an Agilent 7500 CE ICP-MS equipped with a collision cell using He, which removes polyatomic interferences. The data acquisition of the ICP-MS was triggered from the laser ablation software ("New Wave Research Laser Ablation System Version 4.1.0 Build 29 Copyright 2014 New Wave Research").

The Nd:YAG laser operated at a wavelength of 213 nm. Measurements were made with a laser energy output of 60% (11.79 J/cm^2), using a spot size of 30 µm, firing at a rate of 20 Hz. The sample stage was programmed to move at 100 µm per second. The effluent stream from the laser ablation system (He, 800 L/minute) was introduced directly into the ICP-MS torch. Make-up gas, Ar (0.5 L/min), was T-ed into the line immediately upstream of the torch. Elements of interest were quantified with He mode using the collision cell. The ICP-MS was operated with rf power of 1500W and set to measure the following isotopes (dwell time) in this order: ^{34}S (0.1 ms), ^{53}Cr (0.4 ms), ^{55}Mn (0.4 ms), ^{56}Fe (0.1 ms), ^{57}Fe (0.1 ms), ^{60}Ni (0.1 ms), ^{63}Cu (0.1 ms), ^{66}Zn (0.1 ms), ^{208}Pb (0.1 ms). Total cycle time was 1.52 seconds.

4.3.6 *Data processing*

As hair grows, scales are formed on the outside of the strand (Robbins 2012). Hair scales point from the follicle (proximal) end to the distal end of the strand. The directional growth of each

ablated hair strand was checked by 2 scientists inspecting the direction of each strand's scales with a light microscope at 40x magnification. The scientists agreed in all cases and found that two hair strands had been ablated in the wrong direction, i.e., from the distal end rather than from the proximal end. The time line records of these two strands were corrected to match the direction of the hair strands that had been ablated according to the established study procedure, i.e., from the proximal to the distal end.

The normality of $MnH_{(La)}$ and MnA data was assessed with qq-plots and found to be ln-normally distributed. Subsequent analysis was done on ln-transformed concentrations.

Figure 4-1 shows how the data were processed in order to align $MnH_{(La)}$ with MnA. Each conversion step is indicated with a letter. Both time series were converted to the same timescale to facilitate the alignment. Two datasets were created for each hair strand. The first dataset (hair) contained the ablation vectors burn path time stamp and $MnH_{(La)}$. The second dataset (air) contained the data vectors daily air sampling time stamp and MnA. Starting times for $MnH_{(La)}$ ablation were determined through a signal change corresponding to the ^{34}S isotope, which indicates when ablation began. More details are provided in the supplementary information.

As a first step of the alignment, the $MnH_{(La)}$ burn path time stamps were converted to "air sampling" real-time intervals (Figure 4-1: A). Records in the hair data set were stored in the ICP-MS's data acquisition rate of 1.52 second per record. The $MnH_{(La)}$ "real-time" interval was estimated on the basis of the length of the hair strand, the data points obtained per record, and the assumption of a uniform monthly hair growth rate for all hair strands. For example, one hair strand of length 11866.7 µm was found to have 841 $MnH_{(La)}$ points. These data correspond to approximately 2.4 $MnH_{(La)}$ points per day, based on the assumptions of a monthly hair growth rate of 1 cm and 30.42 days in the average month. A sample rate of 2.4 measurements per day is

equivalent to one sample every 10 hrs. In the first dataset, the $MnH_{(La)}$ burn interval of 1.52 sec was replaced with 10 hrs. For example, 1.52, 3.04, 4.56 secs represent the time steps 10, 20, 30 hrs. The earliest $MnH_{(La)}$ value was assumed to reflect the MnA value at the hair sample date. As an example, for the hair sample collected on 2012-08-02 (August 2nd, 2012), each time point for $MnH_{(La)}$ was replaced with a corresponding 10 hr date-time stamp. The values 39.53, 41.05, and 42.57 sec were replaced with the specific dates: August 2nd, 2012 18:00 hrs, August 2nd, 2012 08:00 hrs, and August 1st, 2012 22:00 hrs.

As a second step, the daily MnA exposure profiles for each hair strand were converted from 24 hr to 10 hr time intervals using the "approx()" function of the R-package "stats" V. 3.2.2 (Figure 4-1: B). Starting from the oldest date, the function splits days into 10 hr intervals. The lnMnA estimates for a given day were assigned to all 10 hr values for that day, i.e., the estimate 1.87 ln(µg/m^3) Sep. 19th, 2011 was expanded to 1.87 ln(µg/m^3) Sep. 19th, 2011 10:00 hrs and 1.87 ln(µg/m^3) Sep. 19th, 2011 20:00 hrs. The expansion of the next day 1.87 ln(µg/m^3) Sep. 20th, 2011 continues the time interval of the previous day, i.e. 1.87 ln(µg/m^3) Sep. 20th , 2011 06:00 hrs and 1.87 ln(µg/m^3) Sep. 20th , 2011 16:00hrs.

As a third step, $MnH_{(La)}$ data were linked to the MnA exposure profiles by their date-time stamps (Figure 4-1: C). The combined data set contains 3 vectors: a date-time stamp in 10 hr increments, MnA, and $MnH_{(La)}$. As the two data sets are combined by the 10 hr date-time stamp the $MnH_{(La)}$ data from the ICP-MS get inverted, because they are put into chronological order.

4.3.7 *Statistical analysis*

The combined data set with the date-time stamp in 10 hr increments, MnA, and $MnH_{(La)}$ was assessed with cross-correlation to determine the correlation between MnA and $MnH_{(La)}$. Serial correlation in these time series was suspected, due to the high sampling frequency of the $MnH_{(La)}$

and MnA data. The presence of serial correlation was assessed with the Durbin Watson test (dw-test). A significant dw-test statistic of <2 (positive serial correlated) or >2 (negative serial correlated) indicates serial dependence. Both MnA and $MnH_{(La)}$ data were found to be positively serial-correlated: the dw-test statistic was highly significant (all p-values <0.001) and less than 2 for the 9 $MnH_{(La)}$ and MnA profiles (MnA: 0.20 – 1.59; $MnH_{(La)}$: 0.31- 0.51).

The hair strands were cut above their follicles; consequently, the first ablation (which begins at the proximal end of the hair shaft) data point corresponds to a point in time prior to the hair sampling date. The difference could be as much as 1 month (10 mm). The base of a Caucasian hair follicle is approximately 4.2 ± 0.4 mm from the surface of the skin (Jimenez et al. 2011) and hair cannot be cut at the skin of the scalp with ceramic scissors due to the width of the scissors (~5 mm). Consequently, the MnA and $MnH_{(La)}$ data are not perfectly matched in time. Therefore, the $MnH_{(La)}$ vector was shifted to find the the best cross-correlation $MnH_{(La)}$ and MnA.

For each hair strand, the vector with $MnH_{(La)}$ data was shifted relative to the MnA data in steps of 10 hrs (the interval from one record to the next). The data were shifted over a lag time range of zero time to the maximum time that the data from a particular $MnH_{(La)}$ vector spanned. A cross-correlation was calculated for each lag and the lag with the maximum cross-correlation was considered the best matching lag. The highest cross-correlation was considered the measure of association between $MnH_{(La)}$ and MnA.

