Elemental Analysis of Dried Blood Spots Using Total Reflection X-Ray Fluorescence Spectroscopy

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ABSTRACT

Introduction: Essential elements are required for the proper functioning of the human body and are critical to growth and development. Dried blood spots (DBS), capillary blood collected on specialized filter paper by pricking an individual's finger or heel, are a minimally invasive and cost-effective alternative to venipuncture for multi-element analysis. Nutritional assessments using DBS can help overcome logistical challenges faced by researchers, particularly in terms of data collection efforts in resource-limited regions and newborn screening programs. Although previous studies have used DBS for elemental measurements, technological and practical hurdles remain, such as detection limits and sample volume requirements. In addition, there is currently no widely accepted standard method of analysis.

Objectives: The primary objective of this research was to develop a method for quantifying select essential elements (copper, selenium and zinc) in DBS using Total Reflection X-Ray Fluorescence Spectroscopy (TXRF) and apply this method to a cohort of newborns from the Michigan BioTrust for Health Program.

Methods: The study was conducted in two phases. In Phase 1, method development and validation was established using human whole blood standard reference material (SRM) of known elemental concentrations from the Institut National de Santé Publique du Québec (INSPQ; n=7). The selected protocol on 3mm diameter DBS sub-samples was subsequently used in Phase 2 to analyze essential elements in sub-sampled human DBS from a cohort of newborns from the Michigan BioTrust for Health Project (n=675). Three of the SRMs were selected for inter-assay accuracy (n=38 batches) and precision analysis. The generalized extreme studentized deviate (ESD) test was applied to detect outliers in the cohort. Descriptive statistics, accuracy and precision measures were first calculated, followed by Student's *t*-tests and one-way analysis of variance (ANOVA) to measure inter-assay variation, variation in punch type and storage temperature. All analyses were conducted using JMP®Pro 13.0.0 and Microsoft Excel 15.4.

Results: Accuracy was measured by comparing TXRF DBS concentrations to corresponding whole blood SRM concentrations. Accuracy (mean±SD) for triplicate measurements of copper (Cu), selenium (Se), and zinc (Zn) using a 3mm diameter SRM DBS sub-sample were 99.5±3.4%, 100.2±6.9%, 102.6±4.4%, respectively. Accuracy of the selected SRMs (n=3) analyzed in the Michigan BioTrust Cohort for Cu was 107.8 ± 16.3%, 93.7 ± 14.2% for Se, and 109.0 ± 15.7%for Zn. The coefficient of variation, a measure of precision, of the SRMs across all batches was 13.3% for Cu, 17.3% for Se, and 13.7% for Zn. The Michigan BioTrust Cohort population values for Cu, Se, and Zn in the DBS were 1018.1 ± 269.9 μg/L, 241.0 ±58.2 μg/L, and 4250.0 ±1314.9 μg/L, respectively. High concentrations and high variability of Cu, 818.0 ± 1413.9 μg/L, and Zn, 2005.3 ± 1622.2 μg/L, were detected in the blank filter paper and are a barrier to quantitative analysis of DBS.

Implications: These results imply that elemental analysis of DBS using TXRF provide a semi-quantitative method for measuring select essential elements in sub-sampled DBS. The values obtained are for Cu and Se are comparable to human adult population reference values whereas the Zn values are lower, reflecting physiologically lower Zn concentrations in newborns. Given present limitations in the quantitative analysis of DBS, this method has the potential to be used as a screening tool in the evaluation of essential elements in nutritional research, overcoming many of the challenges associated with venipuncture and current analytical methods–potentially leading to informed health policies and interventions.

RÉSUMÉ

Introduction: Les éléments essentiels sont exigés pour le bon fonctionnement du corps humain. Les taches de sang séchées (DBS), soit du sang capillaire sur du papier filtre spécialisé, sont obtenues en piquant le doigt ou le talon d'un individu. Cette technique est une alternative à la fois peu envahissante et rentable au prélèvement sanguin pour l'analyse multiéléments. Des évaluations nutritionnelles utilisant DBS peuvent aider à surmonter les défis logistiques auxquelles sont confrontés les chercheurs, notamment en termes d'efforts requis pour la collecte de données dans les régions à moyen limitées et les programmes de dépistage pour les nouveau-nés. Bien que des études précédentes aient utilisé DBS pour des mesures d'éléments, des obstacles technologiques et pratiques tels que des limites de détection et d'échantillonnent persistent. Il n'y a présentement pas de méthode standard d'analyse acceptée universellement.

Objectifs: L'objectif est de développer une méthode pour quantifier certains éléments essentiels (le cuivre, le sélénium et le zinc) qu'on retrouve dans les DBS en utilisant la Spectroscopie à Fluorescence X à Réflexion Totale (TXRF) et de l'appliquer sur les DBS d'une cohorte de nouveau-nés du Michigan BioTrust.

Méthodes: Dans la phase 1, le développement et la validation de la méthode ont été établis en utilisant la norme de sang entier (SRM) dont la concentration des éléments est connues par l'Institut National de Santé Publique du Québec (INSPQ; n=7). Dans la phase 2, le protocole choisi dans la phase 1 soit, le protocole avec les DBS d'un diamètre de 3mm, a été utilise sur des échantillons DBS d'une cohorte de nouveau-nés du Michigan BioTrust (n=675) afin d'analyser la concentration des éléments essentiels. Trois des SRMs ont été choisis pour mesurer l'exactitude entre les lots (n=38 lots) et la précision. Le test « generalized extreme studentized deviate (ESD) » a été appliqué pour détecter des valeurs atypiques dans la cohorte. Des statistiques descriptives suivi par le *t*-test de l'étudiant et une analyse de variance a un facteur. Toutes les analyses ont été effectués en utilisant JMP ® Pro 13.0.0 et Microsoft Excel 15.4.

Résultats: L'exactitude a été mesurée en comparant les résultats du niveaux de concentration du TXRF DBS au concentration du sang entier correspondant SRM. L'exactitude (moyenne ± écart type) pour les mesures de cuivre (Cu), sélénium (Se) et zinc (Zn) en utilisant un SRM DBS de 3mm de diamètre était de 99.5±3.4 %, 100.2±6.9 %, 102.6±4.4 %, respectivement. L'exactitude des SRMs sélectionnés (n=3) analysés au "Michigan BioTrust Cohorte" était de 107.8 ± 16.3% pour le Cu, 93.7 ± 14.2% pour le Se, and 109.0 ± 15.7% pour le Zn. Les coefficients de variation, une mesure de précision du SRM à travers tous les lots, était de 13.3 % pour le Cu, 17.3 % pour le Se et 13.7 % pour le Zn. Les résultats pour la population de « Michigan BioTrust Cohorte » pour le Cu, Se et Zn dans les DBS était de 1018.1 ± 269.9 μg/L, 241.0 ±58.2 μg/L et 4250.0 ±1314.9 μg/L, respectivement. Des hautes concentrations et des grandes variabilités de Cu (818.0 ± 1413.9 μ G/L) et de Zn (2005.3 ± 1622.2 μg/L) qui ont été détecté dans les papier filtres sont des entraves a une analyses quantitative complète des DBS.

Retombées: Ces résultats indiquent que l'analyse d'éléments pour les DBS en utilisant TXRF offre une approche semi-quantitative pour mesurer la concentration d'éléments essentiels. Les résultats obtenue pour le Cu and Se sont comparable aux résultats établis, alors que les résultats pour le Zn sont inférieurs aux résultats établis pour les adultes, ce qui reflètent les concentrations de Zn physiologiquement plus faible chez les nouveau-nés. Cette méthode peut être utilisée comme outil de sélection dans l'évaluation des éléments essentiels et permet de remédier à de nombreux défis associés au prélèvement sanguin.

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PREFACE AND CONTRIBUTION OF AUTHORS

This thesis is comprised of one manuscript (Chapter 3), which has been prepared for submission to an academic journal. As the author of this thesis, I was responsible for the development of objectives and hypothesis, literature review, lab work, data management, statistical analysis, interpretation of findings and writing. Dr. Niladri Basu provided guidance, feedback and overall support throughout the thesis.

Manuscript 1. Development and Application of a Novel Method to Measure Essential Elements in Newborn Dried Blood Spots from the Michigan BioTrust for Health
Authors: Jessica Pawly, Niladri Basu

I developed the study design, methods and rationale for the study in collaboration with my supervisor, Dr. Niladri Basu. Samples were analyzed in the lab with the support of Lisa Bidinosti. I conducted the statistical analysis and wrote the first full draft of the manuscript with editorial contributions from Dr. Niladri Basu.