The statistical software R version 3.2.2 was used to conduct the statistical analysis of the MnA and $MnH_{(La)}$ data. The cross-correlation analysis was performed with the “ccf()” function from the R-package stats V. 3.2.2.

The link of $MnH_{(La)}$ to the MnA data was tested for a random observation. A simulation experiment was conducted to determine if the highest found cross-correlation between MnA and

$MnH_{(La)}$ was a chance observation. First, the cross-correlation between MnA and observed $MnH_{(La)}$ was determined. Then the cross-correlation between MnA and randomized $MnH_{(La)}$ was determined. The cross-correlation between MnA and randomized $MnH_{(La)}$ was repeated 10000 times each time with newly randomized $MnH_{(La)}$ data. Cross-correlations were Fisher-z transformed, because this transformation "normalizes" correlation distributions. The proportion of the highest cross-correlations from the randomized data that was higher than the highest cross-correlation from the non-randomized data was calculated for each hair strand. With this proportion, a p-value was calculated to assess the chance of obtaining a cross correlation equal to or "more extreme" than was actually observed.

Finally, a linear mixed model (lme-function; nlme-package V3.1-1.2.1; R V3.2.2) was used to estimate the strength of the association. Daily ln-transformed $MnH_{(La)}$ concentrations were estimated in a linear mixed model using ln-transformed MnA (fixed effect) and individual subject was entered as a random effect to adjust for within-subject variance. The percentage increase of $MnH_{(La)}$ levels for each doubling in MnA and the between- and within-subject variance components were determined with the model. Time and a time-dependent correlation structure were not included in the model because it is beyond the scope of this project. Selecting a suitable time-dependent correlation structure is non-trivial (Chatfield 2003; Zuur et al. 2009) and will be investigated in future research.

In addition, for each 1 cm hair sample (used in Chapter 2) a 30 day time-weighted average Mn exposure was calculated for the 30 days prior (MnA_{30d}) to the sample collection date using the individual's daily estimated exposures. Ln-transformed MnA_{30d} were used in a linear mixed model to estimate monthly average Mn/S (fixed effect), adjusted for individual subject (random effect)

using (R R3.1.1 (32-bit) platform with the lme-function from the nlme-package (version: nlme_3.1-117)).

4.4 RESULTS

The full length of hair strands longer than 3 cm from nine different welding apprentices with corresponding exposure profiles that contain a wide range of exposures were analyzed with LA-ICP-MS. The hair strands had a mean length of 7.2 ± 3.7 cm, ranging from 2.7 to 12.0 cm. On average, 516 ± 260 $MnH_{(La)}$ data points, ranging from 191 to 864 data points, were collected from each hair strand. The average travel speed of the laser was estimated to be 92.4 ± 0.4 µm/min, slightly slower than the set travel speed of 100 µm/min. The sampling frequency of the ICP-MS combined with the translation speed of the laser ablation stage led to an estimated 2.4 ± 0.1 $MnH_{(La)}$ measurements per day (equivalent to a sample frequency of 10 hrs), assuming a nominal hair growth rate of 1 cm/month. However, approximately 10% of scalp hair follicles are in the telogen i.e., resting phase where there is no growth. The telogen phase is estimated to last from to 1-3 months (Tobin 2005b).

The $MnH_{(La)}$ data for the nine hair strands were aligned with the associated MnA profiles. Figure 4-2 and Figure 4-3 each show one example of ln-transformed MnA and $MnH_{(La)}$ profiles. Table 4-2 shows the association between $MnH_{(La)}$ and MnA and the results of the simulation analysis for the observed and randomized cross-correlations. The average observed cross-correlations was -0.184 ± 0.167 and most observed cross-correlations between $MnH_{(La)}$ and MnA were negative. The average cross-correlation of the randomized data was substantially lower (i.e. 0.000 ± 0.014). No observed cross-correlation in the randomized data was significant. The p-value was more than 0.999, except for two hair strands where it was 0.07 and 0.10.

The raw data were more correlated than the shifted data (data not shown). The average observed cross-correlation of the shifted data was 0.093 ± 0.114. The average cross-correlation of the shifted randomized data was 0.093 ± 0.026. One observed p-value was significant and the average p-value was 0.6 ± 0.5. The average lag was -4.4 ± 4.3 months ranging from -0.6 to 11.5 month.

Table 4-3 shows the results of the initial mixed model analysis of the ln-transformed MnA and $MnH_{(La)}$ levels. MnA levels were found to be significantly negatively associated ($p < 0.001$) with an increase in $MnH_{(La)}$, yielding a 4.0% decrease in $MnH_{(La)}$ levels [95% confidence interval (CI): 3.5 to 4.5 %] per doubling in MnA. Most of the remaining variance is between subjects (60.5%) rather than within subjects (39.5%).

A non-significant (p-values = 0.278) positive trend was observed for the 1 cm hair strand for the association between thirty-day MnA levels and 1 cm $MnH_{(La)}$.

4.5 DISCUSSION

Our study does not provide compelling evidence that Mn levels in time resolved individual hair strands can be used as a biomarker of exposure to MnA. This is in contrast to prior studies that have shown an association with Mn exposure and bulk hair samples. For example, positive associations with $Mn(H)_{(B)}$ have been found in children exposed to high Mn levels in drinking water (Bouchard et al. 2007), and in ferro-manganese dust (Menezes-Filho et al. 2011). In occupational studies, MnA has been found to be associated with $MnH_{(B)}$ in workers at dry-cell battery facilities (Bader et al. 1999) and in of workers exposed to welding fume (Grund et al. 1980; Bergert et al. 1982; Gorban et al. 1992; Ramakrishna et al. 1996; Reiss et al. 2015).

MnA exposure concentrations in this study were substantially lower than those reported in other occupational studies. Our mean measured MnA concentration was 34.7 ± 32.2 $\mu g/m^3$

compared to 160 ± 190 μg/m^3 (Hobson et al. 2011), and 543 ± 1530 μg/m^3 (Liu et al. 2011). A modest association between MnA and 1 cm $MnH_{(B)}$ was previously found in our study even at those lower exposure levels (Reiss et al. 2015). Although a more recent study (Reiss et al.; Chapter 3) showed a strong association between $MnH_{(B)}$ and $MnH_{(La)}$ at the low exposure levels indicated above, the current study did not demonstrate a link between $MnH_{(La)}$ and MnA. A modest association between low MnA levels and $MnH_{(B)}$ was found in the study reported in Chapter 2 using approximately 30-50 hair strands total per matched MnA exposures. An average measurement based on 30-50 1 cm hair strands may provide better precision in the biomarker measurement in comparison to a single ablated hair strand.

The low MnA levels of this study may not be detectable with spatially-resolved $MnH_{(La)}$ on individual hair strands, because the low MnA exposures may result in deposition of quantities of Mn in the hair shaft that are insufficient for detection by LA-ICP-MS. Furthermore, the degree to which Mn is retained in hair strands as they are exposed to stresses such as frequent hair washing, is unclear. It is possible that repeated washing of hair leaches Mn from the hair shafts. Alternatively, Mn levels in hair strands might increase if the hair is washed with water containing high levels of Mn (Eastman et al. 2013). For these reasons, Mn levels in long hair strands may be less representative of the airborne exposure than shorter hair strands. Short hair strands would have been exposed to environmental impacts such as washing for shorter periods of time.

We assumed a uniform hair growth rate of 1 cm per month (Tobin 2005a), although a rate of 1 cm in 20 days has also been reported (Chamberlain and Dawber 2003). A different growth rate might result in a higher correlation. In addition, some of the selected hair strands may not have captured the exposure as they may have been in their telogen state while a subject was exposed.

We attempted to improve the association by shifting $MnH_{(La)}$ vectors relative to MnA vectors. This shift was made to account for a potential temporal offset due to cutting the hair at the sample date. Hair profiles were moved substantially (months to years of out range) away from their expected relative position to MnA. As a result, on average, the selected shifting-method decreased the associations. These data are not shown, because this method did not result in positive cross-correlations and shifted the entire $MnH_{(La)}$ vector away from the MnA profile until there was a cross-correlation of zero. The average lag of 4 months indicates a shift of approximately 4cm, which is far beyond any reasonable lag period. This result clearly indicates that the use of ccr with shifting was not a useful technique for our data. It may be a useful tool when the data are positively cross-correlated.

Both Figure 4-2 and Figure 4-3 show that MnA varies less than $MnH_{(La)}$ over time. MnA levels were modeled and assigned to subjects based on their reported welding activity. If a subject reported the same welding activity for several days, the same MnA value was assigned to each day. Given that observed associations between MnA and $MnH_{(B)}$ are moderate, it may not be possible to detect changes in $MnH_{(La)}$ without greater MnA variations. It remains to be determined if the within-hair variability in $MnH_{(La)}$ represents true variability in biological levels of Mn or if it is a property of the analytical method.

Additional research is needed to focus on ways of improving the synchronization of LA-ICP-MS hair measurements with real time. This objective might be accomplished by using information from other air contaminants that occur in much higher concentrations in air in order to identify timelines. Supplementation of subjects' diet with Mn or other metals (e.g. selenium) that can be detected with LA-ICP-MS may be used as time markers.

Little is known about whether Mn is stored in hair over extended periods of time, or how washing, or ingestion of Mn affects levels detected in hair. More research is necessary to identify the temporal storage capacity of Mn in hair. The significant decrease of $MnH_{(La)}$ with increasing MnA could be explained if Mn was removed from hair over time.

Finally, two subjects reported using respiratory protection equipment. While these reports were not validated, the number of samples is too small to conduct a meaningful statistical test to evaluate the effect of respiratory protection. However, it appears that in one case (ID:9) the $MnH_{(La)}$ data are somewhat lower compared to other hair strands. Addressing this aspect in future studies would be beneficial.

4.6 FUTURE WORK

Given the limitations of this study and the points discussed above, four key additional experiments are suggested to determine if $MnH_{(La)}$ can be used to resolve MnA exposures. First and most importantly, a larger contrast in MnA exposures across time is necessary. For example, welders (i.e. part-time maintenance welders), with potentially higher exposures due to lack of experience or lack of controls, sampled over longer periods of time, are recommended as potential test subjects. This group is expected to show considerable variations in exposures with time, potentially resulting in larger $MnH_{(La)}$ signal contrasts. A higher sampling frequency may also help to measure exposures with more contrasts.

Second, the capacity of hair to store Mn over time needs to be determined. Once $MnH_{(La)}$ can be detected following an external exposure to Mn, frequent repeated sampling of hair strands over time, may allow the assessment of a $MnH_{(La)}$ signal degradation of due to washing-in or washing-out Mn, hair color, and or hair treatment.

Third, given the large fraction of resting hair strands and the uncertain hair growth rate, a method for assessing hair growth is needed. This information may be obtained by using subjects who regularly (every one to two months) ingest a supplement that can be readily detected in hair (Tobin 2005b). Testing would be required on several hair strands to ensure that actively growing, not dormant-phase hair is selected.

Four, given several Mn exposure routes, using an external control group from the same location with similar life style is needed to assess a Mn signal due different exposure routes such ingestion and inhalation.

Finally, different statistical tools may be needed to assess this association. Cross-correlation performs better on time series that are "stationary" (Zuur et al. 2009). Optimizing the different times scales i.e. for days, weeks, or months etc. may help to reduce the point-to-point variability. Linear mixed-models with time in the auto-regression structures may allow more accurate modelling of the association between MnA, time, and $MnH_{(La)}$.

4.7 CONCLUSIONS

Time-resolved $MnH_{(La)}$ levels measured along the shaft of individual hair strands were consistently not associated with MnA. These results suggest that LA-ICP-MS Mn measurements in hair strands may not be a suitable measure of Mn air exposure at the relatively low occupational levels observed in this study. Lag optimization did not improve the association between $MnH_{(La)}$ and MnA, and the optimized lags were substantially out of the expected range indicating that using ccr is not suitable for this problem. Although there are indications that hair stores Mn, this study does not provide further evidence to support this conjecture.

4.8 TABLES

Table 4-1 Hair strand characteristics

ID	$MnH_{(La)}$ (cps; AM±ASD)	Length (cm)	Sample points (count)	Exposure coverage (days)
1	8300±4800	7.2	514	214
2	8000±2500	12.0	864	360
3	9300±7600	12.0	841	350
4	2200±670	3.6	274	114
5	2300±1100	2.7	191	80
6	2800±1300	4.5	319	133
7	14000±5600	8.9	643	268
8	9500±8600	10.0	715	298
9	2000±400	4.0	285	119

Table 4-2 Cross-correlation (CC) of MnA and observed $MnH_{(La)}$.

ID	Observed CC	Randomized Data* CC Mean*±SD	p-value**
1	-0.252	0.000 ± 0.0442	>0.999
2	-0.070	-0.001 ± 0.0343	0.09
3	-0.492	0.000 ± 0.0345	>0.999
4	-0.131	0.001 ± 0.0603	<0.001
5	0.058	-0.001 ± 0.0728	0.516
6	-0.155	0.000 ± 0.0569	<0.001
7	-0.268	0.001 ± 0.0397	>0.999
8	-0.319	0.000 ± 0.0377	>0.999
9	-0.032	0.000 ± 0.0592	0.79

*mean of the maximum cross-correlations generated from each of the 10,000 simulations; mean rounded to 3 digits with 1 significant figure
** probability that the maximum correlation observed in each sample is due to chance