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LIST OF ABBREVIATIONS

AAS – Atomic Absorption Spectroscopy
AI – Adequate Intake
DBS – Dried Blood Spot
ESD – Extreme Studentized Deviate Statistical Test
EDXRF – Energy Dispersive X-ray Fluorescence Spectrometry
FAAS – Flame Atomic Absorption Spectroscopy
GFAAS – Graphite Furnace Atomic Absorption Spectroscopy
ICP-AES – Inductively Coupled Plasma Mass Spectroscopy
ICP-MS – Inductively Coupled Plasma Mass Spectrometry
IS – Internal Standard
LA-ICP-MS – Laster Ablation Inductively Coupled Plasma Mass Spectrometry
LIC – Low-Income Country
RDA – Recommended Dietary Allowance
SRM – Standard Reference Material
SF-ICP-MS – Sector-Field Inductively Coupled Plasma-Mass Spectrometry
USAID – United States Agency for International Development

Relevant Elements
As – Arsenic
Cl – Chlorine
Co – Cobalt
Cr – Chromium
Cu – Copper
Fe – Iron
Hg - Mercury
K – Potassium
MeHg – Methylmercury
Mn – Manganese
Mo – Molybdenum
Pb – Lead
P – Phosphorus
S – Sulfur

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CHAPTER 1. INTRODUCTION

1.1 Introduction

Essential trace elements are required for the proper functioning of the human body and quantification of these elements is critical for nutritional research and public health interventions. The development of minimally invasive sample collection methods for elemental analysis, one indicator of nutritional status, facilitates data collection efforts (Boerma et al., 2001; Langer et al., 2010; Mcdade et al., 2007; Mei et al., 2001). This is critical among vulnerable populations, such as infants and the elderly, and resource-limited areas that have inadequate laboratory infrastructure and lack trained professionals (Boerma et al., 2001; Mcdade et al., 2007).

Whole blood is frequently collected as a biological sample for elemental analysis; however, whole blood collection by venipuncture is costly, invasive, and requires trained professionals (Mcdade et al., 2007; Mei et al., 2001). Dried blood spots (DBS)—drops of whole blood collected on specialized filter paper from a simple finger or heel prick—are a minimally invasive alternative for obtaining human biological samples. DBS have several advantages over venipuncture, such as relative ease of sample collection and significantly lower cost of collection, storage and transport (Mcdade et al., 2007).

Despite the benefits of DBS for elemental analysis, there are gaps in our present knowledge and limitations to current analytical methods and technology. Current DBS analysis requires equipment with low detection limits, low sample volume requirements, simultaneous multi-element detection, and extensive sample preparation (Mcdade et al., 2007). In addition, the sample volumes of DBS and the effects of temperature and storage methods on elemental measurements are currently unknown. Furthermore, the elemental contamination of filter paper is a barrier to fully quantitative analysis (Hannon et al., 2003; Langer et al., 2010; Marguí et al., 2017; Mei et al., 2001). Total Reflection X-Ray Fluorescence (TXRF) Spectroscopy, relatively new in the field of elemental analysis, is an instrument capable of overcoming some of these obstacles. TXRF has the ability to detect multiple elements with a lower cost of analysis, simpler sample preparation and lower sample volume requirement as compared to traditional instrumentation, such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Validation of TXRF spectroscopy as an accurate and precise method for DBS multi-element analysis could improve biomonitoring programs and research efforts in vulnerable populations—leading to informed nutrition interventions and programs.

1.2 Research Objectives

The ***main objective*** of this research project was to develop and validate a method of quantifying essential elements in DBS using TXRF spectroscopy. The essential elements analyzed in this thesis are copper (Cu), selenium (Se), and zinc (Zn). The ***overall hypothesis*** is that TXRF spectroscopy measurements of the selected essential elements in DBS will accurately reflect concentrations measured in the whole blood sample, expanding the applicability of DBS as an effective tool for data collection and research. This objective was achieved with the following specific aims:

1. **Development of a novel method to measure essential elements in DBS.** The method was developed using adult human blood standard reference material (SRM, n=7) of known multi-element concentrations from the *Institut National de Santé Publique du Québec* (INSPQ) and evaluated for accuracy and precision.
2. **Application of the method to a cohort of newborns**. The method was used to analyze samples from a cohort of newborns from the Michigan BioTrust for Health Program in the State of Michigan (n=675).

The outcome of this research was the development of a novel method for the simultaneous multi-element analysis of DBS using TXRF spectroscopy. These results contribute to a body of literature investigating the potential to use of DBS as a cost-effective and minimally invasive method for evaluating nutritional status with direct implications for vulnerable populations and resource-limited regions.

1.3 Thesis Overview

This thesis is comprised of five chapters, beginning with an introduction in Chapter 1 and a literature review exploring the benefits and challenges of DBS in Chapter 2. This thesis follows the manuscript-based format, in which the research objectives in Chapter 3 are organized in a manuscript. Manuscipt 1 in Chapter 3 is followed with a broader discussion of the limitations and future directions of the research in Chapter 4 and concluding remarks in Chapter 5.

CHAPTER 2. LITERATURE REVIEW

2.1 Biomarkers in Human Nutrition

Trace elements can generally be divided into essential trace elements and non-essential trace elements. Non-essential trace elements, such as lead (Pb), mercury (Hg), arsenic (As) and cadmium (Cd), are detrimental to human health and can lead to negative health effects. On the other hand, essential elements, such as copper (Cu), cobalt, (Co), chromium (Cr), iron (Fe), manganese (Mn), molybdenum (Mo), selenium (Se), and zinc (Zn), are required for the proper functioning of the human body and have a major role in metabolic activities (Chasapis et al., 2012; Holben & Smith, 1999; Stern, 2010; Vacchina et al., 2014). The dose-response relationship for essential vitamin and mineral nutrients has been shown to be a U-shaped response (Hayes, 2008). In summary, there is a region of homeostasis between thresholds of adverse effects from both deficiency and toxicity (refer to Figure 1 in Appendix) (Hayes, 2008). Thus, quantification of these essential elements is vital in order to detect individuals experiencing either under-nutrition or are at risk for toxicity prior to clinical symptoms as part of program evaluations and interventions in nutrition.

Traditionally, scientists in the field of nutrition rely on health data derived from self-reports or clinical records. These self-reported assessments methods are considered minimally invasive and have been validated in specific populations. However, they can still be costly, time-consuming and difficult in low-income or low-literacy populations (Mcdade et al., 2007). In addition, self-reported data rely on subjective, conscious experiences and are prone to recall bias (Mcdade et al., 2007). Language barriers, politics and cultural factors that define health, for example, contribute to variations in reporting health data. Thus, assessment tools should be validated in a specific context prior to use, limiting the extent to which they can be applied (Podsakoff et al., 2003). Biomarkers, on the other hand, are not susceptible to many of the shortcomings of self-reported health measures and represent more objective indicators of health.

Biomarkers are defined as measurable indicators of an exposure, effect, susceptibility or clinical disease that is observed from outside the participant and can be measured accurately and objectively (Mcdade et al., 2007). Biomarkers do not rely on a participant's ability to recall health information and can assess subclinical physiological processes (Mcdade et al., 2007). They are integral to research programs and are particularly advantageous for health data collection in developing countries, cross-cultural settings, and research with vulnerable

populations, including newborns and children (Boerma et al., 2001). In the field of nutrition, they are critical to identifying populations at risk of deficiencies prior to clinical symptoms and understanding biological pathways through which social, nutritional and environmental factors shape health (Crimmins & Seeman, 2001; Mcdade et al., 2007). Large national surveys, such as the National Health and Nutrition Examination Survey (NHANES) in the United States or the Canadian Health Measures Survey (CHMS) in Canada, use biomarkers to demonstrate population distribution of health and disease and identify trends in overall population health. Epidemiological cohorts provide an in-depth examination of these trends at the individual, household and community-level (Mcdade et al., 2007). The availability of objective data from biomarkers is critical and effective in informing regulations, assessing public health interventions and mobilizing policy makers (Graham, 2010).

Despite the many benefits, biomarkers can be costly to analyze and have both logistical challenges and ethical considerations. There are costs to sample collection, transport, storage, and analysis. Protocols and safety measures are required to maintain sample integrity as well as protect interviewers and personnel handling biospecimens from potential risks of infection. The collection of biological samples may impose additional risks to research participation, potentially affecting recruitment, retention and study design (Mcdade et al., 2007). The risk to participants varies by method of sample collection and the implications of biospecimen collection need to be considered in specific contexts. For example, venipuncture is invasive and challenging for certain populations, such as newborns, pregnant women, malnourished individuals and the elderly.