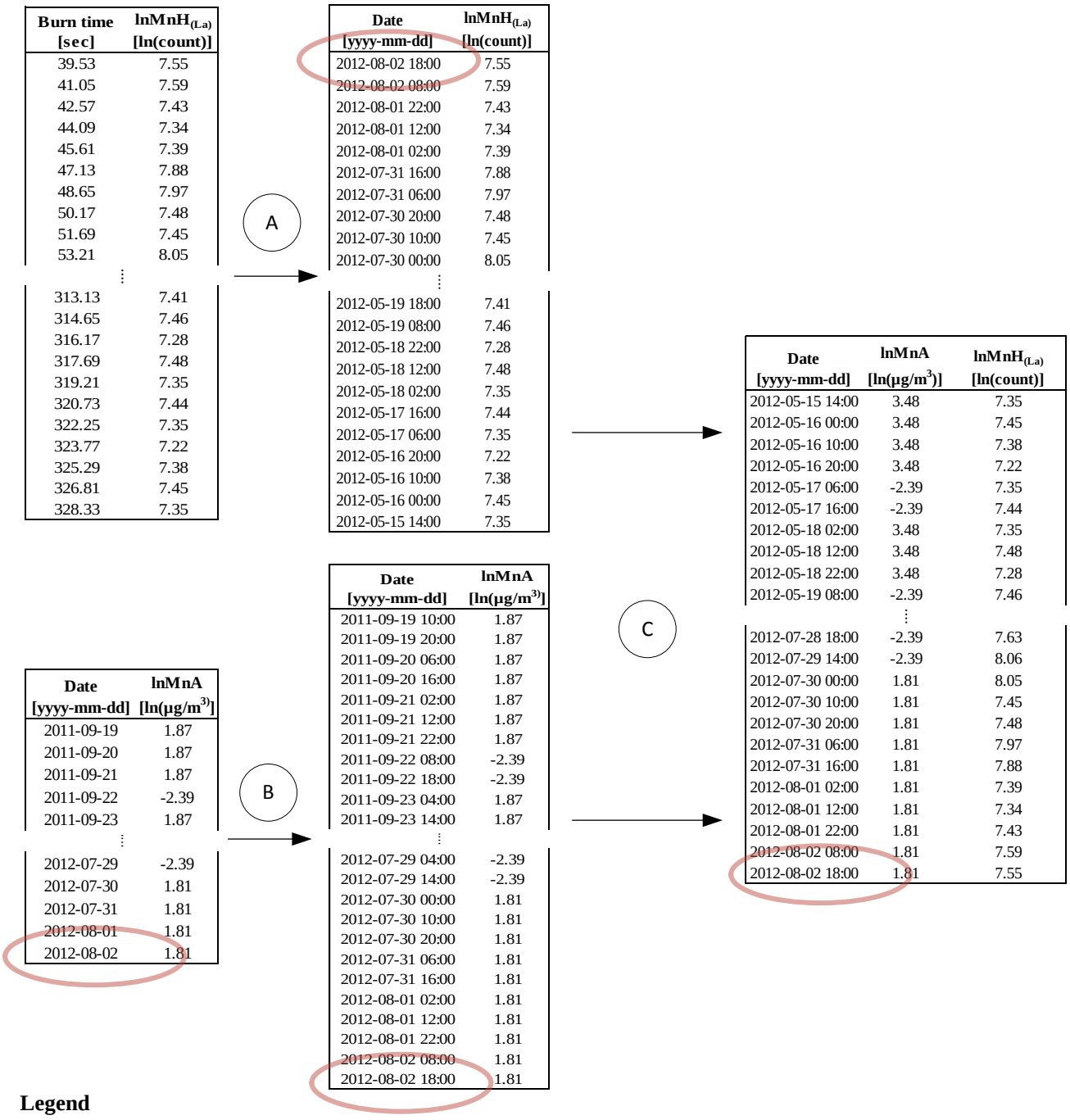
Table 4-3 Relationship between $MnH_{(La)}$ and MnA levels (ln-ln)

	ln-MnA		
Fixed effects	**estimate (95% CI)**	**SE**	**p-value**
Intercept	8.4 (7.9 - 8.8)	0.222	<0.001
ln MnA	-0.059 (-0.066 to -0.052)	0.0035	<0.001

Variance components	**ln MnA**	**Value (%)**
Within hair	0.290	39.5
Between hair	0.444	60.5
Total	0.733	100

4.9 FIGURES

Figure 4-1 Data flow – Sample 5



Legend

A: Convert $lnMnH_{(La)}$ burn interval from 1.52 sec to 10 hrs using hair sample date for first value

B: Interpolate lnMnA daily sample rate to 10 hr

C: Join lnMnA and $lnMnH_{(La)}$ by date-time stamp (inversion of $lnMnH_{(La)}$ data)

: Hair sampling date

Figure 4-2 Sample 5: 80 days measurements of lnMnA and $lnMnH_{(La)}$; units: ln(µg/m^3) and ln(^{55}Mn counts per second)

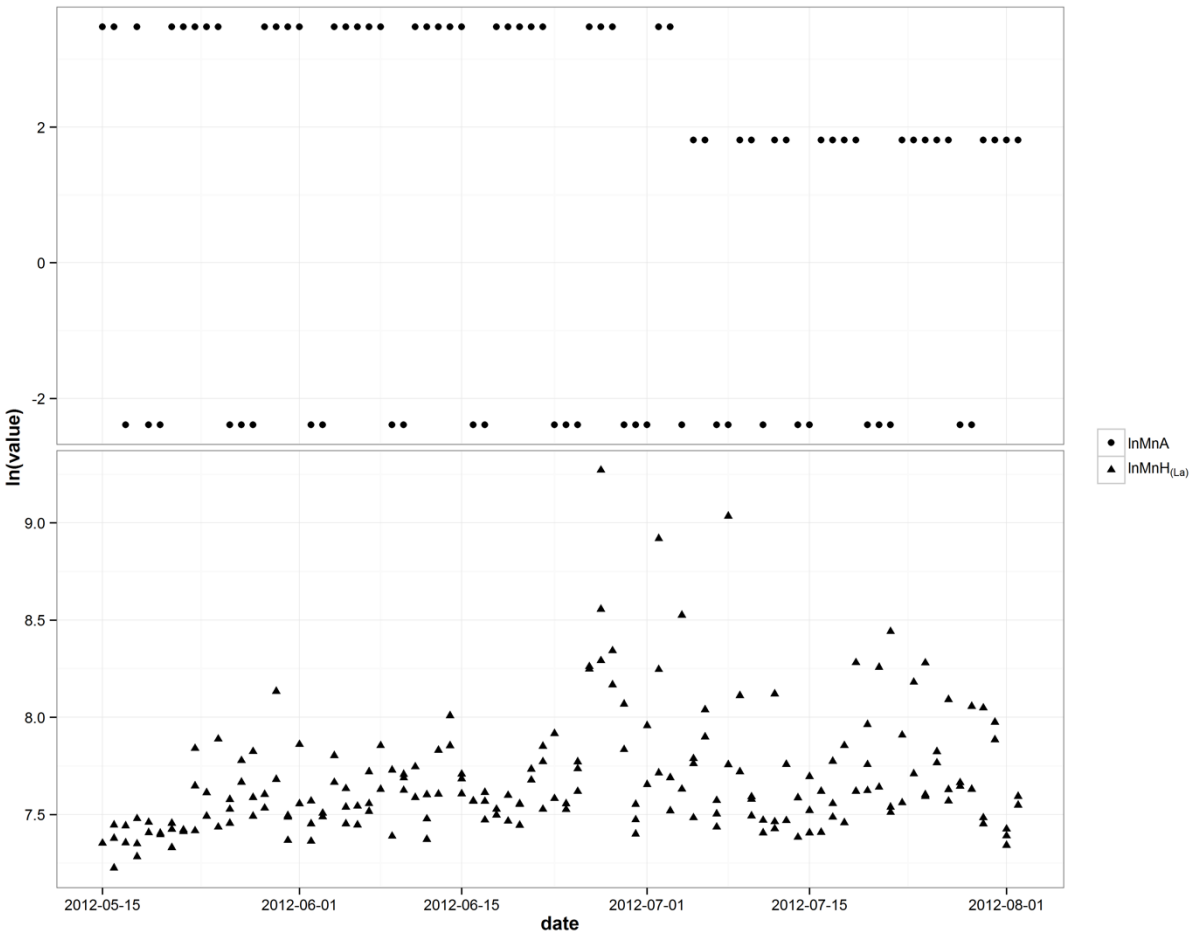
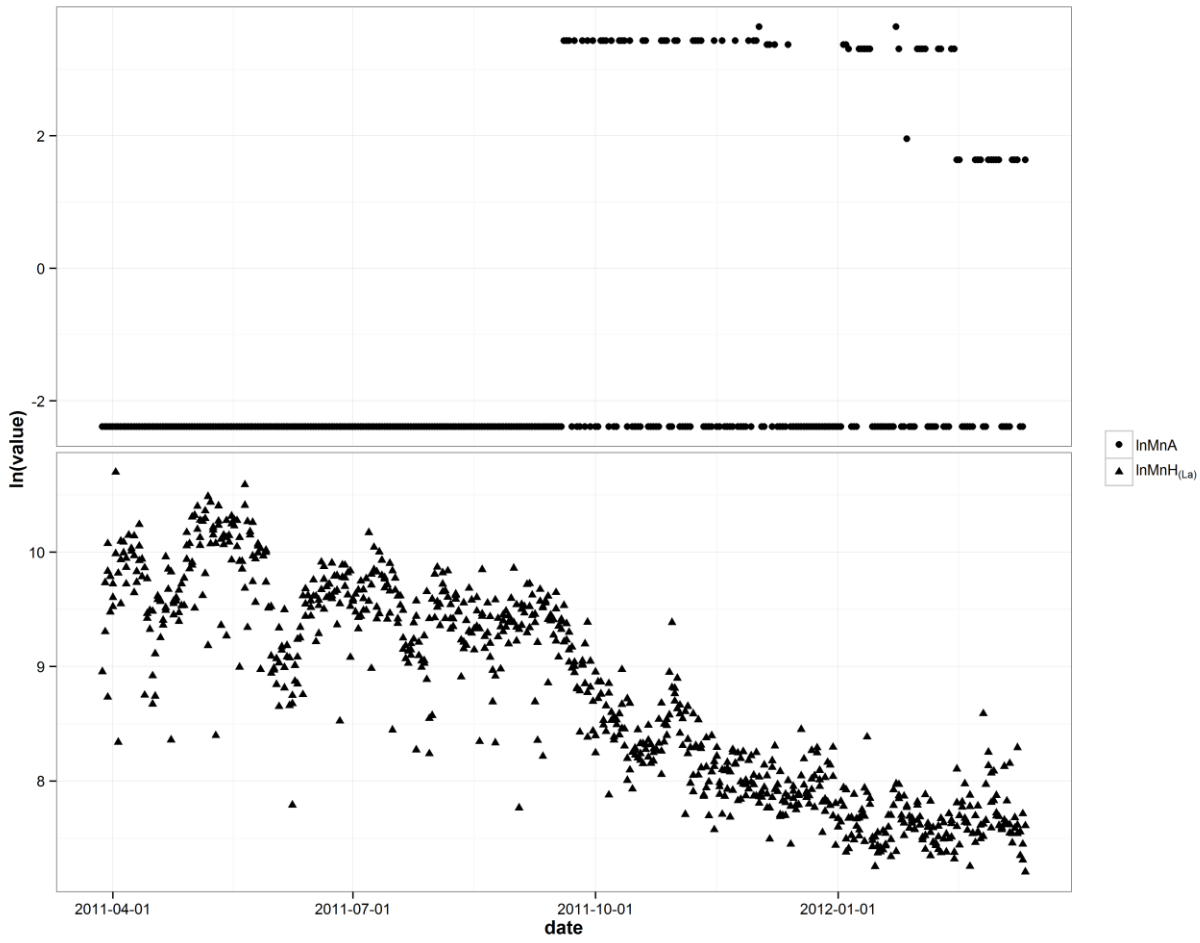


Figure 4-3 Sample 3: 350 days measurements of lnMnA and $lnMnH_{(La)}$; units: ln(µg/m^3) and ln(^{55}Mn counts per second)



Chapter 5. CONCLUSIONS

The primary objective of this research was to test if hair can be used as a biomarker for exposures to airborne manganese. Occupational inhalation exposures to Mn are often chronic and vary substantially from day to day (Flynn and Susi 2009; Hobson et al. 2011; Pesch et al. 2012). Estimating long term exposures using current standard practice, i.e., personal sampling, requires intensive effort and cost. Use of biomarkers for Mn has been considered as a potentially efficient and cost-effective alternative exposure monitoring strategy. Biomarkers may also provide information on internal dose and integrated exposure over time.

Some biomarkers of Mn exposure, such as measurements in blood, nails, and hair, have been implemented and have shown differences between exposed and unexposed groups (Roels et al. 1987; Roels et al. 1992; Apostoli et al. 2000; Myers et al. 2003). However, on an individual basis, these biomarkers have not been completely successful in demonstrating associations with external measures of Mn exposure. While previous health effect studies have used Mn levels in hair as a biomarker of Mn exposure (Bader et al. 1999) and some have shown increasing concentrations of Mn in hair of welders with increased years of exposure (Xie et al. 1995; Lin 2002), individual exposure was not quantified. Hair, however, may be a useful biomarker for individual longitudinal exposure to Mn because it has the potential to record the long-term time course of Mn exposure. In addition, hair can easily be obtained from workers (Legrand et al. 2004; Stadlbauer et al. 2005).

The studies presented here were derived from a 2-year long apprentice welding cohort of 53 students. During this time 154, $MnH_{(B)}$ and 600 MnA samples were collected. MnA samples were used to estimate daily MnA levels for all subjects.

Chapter 2 describes the assessment of the association between MnA and $MnH_{(B)}$. $MnH_{(B)}$ was linearly related to MnA on a ln-ln scale. A time integrated quantitative estimate of exposure over the previous 30 day period was associated with $MnH_{(B)}$ in the first centimeter of hair proximal to the scalp. This length of hair is nominally grown over a period of about a month. Two washing procedures were used and an offset between them was found. Although the associations found between MnA and $MnH_{(B)}$ were moderate, the results described in this chapter provide evidence to indicate that Mn in hair could be used as a biomarker for exposure.