The challenges associated with biospecimen collection is driving a demand for alternative methods to measure biomarkers. The development of minimally invasive methods for sample collection addresses some of the logistical and ethical challenges, facilitating the integration of biomarkers into nutritional research in addition to other fields. This is particularly important for research in vulnerable populations, such as with newborns, and in developing countries, where low rates of health service use, inadequate laboratory infrastructure and logistical obstacles associated with sample collection and transport are critical barriers to health surveillance efforts (Boerma et al., 2001).

2.2 Essential Elements

Meeting nutritional needs is critical to early childhood development and overall health in all stages of life. Essential elements, in particular, are crucial for human biological activity. Essential trace elements are elements required in small quantities for physiological function, include copper (Cu), cobalt, (Co), chromium (Cr), iron (Fe), manganese (Mn), molybdenum (Mo), selenium (Se), and zinc (Zn). These trace elements, also known as micronutrients or microminerals, are of particular concern for low-income countries (LIC), where there are not many available sources of nutrients in their diet (Eicher-Miller et al., 2009). Lower-income regions are often associated with food insecurity and a decreased intake of calcium, iron and other essential nutrients (Eicher-Miller et al., 2009). In middle- and higher-income countries, there is significant variability at a neighborhood level. Children in low-income urban areas have been shown to have poorer nutritional status than children in more affluent suburban communities (Fahlman et al., 2010). Deficiencies and exposure are also critical to fetal development, with certain developmental stages disproportionally impacted by nutritional deficiencies and toxicities. Embryo development is a critical stage of growth that is prone to teratogenesis, defined as the production of birth defects. Classes of teratogens, agents that cause malformation of an embryo, include both nutrient deficiencies and excesses, as well as exposure to environmental agents (Otten, Hellwig, & Meyers, 2006).

The essential elements copper (Cu), selenium (Se), and zinc (Zn) will be the focus of this research due to their importance for physiological function, need to measure effectively at a population and global level, as well as feasibility within the scope of this study and analytical instrument. As explored further throughout Chapter 2, there are existing analytical challenges to elemental analysis of dried blood spots, particularly with trace elements at low concentrations in limited sampe quantity, and the instrument detection limit is a key consideration when selecting elements. This research project is a preliminary investigation and development of a method for elemental analysis of total concentration of select elements, which can be expanded to include additional elements in future studies. There is currently sparse data for indicators of nutrient adequacy in infants and there is insufficient scientific data from observed and experimental studies to set Recommended Dietary Allowances (RDA) for micronutrients for infants (Otten et al., 2006). Thus, adequate intake (AI) levels are proposed for infants to age 1 and there remains a need to effectively measure deficiencies and toxicity in this age-group (Otten et al., 2006).

2.2.1 Copper (Cu)

Copper (Cu) has been recognized as an essential element for many years due to its role in enzymes and proteins (Bazzi, Nriagu, & Linder, 2008; Institute of Medicine, 2001). Cu is a trace element involved in the function of several cuproenzymes that are essential for life and are critical during pregnancy and infant growth (Bermúdez et al., 2015; Institute of Medicine, 2001; Iyengar & Woittiez, 1988). It works to catalyze the activity of many copper metalloenzymes that act as oxidases to achieve the reduction of molecular oxygen (Institute of Medicine, 2001). It is necessary for the proper development of cognitive tissue, myelin and melanin. Pregnant women are at risk of copper toxicity, which has been shown to result in growth retardation in rat fetuses and may have other negative effects on developmental outcomes (Bermúdez et al., 2015). A negative association has been observed between Cu concentrations in the umbilical cord and birth weight of human newborns (Bermúdez et al., 2015).

Although Cu deficiency in humans is rare, deficiency symptoms include normocytic and hypochromic anemia, leukopenia and neutropenia (Institute of Medicine, 2001). A deficiency in Cu could lead to defects in connective tissue that lead to vascular and skeletal problems, specifically aspects of central nervous system dysfunction (Institute of Medicine, 2001; Turnlund et al., 1998). Genetic inborn errors of Cu metabolism, also known as Menkes kinky hair syndrome, can lead to major developmental abnormalities in the brain, bones and blood vessels (Institute of Medicine, 2001). Cu deficiency has also been observed in premature infants fed milk formulas, infants recovering from malnutrition and patients with prolonged total parenteral nutrition (Institute of Medicine, 2001). The AI for infants 0 to 6 months is based on the usual intake from human milk. The extent of copper absorption varies with dietary copper intake and nearly 2/3 of body copper content is located in skeleton and muscle. Cu homeostasis is affected by the interaction with Zn, Fe and Mo and the concentrations of Cu and Zn are known to correlate (Grandjean et al., 1992; Institute of Medicine, 2001). The liver has been shown to be a site for maintaining plasma Cu concentrations in studies with stable isotopes (Olivares & Uauy, 1996; Turnlund et al., 1998). As a result, liver Cu stores are high and serum Cu concentrations are low in newborn infants (Salmenpera et al., 1986). Indicators to measure Cu include plasma Cu and ceruloplasmin concentrations, erythrocyte superoxide dismutase activity, and platelet Cu concentrations in controlled human depletion or repletion studies (Institute of Medicine, 2001).

However, these methods are expensive and require venipuncture to collect biological samples, raising the costs of sample processing and analysis in addition to the ethical challenges.

2.2.2 Selenium (Se)

Selenium (Se) is an antioxidant and is critical for enzyme function (Institute of Medicine, 2000; Iyengar & Woittiez, 1988). Selenium is an essential element that primarily exists in two forms: inorganic and organic (selenomethionine and selenocysteine) (Risher et al., 2003). Most Se found in animal tissue is in the form of selenomethionine, the major dietary form, or selenocysteine. Both of these forms are well absorbed. Se has constitutive roles in antioxidant enzymes and thyroid hormone biosynthesis (Institute of Medicine, 2000; Risher et al., 2003). Several neurologic, reproductive, cardiovascular diseases and cancers have been associated with impaired selenium homeostasis (Rayman, 2000; Risher et al., 2003). Selenium is also needed for the proper functioning of the immune system and may reduce miscarriages in pregnant women (Rayman, 2000). Furthermore, Se is associated with a reduction of the toxicity of methylmercury (MeHg) due to strong binding between Se and MeHg (Dyrssen & Wedborg, 1991). This binding can create insoluble and non-toxic complexes in joint exposure and deposit in tissue, thus protecting against MeHg's toxicity (Ceccatelly & Aschner, 2012). Se is highly regulated in the body because of its incorporation in selenoproteins. However, Se deficiency may lead to biochemical changes that can predispose a person to illnesses associated with other stressors, such as Keshan disease and Kashin-Beck disease (Institute of Medicine, 2000). People in countries where the diet consists of vegetables grown in low-Se areas are at risk of deficiency (Institute of Medicine, 2000). On the other end, reported symptoms of selenosis, the chronic toxicity of Se, are hair and nail brittleness (Institute of Medicine, 2000). Variations observed in Se concentrations of whole blood in different populations reflect geographic differences (Iyengar & Woittiez, 1988).

2.2.3 Zinc (Zn)

Zinc (Zn) is associated with catalytic activity of more than 200 enzymes and regulatory proteins, including many transcription factors. It functions through the catalysis of various enzymes, the maintenance of structural integrity of proteins, and the regulation of gene expression and cellular growth (Black, 1998; Hambidge, 2000; Institute of Medicine, 2001). It is

crucial for proper growth and development, facilitating several enzymatic processes related to the metabolism of protein, carbohydrates and fats (Institute of Medicine, 2001). It provides a structural function for some enzymes, the most notable of which is copper-zinc superoxide dismutase (Institute of Medicine, 2001). The majority of the body's total Zn is stored in skeletal muscle and bone and only 0.1% of the total body Zn is found in the plasma (Institute of Medicine, 2001). The body tightly regulates plasma Zn concentrations, keeping them steady at about 10-15 μmol/L (Institute of Medicine, 2001). This tight regulation means that small amounts of Zn are more efficiently absorbed and people with poor Zn status can absorb the nutrient more efficiently (Institute of Medicine, 2001). Thus, Zn deficiency is rare and the symptoms of mild Zn deficiency are diverse and likely impaired in all tissues due to its extensive involvement in metabolic processes (Hambidge, 2000). However, measuements of zinc concentratiosn in plasma lacks the sensitivity to be a strong biomarker of zinc status and there is a lack of confirmation and application of proposed alternative biomarkers (Hambidge, 2000). The lack of adequate biomarkers and attributable clinical features to zinc deficiency has delayed our understanding of the effects of zinc deficiency and toxicity (Hambidge, 2000).

Impaired growth rate is the primary clinical feature of zinc deficiency and can be corrected with Zn supplementation (Hambidge, 2000; Institute of Medicine, 2001). There is a demonstrated increase in growth velocity associated with dietary zinc supplements, primarily in low-income countries (Hambidge, 2000). In addition, individuals with malabsorption syndromes, including sprue, Crohn's disease, and short bowel syndrome are at risk of Zn deficiency, among other nutrient deficiencies, due to malabsorption of Zn and increased urinary Zn losses (Institute of Medicine, 2001). Zinc supplementation is also effective in reducing the duration and severity of acute and persistent diarrhea (Hambidge, 2000). Other factors such as stress, fasting, medication or pregnancy also influence serum Zn values (Iyengar & Woittiez, 1988). A review by Black (1998) discusses the association between zinc deficiency with deficitsy in activity, attention and motor development common among nutritionally deficient children. Children are particularly vulnerable to deficiencies during periods of rapid growth and development, such as infancy and adolescence (Black, 1998). On the other hand, adverse effects associated with chronic intake of supplemental Zn include suppression of the immune system, decrease in high density lipoprotein cholesterol and reduced Cu status (Institute of Medicine, 2001).

2.2.4 Reference Values in Newborns

Among other indicators of health and nutrition, reference values allow investigators to interpret results generated in clinical laboratories and compare them to values in a normal, healthy population. Reference values reflect the findings in a well-defined group of individuals and are expected to reflect population concentrations, although age, sex, environment and diet influence the concentrations of certain trace elements (Iyengar & Woittiez, 1988). These reference values are essential to understand and compare individual or population values, with implications in public health programs and identification of at-risk communities. Despite extensive research in nutrition, data is sparse for indicators of nutrient adequacy in infants and toddlers due to the difficulty of data collection and ethical concerns.

Reference values have been proposed for the total concentrations of Cu, Se, and Zn in the whole blood of adults by Iyengar & Woittiez (1998). The review paper compiled data from the literature to determine baseline values for adults. As discussed in Iyengar & Woittiez (1998), many of the essential elements, including Cu and Zn, are homeostatically controlled and follow a normal distribution in the various biological samples such as whole blood, serum, and hair (Iyengar & Woittiez, 1988). On the other hand, Se concentrations are influenced by dietary availability and, thus, demonstrate a wider distribution. Although the publication is relatively old, the instruments used to analyze the various biological specimen are comparable to current technology and the report compiles data from a substantial number of publications, reflecting a wide population and external validation. In addition, it reports reference values for elements in whole blood whereas more recent research focuses on concentrations of essential elements in plasma or serum. The article also reports on total elemental concentrations and not speciation of elements, providing a framework for comparison. Thus, the baseline values reported are the most relevant to the analysis conducted in this research project. However, one critical limitation is the reference values are from an adult population and this research is conducted in a newborn cohort.

Krachler et al. (1999), alternatively, measured and compared trace element concentrations in the serum of umbilical cords (n=20), young infants (n=5) and adults (n=20) using ICP-MS. They found that serum Cu was lower in early infancy and increased significantly with age. However, they could not establish age-dependence for the other trace elements (Krachler, Rossipal, & Micetic-Turk, 1999). Similarly, Galinier et al. (2005) aimed to establish reference ranges for essential elements and other micronutrients in a larger cohort of infants

(n=510). All selected infants had appropriate birth weight for gestational age and the objective of the study was to establish reference ranges according to gestational age (Galinier et al., 2005). However, elements in both of these studies were measured in the serum of the umbilical cord of infants and cannot be compared to whole blood concentrations. One study by Custodio et al. (2005) measured essential elements directly in maternal blood samples following delivery and newborn cord blood samples using Energy Dispersive X-ray Fluorescence Spectrometry (EDXRF). Cu concentrations ranged from 3 to 13 μg/L for both the mother and newborn and Zn concentrations ranged between 10 and 40 μg/L (Custódio et al., 2005). Custodio et al. (2005) found Cu and Zn values were higher in maternal samples than umbilical cord whole blood. However, this study did not measure Se and samples a small population. Furthermore, the results reported in this study are divided into groups based on maternal age, newborn weight, or gestational age and overall values for the population are not reported as the objective of the research was to determine elemental correlations between maternal and newborn blood at birth.

To my knowledge, there is no alternative article equivalent to the population scope of the Iyengar & Woittiez (1998) review or determines baseline values of essential elements in the whole blood of newborns. This makes it challenging to compare elemental concentrations across different studies. However, minimally invasive sample collection techniques can facilitate future research in the newborn population and expand the current knowledge of baseline values in addition to working towards determining nutrient requirements and effective interventions.

2.3 Whole Blood

The collection and analysis of biological specimen can add an objective component to research in nutrition and public health. However, logistical constraints remain an obstacle to incorporating biomarkers into research and may impose unnecessary risks to research participants. Conventional methods of elemental analysis involve collecting whole blood via venipuncture, but this is invasive and ethically challenging, especially with regards to the health status and age of an individual. Collecting whole blood via venipuncture is more difficult in vulnerable populations, such as infants, pregnant women and the elderly as it may be difficult to collect several milliliters of blood from an individual, hindering analytical assays of interest (Vacchina et al., 2014). Furthermore, in resource-limited communities, whole blood collection is

challenging due to a need for specialized collection supplies, clinical settings, cold-chain requirements and trained personnel (Garrett et al., 2011). The collection of venous blood frequently requires processing prior to analysis in a designated laboratory; liquid blood samples must first be anti-coagulated with appropriate reagents after collection and stored at low temperatures (Garrett et al., 2011). Additional steps, such as centrifugation, are required to process the whole blood to serum or plasma and requires added equipment. A "cold-chain" needs to be maintained from the point of collection until analysis of the samples, increasing requirements for transportation and storage (Garrett et al., 2011; Mcdade et al., 2007). Altogether, this adds substantial costs and barriers to analysis of blood biospecimens.

The logistical constraints of collecting and analyzing whole blood, particularly in resource-limited regions, are a barrier to incorporating biological data in research and warrant the need to explore alternative methods (Garrett et al., 2011; Mcdade et al., 2007). Finger-prick blood collection methods, such as dried blood spots, have gained interest in nutrition and other fields as a cost-effective alternative to venipuncture.

2.4 Dried Blood Spots

Dried blood spots (DBS) provide a minimally invasive and low-cost method for collecting blood samples in both clinical and nonclinical settings, overcoming many constraints and expanding the integration of biomarker data into research. There are also many ethical, practical and economical advantages to using DBS in health research. DBS are capillary blood collected on specialized filter paper by pricking an individual's finger or heel. The DBS are first allowed to dry, then they are stacked, stored and shipped for analysis or storage. The ease of sample collection, transport and storage make DBS a cost-effective alternative to venipuncture for use in research and clinical applications. Many new tests, including those for human immunodeficiency virus (HIV), micronutrients, and infectious diseases, are increasingly being validated for use with DBS and the application of tests using DBS has revolutionized the capacity to obtain biomarkers in field settings (Garrett et al., 2011).

DBS have become an appealing alternative for classical venous blood sampling. The sample collection is relatively non-invasive and requires minimal equipment. Thus, it can be conducted in a participant's home by individuals who are not medically trained and, in some

cases, the participants themselves. In addition, DBS do not need a cold chain and refrigeration of specimen in the field, reducing the complexity of storage in remote areas and transport to the laboratory (Garrett et al., 2011). Depending on the analyte of interest, they remain stable for long periods of time and can be re-analyzed as new biomarkers and analytical methods emerge (Mcdade et al., 2007). The whole blood does not need to be centrifuged, separated or frozen after collection. As a result, DBS are particularly useful in resource-limited areas with logistical obstacles associated with staffing, sample collection and transport (Boerma et al., 2001; Garrett et al., 2011). Furthermore, the small volume of sample and method of collection are a relatively low burden on participants, which is particularly advantageous for work with infants, children and the elderly where venipuncture can be difficult (Mcdade et al., 2007). Frequently, a single finger-prick can provide capillary whole blood for spots on filter paper and also other portable point-of-care instruments, providing key results on-site for participants. The primary advantage of this method is access to physiological information that would otherwise not be attainable in a non-clinical setting.

The ability to archive samples is another key advantage to DBS sample collection as the filter paper has been shown to have a stabilizing effect on multiple analytes (Bowen et al., 2010; Stove et al., 2012). They offer the possibility of preserving small amounts of sample in an economical way (Stove et al., 2012). Analysis of stored samples following newborn screening analytical tests have been proposed as a novel source of biological specimen, opening new possibilities in human health research (Di Martino et al., 2004; Mcdade et al., 2007).