Chapter 3 describes the use of different calibration approaches to assess the association between $MnH_{(B)}$ and $MnH_{(La)}$ using $MnH_{(B)}$ samples to calibrate Mn levels in individual spatially-resolved 1 cm long hair strands. In addition, Mn in gelatin ($MnG_{(B)}$) was used to calibrate LA-ICP-MS measurements ($MnG_{(La)}$) in order to evaluate MnG as an alternative calibration standard for $MnH_{(B)}$. Both, $MnH_{(La)}$ and $MnG_{(La)}$ were highly correlated with their respective bulk standards $MnH_{(B)}$ and $MnG_{(B)}$. The slopes for both calibrations were very similar and statistical non-distinguishable. A small, non-significant offset between the slopes was identified. The results of this study indicate that there is a strong association between $MnH_{(B)}$ and $MnH_{(La)}$. Gelatin shows strong promise as a proxy calibration standard for measuring Mn levels in hair strands.

Chapter 4 describes the assessment of the association between time-resolved $MnH_{(La)}$ levels measured along the shaft of individual hair strands and MnA. These measures were negatively associated. Lag optimization did not improve the association between $MnH_{(La)}$ and MnA and yielded lag periods that were substantially out of the expected range. The results of Chapter 4 alone suggest that LA-ICP-MS Mn measurements in hair strands may not be a suitable measure of Mn air exposure at the relatively low occupational levels observed in this study.

The results of this work provides support for existing evidence that $MnH_{(B)}$ may be a useful exposure biomarker in similar settings. Although the results from Chapter 2 and Chapter 3 indicate that hair stores Mn, and that $MnH_{(La)}$ levels in individual hair strands were strongly associated with $MnH_{(B)}$ concentrations, the analysis of LA-ICP-MS measurements described in Chapter 4 did not provide evidence that Mn in individual hair strands is correlated to MnA profiles. There are many potential factors particular to this latter study that could have contributed to the outcome. For example, exposure levels in this study were substantially lower than those identified in cohorts with full time welders (Hobson et al. 2011; Liu et al. 2011; Pesch et al. 2012) and the number of samples was arguably very small.

Future research may resolve the issues raised in this work. First and most importantly, a larger MnA contrast is required in order to identify a suitable detection limit for using $MnH_{(La)}$ as a measure of MnA levels. This could be obtained by recruiting a larger number of subjects with an increased range of exposure. Sampling welders with little experience and little exposure control, over longer periods of time may provide the data needed to determine the usability of $MnH_{(La)}$ measurements. These welders are expected to have higher exposures, potentially creating larger signal contrast in $MnH_{(La)}$ measurements and showing variations in exposures with time.

Second, it is necessary to determine the capacity of hair to store Mn over time. As hair grows, Mn levels may be influenced by the environment in which a person lives. For example, daily hair washing, or diet, may increase or decrease the levels of Mn in hair.

Third, given the large fraction of dormant hair strands and the uncertain hair growth rate, a test for assessing hair growth is needed. It may be necessary for test subjects to ingest a supplement that can be readily detected in hair, every few months, followed by analysis of hair

over time, in order to determine hair growth rates. Testing on several hair strands would be required to ensure that actively growing, not dormant-phase, hair is selected.

Finally, different statistical tools may be needed to better assess this association. Linear mixed-models with time in the auto-regression structures and lag as a random effect may allow more accurate modelling of the association between MnA, time, and $MnH_{(La)}$.

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APPENDIX

A HAIR MANGANESE AS AN EXPOSURE BIOMARKER AMONG WELDERS

A.1 HAIR WASHING PROCEDURE EVALUATION OVERVIEW

We evaluated four washing procedures to test the effectiveness of detergent (acetone and Triton X-100), mechanical agitation (vortex and sonication), and rinse solvent (ultrapure water, acetone, or water followed by acetone) on human hair contaminated with manganese from Shield Metal Arc Welding (SMAW) fume.

A.1.1 Sample collection and preparation

A sample of human hair was obtained from a local barbershop, and separated into two loose bundles where one was kept 'uncontaminated'. Bundle two was attached with a tie-wrap to a wooden pin that was locked into the chuck of a power drill. Inside a welding booth the power drill-hair assembly was clamped onto a post so that the hair reached into the welding plume during welding. The power drill was activated at the lowest possible speed to rotate the hair in the welding plume allowing a more homogeneous hair surface contamination. After hair was exposed for 30 minutes to SMAW welding fume the contaminated hair was put into a zip lock bag and mixed by hand to create a mixed contaminated bundle.

The contaminated and not contaminated bundles were divided, cut into 1cm samples, electrically discharged (Mettler Ionizer Antistatic System Model 11238-354), and weighed on an analytical balance (Mettler Toledo, Model AG285; limit of quantification: 0.1 mg). Hair was then

washed as shown in Table A-1. For each treatment, triplicate analyses were done, except for the contaminated, unwashed samples, for which five samples were run.

Wash and rinse fluids were removed with a fritted glass pipette attached to a vacuum suction system. The fritted glass pipette was flushed with ultra-distilled water or acetone between samples to avoid contamination from sample to sample. After the final rinse was completed the samples were dried at approximately 80°C in a vacuum oven for approximately 1 hour.

Table A-1 Sample parameters for washing procedure evaluation

N		Detergent	Volume of detergent (ml)	Mechanical Agitation (30 min)	Rinse	Rinse volume (ml)	Number of rinses
C+	C-						
3	**3**	T	40	S	W	210±5	6
3	**3**	T	40	V	W	210±5	6
3	**3**	T	40	S	W+A	210±5, 45±1	8 (6^{W} +2^{A})
3	**3**	A	30	S	A	25±1	1
5	-	-	-	-	-	-	-

N: Number of samples; C+: Contaminated; C-: not contaminated
Detergent: A: Acetone(Fischer scientific: Optima A929-4 ultragrade); T: 0.5 ml 1% Triton X-100 + 930mg EDTA + 50 ml ultra distilled water
Mechanical agitation: S: sonication; V: Vortexing in nitric acid-washed 50 ml test tube
Rinse: W: Ultra-distilled water (≥ 18 Mohm; NanoPure, Barnstead); A: Acetone (Fischer scientific: Optima A929-4 ultragrade); Triton X-100 samples were rinsed until hair samples were not sudsy anymore.

Hair samples were prepared following the method of (Puchyr et al. 1998). A recovery standard of 50 ppb Terbium (Tb) was added to each sample. The hair was then digested with 4 ml of a 1:1 mixture of concentrated nitric acid (trace metal grade, Fisher Scientific) and deionized water (≥ 18 Mohm; NanoPure, Barnstead) using microwave-assisted open-vessel digestion (MarsXpress, CEM corp., Mathews, NC). The acid digested samples were analyzed with inductively-coupled plasma mass spectrometry (ICP-MS) Agilent 7500-CE, based on method EPA 6020a Rev.1 (United States Environmental Protection Agency 2007).

^{55}Mn was quantified in He mode, in order to reduce polyatomic isobaric interferences. ^{45}Sc was used as the internal standard. Matrix spike recovery was performed with each batch of hair samples (mean recovery ± sd: 103 ± 6%, N = 31). Process blanks were run with each batch and were never higher than the reporting limit for Mn of 1–4 ng. The reporting limit varied because it was set to the lowest valid calibrant in each sample batch, which in turn varied slightly over the course of the project. The highest process blank for Mn was ~0.015 ng.