Despite the many advantages, there are challenges in the analysis of DBS, particularly with the quantification of select analytes. As the majority of standard laboratory protocols require whole blood or serum, analytical assays need to be developed specifically for DBS and validated for accuracy, precision, reliability and limits of detection (Mcdade et al., 2007). Currently, standard clinical assays are performed on automated and high-throughput analyzers designed for serum or plasma samples. The standard equipment cannot currently accommodate DBS analysis and the lack of high-throughput analyzers is a barrier to widespread use of DBS. Few labs have direct experience with DBS analytical methods (Mcdade et al., 2007). Furthermore, quantification of certain analytes may not be possible due to small sample volume, contamination of filter paper or potential instability of the analyte in the filter paper. The small quantity of sample may remain an insurmountable limitation for select analytes and equipment.

The results from DBS samples also represent concentrations in whole blood and may not be directly comparable with those derived from serum, plasma or other clinically relevant results (Mcdade et al., 2007).

The filter paper is manufactured from high purity cotton linters and has been used to collect blood for public health interventions for over 40 years (Mei et al., 2001). The pre-printed circles on the filter paper provide a gauge for the volume of blood applied to a blood spot in routine collection (Mei et al., 2001). It is manufactured to give accurate and reproducible absorption of blood according to National Committee on Clinical Laboratory Standards (NCCLS) specifications (Hannon et al., 2003). Despite standardization of the filter paper, there are several parameters that affect the volume of a sample within a pre-printed circle. Thus, punching of a fixed-size DBS disk is a critical step in the analysis of DBS as it provides a simple and consistent quantitative sampling technique. The volume of a sample depends on quantity of blood in the spot, the size of the spot (i.e the punch diameter) and the distribution of the sample across the spot (Wong & James, 2014). Factors that affect the volume of a punch include hematocrit level, paper batch-to-batch differences, volume of blood added to paper and location of punch (Chaudhuri et al., 2009; Mei et al., 2001; O'Mara et al., 2011). Increasing the volume of blood applied to the filter paper will increase the amount of blood contained within the punch (Mei et al., 2001). O'Mara et al. (2011) has shown that there may be significant differences in analyte distribution between the perimeter and the center of the blood spot, stating that analyte concentration in perimeter punches was greater than center punches (O'Mara et al., 2011). Perkins & Basu (2018) have shown that total mercury concentrations of punches removed from the edge of the DBS were significantly higher than punches from the interior (Perkins & Basu, 2018). However, it remains unclear if this is representative of all analytes in the card or just the analytes studied. With regards to multi-element analysis, elements bound to proteins compared to those bound to red blood cells may interact with the filter paper differently (Cizdziel, 2007). Although the influence of punch position needs to be tested for specific analytes, the effect has been shown to be minimized by consistently sampling blood from the center or periphery of the DBS (Wong & James, 2014). The difference in card lot could also cause some variability in analyte recovery and lot-to-lot variability of DBS cards should be considered when validating methods (Chaudhuri et al., 2009; Wong & James, 2014). Another challenge is sample volume as punching a sub-sample results in a small volume of the total sample of analysis, which can limit

assay sensitivity. A larger diameter of blood spot, using multiple spots or concentrating the final extract may increase the sensitivity and overcome this analytical challenge but may affect other benefits of DBS.

Some argue that the hematocrit effect is the most prominent limitation as variation in hematocrit affects the viscosity of whole blood and, thus, the spread of the blood on the filter paper and subsequent analysis (Mei et al., 2001; Wong & James, 2014). Hematocrit is the percentage of blood cells in whole blood by volume and ranges from 41% to 50% in male adults and 36% to 44% in female adults (Wong & James, 2014). For infants under two years of age, hematocrit has greater variability and ranges from 28% to 55% (Wong & James, 2014). Furthermore, the hematocrit level of a disease population may differ from a healthy population and may be altered by specific drug treatments (Wong & James, 2014). The volume of whole blood per spot is proportionally related to the hematocrit of the blood as blood samples with higher hematocrit levels are more viscous and have a smaller spot diameter as a result (Adam et al., 2000). These parameters could lead to inaccuracy in the quantification of analytes in a given sample. To overcome the effects of variable hematocrit, the entire DBS can be sampled for analysis. However, the total sample volume remains unknown as the volume of a drop of blood varies by individual and this method may not be feasible in certain applications, such as analysis of residual newborn screening samples.

An additional obstacle to elemental quantification of DBS is the potential contamination and variability of the contamination in the filter paper, requiring adequate control of blank filter paper and quality assurance measures. This may be adjusted by analyzing multiple replicates, although this is may not be possible given the small sample volumes and analysis of residual samples (Cizdziel, 2007, Chaudhuri et al., 2009, Resano et al., 2007, Stove et al., 2012). Furthermore, the variability in the contamination of the filter paper poses concerns of the feasibility of quantification (Chaudhuri et al., 2009; Marguí et al., 2017).

2.5 Current Use of Dried Blood Spots

DBS were first used in early 1960s when Dr. Robert Guthrie began collecting heel-prick blood spot samples from newborns to measure phenylalanine for the detection of phenylketonuria (Guthrie & Susi, 1963). This novel application led to the population screening

of newborns for the detection of inherited metabolic diseases and other disorders, with samples currently collected from >98% of newborns in the United States (Mei et al., 2001; Olshan, 2007; Stove et al., 2012). Newborn screening programs are now being implemented across the world (Mei et al., 2001). With the exception of 'positive' cases, only a limited amount is used for tests as part of the newborn screening program. Several public health departments archive residual samples and make them available for additional research following ethical approval, including exposure assessments or epidemiological studies (Olshan, 2007; Stove et al., 2012). However, it is only recently that both academia and industry have recognized the many advantages that DBS sampling may offer (Stove et al., 2012). In addition to newborn screening programs, DBS sampling and analysis has been incorporated into other data collection and disease-surveillance efforts in developed and developing countries, with applications rising rapidly in the last few years to include the analysis of DNA, proteins (e.g enzyme activity or antibody-based analysis), small molecules (endogenous or exogenous, e.g amino acids or therapeutic drugs) as well as trace elements (Stove et al., 2012).

DBS samples have facilitated research in human biology and health in remote settings around the world (Boerma et al., 2001). In low-resource settings, field-friendly tools to assess the effect of micronutrient interventions and nutritional programs are underutilized or not readily available when needed (Garrett et al., 2011). As discussed, the recovery of several trace essential elements from DBS is challenging due to low physiological concentrations and small sample volumes. Thus, studies focusing on multi-element detection are scarce as, frequently, only one element is evaluated, only toxic elements are investigated or semi-quantitative data is obtained. Key articles that measure the relevant essential elements to this research project (Cu, Se, and Zn) in DBS are summarized in Table 1 in the Appendix. Given that the focus of this research is multi-element detection, five of the articles outlined in Table 1 that quantify more than one of the selected elements in DBS will be discussed in-depth.

Hsieh et al. (2011) used laser ablation coupled with ICP-MS (LA-ICP-MS) to quantify 13 elements in whole blood, including Cu and Zn. This technique is unique as it does not require sample pre-treatment and, thus, has a reduced risk of contamination and sample loss. In addition, the use of LA-ICP-MS enables the determination of the spatial distribution of elements in the DBS. Hence, the whole blood in the DBS and adjacent filter paper can be simultaneously analyzed on the same sample and for every sample. Additional benefits to this technique include

shortened sample preparation times, minimal sample consumption, reduced sample contamination and minimal waste production (Hsieh et al., 2011). However, the drops of blood were dried on hydrophobic filter membranes for this study and not the standard Whatman©903 filter paper cards widely used in newborn screening programs. Thus, this method will need to be validated for use on residual newborn blood samples or other types of filter paper.

Cizdziel (2007) used a similar technique to Hsieh et al. (2011). Cizdziel (2007) used laser ablation inductively coupled plasma time-of-flight-mass spectrometry (LA-ICP-TOF-MS) to primarily quantify Pb and also monitored additional elements of relevance in the DBS. They established that additional elements conducive to potential quantification included Ca, V, Fe, Cu, and Zn. However, Se was not evaluated in this study. As the primary focus of the study was measurement of Pb, this technique may be used as a screening tool for Pb exposure, primarily to identify samples above a set threshold, but validation for other elemental measurements needs to be conducted. This is important as elements bound to proteins may interact with the filter paper matrix differently from those that have different speciation (Cizdziel, 2007).

In contrast to Hsieh et al. (2011) and Cizdziel (2007), Vacchina et al. (2014) analyzed the samples with ICP-MS following acidic digestion of the DBS. This study explored all three elements included in this research and uses matrix-matched calibration curves to quantify the elements. The method was validated for the following elements: As, Cd, Cu, Pb, Mo, Se, and Zn, with repeatability and reproducibility generally below 15% and recovery levels ranging from 96% to 117%. However, Vacchina et al. (2014) evaluated different types of filter paper and concluded that the best results were obtained on a decontaminated polyvinylidene fluoride (PVDF) filter paper. Although this overcomes many of the current barriers in DBS analysis, it limits the applicability of this method to residual samples from newborn screening and epidemiological studies. To validate their method, they recruited two adult subjects to test analytical procedures under real sampling conditions. In addition to the small sample size, there are significant physiological differences between adults and newbors, of which the most relevant is differences in hematocrit. The method needs to be further investigated prior to validation in a newborn population as this will likely increase the heterogeneity of the DBS samples.

Margui et al. (2017) used energy dispersive X-ray fluorescence spectrometry (EDXRF) for direct, non-destructive multi-element analysis of DBS. They used matrix-matched blood samples of known analyte concentrations to quantify the elements in DBS. They measured both

Cu and Zn, but did not measure Se. However, they used an entire spot of DBS, not a sub-sample, which is a barrier to applications with residual newborn samples and other difficult sampling conditions. Furthermore, they concluded that Cu and Zn concentrations could not be quantified because of significant elemental peaks identified in the blank filter paper, which affects quantification at human blood concentrations. They concluded that other elements of nutritional relevance, such as P, S, Cl, K and Fe, could be quantified using this method.

Langer et al. (2010) is a key scientific study in the field of DBS as they measure elements in Whatman©903 filter paper, widely used in newborn screening programs, and also applied their method to newborn DBS samples (n=150). They developed an extraction and analysis protocol for elemental detection specifically for newborn DBS using sector-field inductively coupled plasma-mass spectrometry (SF-ICP-MS). They optimized a 5% (v/v) nitric acid and 0.1% (v/v) Triton X-100 element extraction protocol and measured 28 elements in both filter paper and newborn DBS. Similar to other studies, they concluded that filter paper median levels were highly variable for most elements. However, as opposed to Margui et al. (2017), they found the filter paper contributed very little to the quantification of Cu, Se, and Zn. Despite this finding, they established greater variability in Zn measurements than the other elements, a finding in accordance with Margui et al. (2017), Vacchina et al. (2014), and Hsieh et al. (2011). They conclude that their method could be used for semi-quantitative measurement of elements to classify individuals to exposure categories or screen for highly elevated levels of certain trace elements. Further method development is required for quantification of elements.

These key scientific articles have strengths and limitations in their methods and study design, as outlined above. Each method has been validated for quantitative or semi-quantitative analysis for select elements using SRMs and matrix-matched calibration curves. However, certain themes emerge from the litertuare. First of all, the variability in the filter paper remains a key limitation in elemental quantification in DBS. Hsieh et al. (2011) and Vacchina et al. (2014) both used different filter paper than the standard Whatman©903 filter paper used in newborn screening programs and were able to circumvent the key issue of contamination and elemental variability. Other variations and limitations that emerge across the literature are the size of the punches analyzed, matrix effect of sample, hematocrit effect, and unknown sample volume. In addition, Langer et al. (2010) was the only multi-element method applied to larger cohort of newborns for the analysis of essential elements, whereas other articles applied their method to a

few healthy adults. It is evident that there is no standard method and there remains significant uncertainty in the field of DBS elemental analysis.

2.6 Collection, Storage and Analysis of Dried Blood Spots

Appropriate collection and storage is important for maintaining sample integrity and minimizing variation and error. Standard protocols have been developed for the collection of DBS that require minimal training and can be used in both clinical and non-clinical settings. The United States Agency for International Development (USAID) Biomarker Field Manual provides standardized steps to collect DBS samples (USAID, 2012). Following instructions in the Biomarker Field Manual, the side of the participant's second, third or fourth finger is warmed and cleaned with isopropyl alcohol and then pricked with a sterile, disposable lancet. The lancets are designed to deliver a controlled, uniform puncture that stimulates blood flow with minimal injury (Mcdade et al., 2007; Mei et al., 2001). The initial drop of blood is wiped away with sterile gauze because of possible contamination with alcohol, cell debris and tissue fluid (Bond & Richards-Kortum, 2015). The subsequent drops of blood are formed and dropped onto the specialized filter paper without any direct contact between the finger and paper. The drop should not be blotted or smeared onto the paper or placed on top of a previous drop. Poor spotting technique can also lead to unreliable analytical results (Wong & James, 2014). The pre-printed circles are guides to standardize the sample volume, containing approximately 50 μL of whole blood when filled.

The DBS samples are dried for a minimum of 4 hours, then stacked and stored in re-sealable bags or plastic containers. During the drying process, samples should be kept away from direct sunlight and should not be heated, stacked or allowed to touch other surfaces (Mei et al., 2001). It is important to dry DBS samples completely before storage or transport as moisture may harm the specimen. It is recommended that they are stored in low gas-permeable zip-closure bags with desiccant and a colored humidity indicator to show when it should be replaced, assuring the stability of analytes (Hannon et al., 2003; Mei et al., 2001). Refrigerating or freezing samples after drying is also recommended to minimize degradation but filter paper provides stability to most analytes and, thus, creates flexibility in sample collection and transport (Mcdade et al., 2007). For long-term storage, samples should be packed with desiccant and frozen in a

reliable laboratory-grade freezer to ensure sample integrity. As with any biological sample, repeated cycles of freezing and thawing are to be avoided although the filter paper matrix provides additional analyte stability (Bowen et al., 2010; Mcdade et al., 2007; Stove et al., 2012). Despite the standard protocols developed, considerable variability remains with how DBS are stored, length of storage time and access to specimen for research (Olshan, 2007).

A standard hole punch is typically used to cut discs of whole blood of uniform size for analysis, as discussed in *Section 2.5*. The DBS card is placed on top of a punching mat and a punching tool is pressed down at the appropriate location of the DBS. Carryover may be a concern and can be mediated by punching samples in order from low to high concentration, punching a blank card between samples, or cleaning tools with an appropriate solution between cards. The DBS is cut then placed into the suitable tube for extraction. Often, the disk is extracted with a solvent or buffer solution to yield a liquid sample for analysis (Wong & James, 2014). The optimal combination of elution duration, mixing, temperature and buffer constituents that maximizes the efficacy of elution needs to be developed and evaluated for a given assay (Mcdade et al., 2007). Different assay protocols and specific analytes will vary in their sensitivity to interference and additional processing prior to analysis may be required (Funk et al., 2015). Typically, an internal standard (IS) is added to the extraction solution to compensate for the matrix effect, loss of sample handling and the drift in detector response (Wong & James, 2014). Extraction and analysis of DBS present various challenges due to the unique attributes of the filter paper matrix.

2.7 Analytical Technology

Given the low physiological concentrations of trace elements in biological samples, the recovery from DBS is challenging and requires equipment with low detection limits. A variety of instrumental techniques are available to measure elements in human biological material, including single-element equipment such as flame atomic absorption spectroscopy (FAAS) and graphite furnace atomic absorption spectroscopy (GFAAS) and multi-element tools such as inductively coupled plasma atomic emission spectroscopy (ICP-AES) and inductively coupled plasma mass spectroscopy (ICP-MS). ICP-MS has led to significant advances in the field of clinical biology, particularly in toxicological analysis, and is considered by many to be the ‘gold

standard' (Goullé et al., 2005; Hsieh et al., 2011). ICP-MS is widely used for the measurement of trace elements in biological samples due to its capacity to quantify multiple elements, low detection limits and relatively high-throughput (Langer et al., 2010; Vacchina et al., 2014). Whole blood samples are analyzed directly after sample dilution and decomposition of the organic matrix (Hsieh et al., 2011).

However, there are limitations to the use of ICP-MS to analyze DBS. The amount of whole blood collected for elemental analysis must be at least several milliliters, an obstacle in the analysis of DBS. Furthermore, the sample matrix guides the precision and accuracy of the method, presenting an analytical challenge (Langer et al., 2010). As the blood is dried on filter paper, it needs to be extracted first. Langer et al. (2010) define the key challenge as the development of an extraction protocol with adequate recovery rates and minimum contribution of background element contamination to the measurement from the filter paper. This leads to complex sample preparation requiring trained personnel and increased costs.