A.1.2 Statistical analysis

Robust regression was used to determine if the washed contaminated hair was significantly cleaner than the unwashed contaminated hair, and if residual manganese content is present after washing hair. ANOVA was used to test whether the four washing procedures differed from each other in residual Mn content.

A.1.3 Results

Table A-2 shows the results of the hair washing procedure. Three (3) samples were below the limit of detection of 0.2 µg/g of hair, all among the washed non-contaminated samples. Washing the contaminated hair produced on average 80% lower MnH levels than unwashed hair ($p<0.001$). The residual Mn content in hair after washing was 0.8 ± 0.5 µg/g for the contaminated hair and 0.6 ± 0.7 µg/g in the uncontaminated hair: the difference was not significant (t-test: $p=0.322$). There was no significant differences among the levels associated with the four washing procedures (ANOVA: $p=0.302$, F-value:1.3).

Table A-2 Mn concentrations after washing contaminated or uncontaminated hair (µg/g)

Contaminated	Washing procedure (detergent, agitation, rinse)	N	Mean(SD)	GM
No	Washed (all procedures)	12	0.55(0.70)	0.36
Yes	Not washed	5	4.04 (1.72)	3.79
Yes	Acetone, Sonication, Acetone (ASA)	3	0.73 (0.67)	0.56
Yes	Triton X-100, Sonication, Acetone (TSA)	3	1.03 (0.77)	0.86
Yes	Triton X-100, Sonication, Water (TSW)	3	0.66 (0.05)	0.66
Yes	Triton X-100, Vortexing, Water (TVW)	3	0.82 (0.68)	0.65

A.1.4 Conclusion

Based on these findings, all of the wash procedures used are equally effective in substantially cleaning Mn surface contamination, though residual Mn contamination is present after washing.

B CALIBRATION OF LASER ABLATION INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY FOR QUANTIFICATION OF MANGANESE IN HUMAN HAIR.

B.1 PROCESSING OF LA-ICP-MS DATA

Mass spectrometer signal intensities were exported to .csv files and then further processed in R. The ICP-MS acquired data before, during and after ablation of each hair sample, so that it would be possible to monitor stability over time of the ICP-MS background signals for ^{55}Mn and ^{34}S, in addition to measuring those signals when hair was being actively ablated. An automated routine was used to detect periods when the hair was being actively ablated, and to post-process the signals from those periods, as follows.

A threshold on the background ^{34}S signal was used to detect when laser ablation began. The threshold was calculated as the mean + 5 sd of the first 10 records of the ^{34}S signal. The first data point in the subset of data consisting of indices 10-60 that exceeded the threshold was used as the starting point of laser ablation. The time axis was then adjusted to set this as time zero. The period prior to laser ablation consisted of all data points with time-indexes less than -5 seconds (all data points prior to point A in figure S1). A fixed stabilization buffer of 5 data points (~ +5 seconds) after the start of ablation was used to define the beginning of sample collection (point B, beginning of green segment in figure S1).

The automated detection scheme searches for abrupt changes in intensity; to prevent the program from failing, very large outliers were removed. The sulfur signal between the 75^{th} and 95^{th} percentiles was used to calculate a mean and standard deviation. This quantile range corresponds to the part of the signal during active laser ablation. Individual sulfur excursions in excess of the mean + 7 sd were used to censor the entire row of data associated with that point.

The end time of active ablation was most reliably captured by changes in the rolling standard deviation of the ^{34}S signal with a 5 data point window. The maximum change in in the rolling standard deviation calculated on the subset of data that begins 20 entries after point B in the trace to the end of the collection was used to define the end-of-ablation time. A fixed time period of 7 data points (6 seconds) was used to capture the transition period between the end-of-ablation time (point C, end of green segment), and the return to background. The post-ablation period is all points after the end of ablation (red segment beginning at point D).

After the pre, post, and data collection periods were defined using the ^{34}S signal, we computed the mean and standard deviations of the ^{34}S and ^{55}Mn signals for each of those time periods. Additionally, the mean and standard deviation of the ratio of Mn to S was determined for each time period.

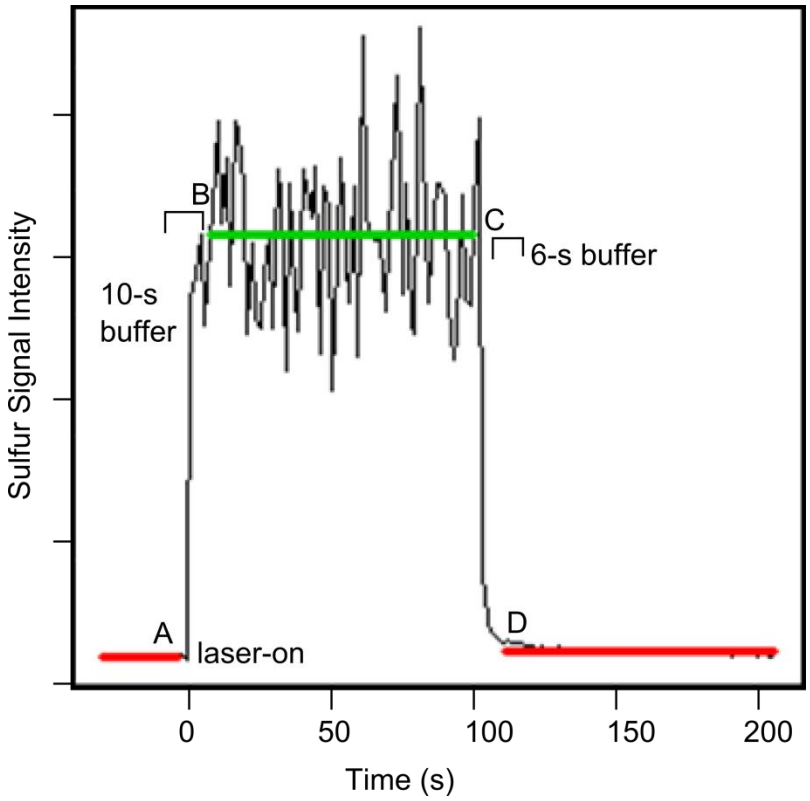

Figure S1: Schematic illustrating definition of pre, post and during ablation periods.

Using data from the compressed hair samples, we explored two approaches to using the ^{34}S signal as an internal standard to adjust the ^{55}Mn signal. The first approach was to divide the mean ^{55}Mn signal by the mean ^{34}S signal from the time series (i.e. <Mn>/<S>). The second approach was to divide each data point in the Mn time series by the corresponding sulfur signal, and then averaging (i.e. <Mn/S>). The latter method would reduce variability if the signal intensities within the time series are positively correlated, such as, if the laser did not efficiently ablate a portion of the hair due to challenges with mounting the strands.