Thus, there is a need to develop and innovate tools for the analytical measurement of DBS as conventional approaches are expensive and require skilled technicians. Total Reflective X-Ray Fluorescence (TXRF) Spectroscopy is a viable analytical alternative for quantitative multi-element analysis and may overcome many of the challenges in the elemental analysis of DBS. Comparisons with standard atomic absorption spectroscopy (AAS) technique, ICP-OES and ICP-MS, show that TXRF is a practical, accurate and reliable technique (Borgese et al., 2009; Stosnach, 2005; Stosnach & Mages, 2009). It is considered a well-established technique to investigate elemental concentrations in different biological fluids and tissues (Khuder et al., 2007). TXRF is a versatile apparatus that allows for more rapid and simultaneous element detection. There are several advantages to using the TXRF, primarily multi-element detection, low sample volume, and simplicity of instrument use. TXRF only requires small sample amounts and the analysis, including sample preparation and quantification, is simpler than other analytical chemistry instruments. Most importantly, the matrix effect is minimized as samples are dried on a quartz sample carrier, reducing absorption or secondary excitation (Towett et al., 2015).

The S2 PICOFOX Spectrometer (Bruker AXS Microanaylysis GmbH, Germany) is the instrument used in this study. It is a portable TXRF instrument with an air-cooled low power X-ray metal-ceramic tube and a molybdenum target (Bruker Nano GmbH, 2011; Bruker Nano GmbH, 2007). Specifications of the instrument are found in Table 2 of the Appendix. Samples

are first brought to solution and an internal standard is added to the sample solution volume for quantification. For example, gallium (Ga) is often selected as the internal standard when analyzing whole blood because it is not naturally present in blood. As a result, external calibration routines are not required. After the addition of the internal standard, all solutions are thoroughly homogenized prior to pipetting. TXRF analysis requires a very small quantity for each sample – a maximum of 10 μL of sample solution is deposited on the quartz sample carrier (Bruker Nano GmbH, 2011). The sample is dried as a thin layer prior to analysis, reducing the potential matrix effects (Towett et al., 2015). In the instrument, a monochromatic x-ray beam is directed onto the sample at an angle less than the critical angle of external total reflection for x-rays (<0.1 degrees) (Bruker Nano GmbH, 2007), leading to the excitation of the sample. Since the wavelength and energy of the fluorescence radiation are specific for each element, TXRF quantification is possible using the intensity of fluorescence radiation and an internal standard (Bruker Nano GmbH, 2007). An energy-dispersive detector determines the fluorescence radiation emitted by the sample and the intensity is measured by means of an amplifier coupled to a multichannel analyzer (Bruker Nano GmbH, 2007). The limits of detection are specific to each element and generally in the low parts per billion (ppb or μg/L) range. The various challenges to DBS elemental quantification, such as unknown sample volume and variability across samples, remain present but analyzing essential elements by TXRF offers the capability of simultaneous and cost-effective multi-element analysis with a relatively small sample size. Despite the potential of using TXRF to measure elements in DBS, this has not yet been done in a comprehensive manner to our knowedge.

CHAPTER 3. MANUSCRIPT 1

Development and Application of a Novel Method to Measure Essential Elements in Newborn Dried Blood Spots from the Michigan BioTrust for Health

Role of Manuscript 1

In this chapter, I address the key objectives of my thesis. First, a method was developed to measure select essential elements in sub-sampled DBS using SRMs from the INSPQ (n=7) with varying concentrations of elements. Following analysis of certain validation criteria, such as accuracy and precision, the selected protocol was applied to a newborn cohort from the Michigan BioTrust for Health (n=675). In addition to population measures, these results were also analyzed for variability across punch type, storage method, and, last of all, inter-day variability of entire batch, including both the newborn samples and SRMs, for quality control. This chapter provides the development and application of a novel and cost-effective method for the multi-element analysis of human DBS using TXRF and compares the method to existing methods. A version of this manuscript is in preparation for submission to an academic journal, reflecting standards in the field of analytical chemistry.

3.1 Introduction

Essential elements are required for the biological activity and proper functioning of the human body. Essential trace elements, or elements required in small quantities for physiological function, include copper (Cu), cobalt, (Co), chromium (Cr), iron (Fe), manganese (Mn), molybdenum (Mo), selenium (Se), and zinc (Zn). Quantification of these elements is critical for nutritional research and public health interventions. Biomarkers, objective measures of a biological state, are integral to evaluating physiological function (Mcdade et al., 2007). However, logistical constraints on the collection and analysis of biological samples remain an obstacle to incorporating biomarkers into human health research . Whole blood, in particular, can provide various indicators of nutritional status, yet established blood collection protocols via venipuncture require extensive supplies, trained professionals and a cold chain with minimal freeze-thaw cycles for sample transport and storage (Boerma et al., 2001; Garrett et al., 2011; Mcdade et al., 2007). For research conducted at remote locations or in resource-limited regions, the equipment, collection and transportation costs are frequently logistically prohibitive (Boerma et al., 2001; Garrett et al., 2011). Furthermore, blood sampling is invasive and ethically challenging for vulnerable groups, such as newborns, children and the elderly.

Dried blood spots (DBS), a method of collecting capillary blood on specialized filter paper by pricking an individual's finger or heel, provides an alternative method of collecting biological samples to venipuncture. DBS were first used in the early 1960s when Dr. Robert Guthrie began collecting heel-prick blood spot samples from newborns to measure phenylalanine for the detection of phenylketonuria (Guthrie & Susi, 1963). They are now used in the detection of inherited metabolic diseases and other genetic disorders across the world (Mei et al., 2001; Olshan, 2007; Stove et al., 2012). There is increasing interest in using DBS in wider applications due to their practical and economic advantages. They provide an inexpensive and minimally invasive method requiring minimal equipment for collecting blood samples in both clinical and nonclinical settings, overcoming many constraints in biospecimen collection (Mcdade et al., 2007; Stove et al., 2012). DBS are particularly useful in resource-limited areas with logistical obstacles associated with staffing, sample collection and transport and when working with vulnerable populations (Boerma et al., 2001; Garrett et al., 2011).The whole blood does not need to be centrifuged, separated or frozen after collection. Thus, sample collection can be conducted in a participant's home by individuals who are not medically trained and even participants

themselves (Mcdade et al., 2007). Following blood collection, DBS are allowed to dry, then they are stacked, stored and shipped with relative ease. They do not require a cold-chain or refrigeration of specimen in the field, reducing the complexity of storage and transportation requirements in remote areas (Garrett et al., 2011). Furthermore, the filter paper provides stability to analytes of interest for long periods of time, although this varies by analyte (Bowen et al., 2010; Mcdade et al., 2007; Stove et al., 2012). The ability to archive samples is a key advantage to DBS sample collection. Stored samples remaining after newborn screening analytical tests have been proposed as a novel source of biological specimen, opening many possibilities in human health research (Di Martino et al., 2004; Mcdade et al., 2007). Last of all, the relatively small samples of volume and method of collection are a lower burden of sampling on participants, which is advantageous for studies with infants, children and the elderly where venipuncture can be difficult (Mcdade et al., 2007).

Despite the benefits of DBS in research, there are gaps in our present knowledge and analytical challenges to elemental quantification. As the majority of standard laboratory protocols require whole blood or serum, analytical assays need to be developed specifically for DBS and validated for accuracy, precision, reliability and limits of detection (Mcdade et al., 2007). The small sample volume is a benefit when collecting blood from vulnerable populations but is a disadvantage with regards to technological limits, requiring technology with very low detection limitis. The small quantity of sample may be an insurmountable limitation for some analytes and equipment. In addition, quantification of select analytes may not be possible due to contamination of filter paper or instability of the analyte in the filter paper. The filter paper is manufactured from high purity cotton linters and has been standardized with pre-printed circles for routine collection, giving accurate and reproducible absorption of blood according to the National Committee on Clinical Laboratory Standards (NCCLS) specifications (Mei et al. 2001, Hannon et al. 2003). However, there are several parameters that affect the volume of a sample within a pre-printed circle, including hematocrit level, paper batch-to-batch differences, volume of blood added to paper and location of punch (Chaudhuri et al., 2009; Mei et al., 2001; O'Mara et al., 2011; Wong & James, 2014).

The current literature on multi-element quantification of DBS is limited as a result of challenges in method development, technology and intrinsic barriers to the analysis of DBS. Some instruments are limited to as single-element analysis or have detection limits that are too

high for elemental quantification at low biological concentrations or in low sample volumes. Inductively coupled plasma mass spectrometry (ICP-MS), however, has led to significant advances in the field of clinical biology and toxicology. It is considered by many to be the 'gold standard' (Goullé et al., 2005; Hsieh et al., 2009, 2011). However, ICP-MS sample preparation is complicated and expensive and there are barriers to analyzing DBS. The amount of whole blood collected for elemental analysis must be at least several milliliters, presenting an obstacle in the analysis of very small volumes present in DBS. As the blood is dried on filter paper, it needs to be extracted first. Langer et al. (2010) outline the key challenge as the development of an extraction protocol with adequate recovery rates and minimum contribution of background element contamination to the measurement from the filter paper. Most importantly, the sample matrix guides the precision and accuracy of the method, presenting an analytical challenge in analyzing blood, which has a complex matrix (Langer et al., 2010).