For the <Mn>/<S>, the error associated with the individual <Mn> and <S> must be taken into account as follows:

$\frac{\widehat{\sigma_z}}{z} = \sqrt{\left(\frac{\widehat{\sigma_x}}{\bar{x}}\right)^2 + \left(\frac{\widehat{\sigma_y}}{\bar{y}}\right)^2}$, where $z = \bar{x}/\bar{y}$

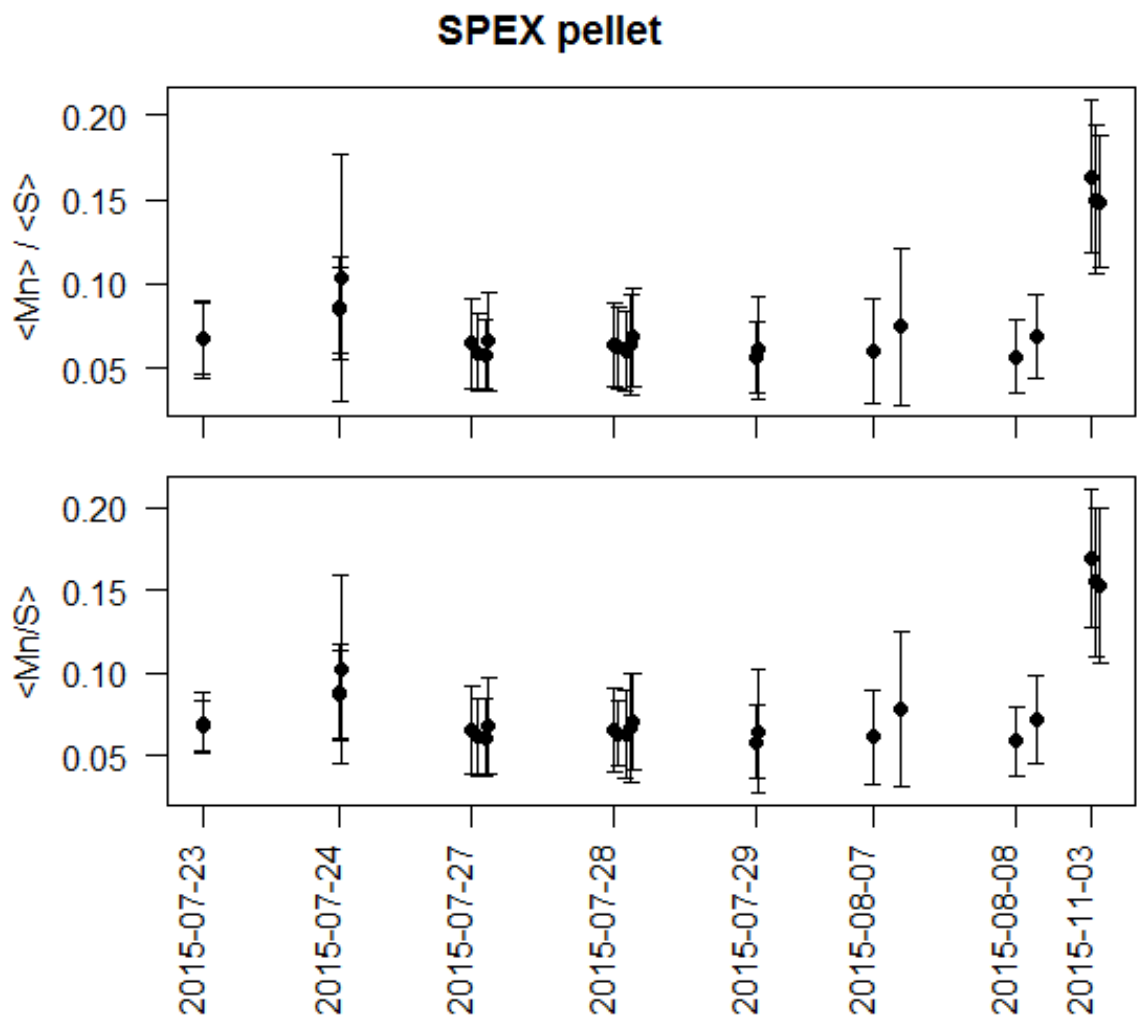

Figure S2: Comparison of two computational approaches for using ^{34}S as an internal standard to adjust ^{55}Mn response.

Figure S2 illustrates that the two approaches for using ^{34}S to adjust the ^{55}Mn signal yield almost identical results. To compare the within sample variability using the two normalization techniques we can compare the percent standard deviation of the means for each sample (Figure S3). If one method consistently out performs the other (has smaller error) then the data would be

primarily grouped on one side of a line with slope 1. Figure S3 confirms that the within sample variability is similar using both approaches.

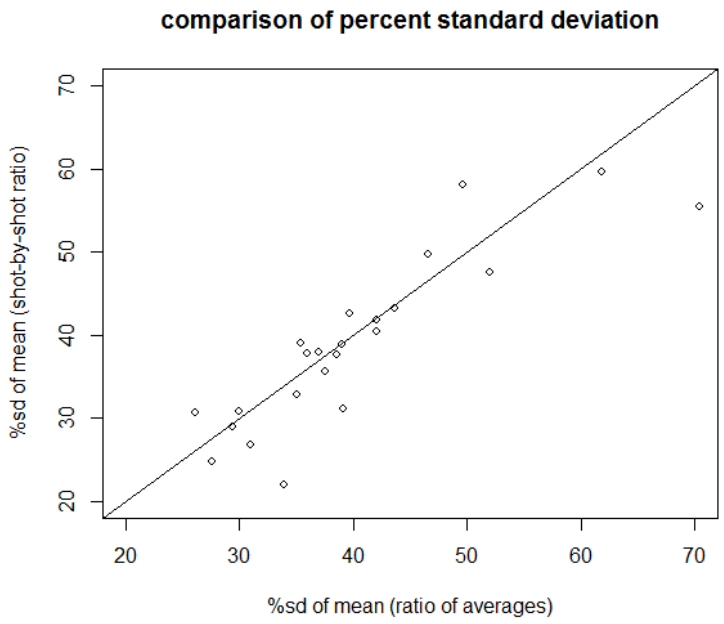

Figure S3: Comparison of coefficient of variation for two computational approaches to using ^{34}S as an internal standard to adjust ^{55}Mn response.

For adjusting the ^{55}Mn data in the subsequent analyses of the gelatin samples and single hair strands we elected to calculate the Mn/S ratio on a point-by-point basis.

VITA

Prior to his Ph.D. Boris Reiss earned an M.Sc. in (Atmospheric) Chemistry from York University, Toronto, Canada in 2000 and an undergraduate degree in Environmental and Radiation Safety Sciences in Karlsruhe, Germany in 1995. He has worked in different areas in industrial hygiene including research, academia, and industry. Boris has evaluated numerous hazards ranging from food sciences to oil and gas exploration. His projects ranged from one-person residential indoor air quality assessments in Canada to managing a large-scale retrospective exposure assessment study in China with 100 employees. He has conducted major projects in Canada, China, Germany, and The Netherlands, and shorter stints in Austria, Qatar, and United Arab Emirates. Boris Reiss research interests lie in exposure assessment. He is in particular interested in time resolved exposure assessment using biomarkers such as hair.