The Total X-Ray Reflection Fluorescence Spectroscopy (TXRF), a spectrometer for quantitative multi-element analysis, is considered a viable analytical alternative and overcomes many challenges in the elemental analysis of DBS. Comparisons with standard atomic absorption spectroscopy (AAS) technique, ICP-OES and ICP-MS, show that TXRF is a practical, accurate and reliable technique (Borgese et al., 2009; Stosnach, 2005; Stosnach & Mages, 2009). TXRF has the ability to detect multiple elements, lower the cost of sample analysis, simpler sample preparation and a lower sample volume requirement. In addition, the matrix effect is minimized as samples are dried on a quartz sample carrier, reducing absorption or secondary excitation (Towett et al., 2015). This is particularly advantageous for the analysis of whole blood. Validation of TXRF spectroscopy as an accurate and precise method for DBS multi-element analysis could increase integration of biomarkers into health programs and research efforts in vulnerable populations—leading to informed nutrition interventions and policies.

The main objective of this study was to develop and apply a new method of quantifying essential elements in DBS using TXRF spectroscopy. The elements selected for this study are copper (Cu), selenium (Se), and zinc (Zn). Meeting nutritional needs is critical in early childhood development and these three essential trace elements are required for physiological function and growth. Following method development and evaluation of key analytical parameters, we applied the method to measure essential elements in DBS obtained from newborns (n=675) from the State of Michigan. In Michigan, the samples are stored and managed by the Michigan BioTrust

for Health. The ability to analyze these residual DBS creates new opportunities to answer critical health questions and incorporate objective data into health research and biomonitoring efforts. In this paper, the method is described and applied to a cohort of newborns, followed by results and a discussion of the advantages and limitations.

3.2 Materials and Methods

3.2.1 General Overview

This study was conducted in two phases. Initial method development was conducted using human whole blood standard reference material (SRM) of known elemental concentrations. This aim was accomplished by created DBS in the laboratory and testing a variety of experimental methods, which were evaluated for accuracy and precision in order to select a suitable analytical method (refer to Table 3 in Appendix for the variables tested). The selected protocol is the focus of this study. Following development and validation, we applied the method to measure essential elements in DBS from newborns (n=675) from the Michigan BioTrust for Health program. These analyses spanned 38 batch runs, each of which contained a maximum of 24 samples, including a range of quality control samples, discussed in *Section 3.2.4*. Institutional Review Board (IRB) approval for this work was obtained from McGill University (A06-M29-16B), the University of Michigan (HUM000771006), and the Michigan Department of Health and Human Services (201212-05-XA-R).

3.2.2 Dried Blood Spot Processing

Human whole blood SRM (n=7) of varying elemental concentrations (refer to Table 4 in Appendix) from the *Institut National de Santé Publique du Québec* (INSPQ) was used for method development. A 60 μL sample of whole SRM was pipetted onto Whatman©903 filter paper (GEO Healthcare Services, Mississauga, ON, Canada), used for all DBS in this study unless mentioned otherwise, and dried overnight at room temperature in a Class 100 ISO Cleanhood. After drying, the DBS cards were stored in plastic Ziploc bags at ambient temperature until analysis. The 60 μL DBS was sub-sampled using a 1/8" or 3mm punch (Harris, Corporation, Melbourne, FL, USA). The volume of this punch is assumed to contain 3.1 μL of blood based on a study by Li and Lee (2014). To optimize the best extraction procedure, different solutions were tested and evaluated. Blank filter paper adjacent to the blood that did not contain blood was also analyzed from approximately 10% of all DBS cards. Punched samples were placed in a metal-free microcentrifuge tube for analysis (Rose Scientific Ltd.).

Since the blood sample has been dried on filter paper, analytes must first be brought into solution. For multi-element analyses via TXRF spectroscopy, 15 μL of concentrated HCl with 5 mmol EDTA was added to the 1/8" punch in the microcentrifuge tube. The sample was then

vortexed thoroughly and digested for 1.5 hours at 55°C. Following the digestion, the sample was centrifuged for 15 minutes at 25°C at 12000 rpm. An 8 μL portion of the extraction fluid was removed and placed into a second microcentrifuge vial, to which a 4 μL solution containing a mixture of gallium (internal standard, 100 μg/L final concentration), lead (spike, 20 μg/L final concentration), and polyvinyl alcohol (PVA, 1%) was added. This solution was mixed, and then an 8 μL aliquot was placed onto a Serva-conditioned quartz sample carrier. All quartz sample carriers are tested for contamination prior to the addition of the sample. The sample is covered and allowed to dry overnight in a lab oven set at 55°C to allow the acid to evaporate prior to analysis in the instrument. Each batch contained a maximum of 24 samples composed of 18 individual newborn DBS samples, 3 DBS SRMs, 2 method blanks and 1 DBS sample from which a duplicate punch is analyzed.

3.2.3 Multi-Element Analysis

Following acid digestion and drying, multi-element measurement was carried out using TXRF spectroscopy. All measurements presented in this paper were performed with the benchtop TXRF spectrometer "S2 PICOFOX" (Bruker AXS Microanalysis GmbH, Germany); technical specifications are in Table 2 of the Appendix. This portable TXRF instrument has an air-cooled low power X-ray metal-ceramic tube, a molybdenum target, working at 50 W of max power, and a liquid nitrogen-free Silicon Drift Detector (SSD) (Bruker, 2007b). A monochromatic x-ray beam is directed onto the sample at an angle less than the critical angle of external total reflection, which leads to excitation of the sample (Bruker, 2007a). The preparation of samples as a thin layer largely precludes matrix effects such as absorption or secondary excitation (Towett et al., 2015). Since the wavelength and energy of the fluorescence radiation are specific for each element, TXRF quantification is possible using the intensity of fluorescence radiation and an internal standard (Bruker, 2007a). Samples were read for 2,500 seconds and the results were analyzed using the instrument's software, *Spectra 7* (Bruker AXS Inc.). A longer total analysis time was essential to capture the lower concentration of elements at physiologic levels. Elemental concentration was quantified using a gallium internal standard with a final concentration of 100 μg/L and a seven-point matrix-matched calibration curve for each analyte. The instrument detection limits were determined to be in the low μg/L range for each element.

3.2.4 Assay Validation and Quality Control

Each batch contained a maximum of 24 samples, including a range of quality control samples, specifically 18 individual newborn DBS samples, 3 DBS SRMs, 2 filter paper blanks and 1 DBS sample from which duplicate punches were taken and run separately. The punched DBS made from SRMs (n=7) were used to establish matrix-matched calibration curves and assess analytical accuracy, precision, linearity and range. Analytical percent accuracy was calculated as the difference between the observed value and the accepted concentration value of the SRM, using the following equation:

$$\%\ Accuracy = \frac{\left|V_{accepted} - V_{observed}\right|}{V_{accepted}} \times 100\%$$

Assay precision was assessed by running DBS samples from the Michigan BioTrust for Health cohort in duplicate (n=37). Precision was calculated as the relative percent difference when assessing two samples, where V_1 refers to one sample and V_2 refers to the second, as per the following equation:

$$\%\ RPD = \frac{|V_1 - V_2|}{\left(\frac{V_1 + V_2}{2}\right)} \times 100\%$$

To measure precision with multiple samples, the coefficient of variability(%CV) was used. which is the standard deviation divided by the mean and multiplied by a percentage; calculated by the following equation:

$$\%\ CV = \frac{SD}{\bar{x}} \times\ 100\%$$

In order to determine background levels of elements of the filter paper, we analyzed blank filter paper removed from adjacent areas to the spots of blood of select DBS samples for quality control. The elemental concentrations of the filter paper were not subtracted from the results of the accompanying blood spot, as discussed in *Section 3.3.4*. To assess the assay reliability and precision, inter-assay variance of the entire batch was also evaluated across the 38 batches. Batches that failed quality control could not be re-run for this project due to limited quantities of sample available and, thus, no possibility to repeat.

3.2.5 Michigan BioTrust for Health Samples

The samples selected for this application are from a larger epidemiological study of risk factors for newborn hearing loss. The newborn DBS were collected from 2003 to 2015 in the State of Michigan. For batches #1-10 (n=180), the DBS were stored at ambient temperatures following data collection. The rest of the samples (n=495) were stored at -20°C between collection and analyses. For the first 12 batches analyzed, one punch (3mm diameter) was taken from the edge of a single spot of a DBS card following the same protocol used to process the SRMs outlined in *Section 3.3.2*. For the remaining batches, the Michigan BioTrust for Health Project provided rectangular punches of 2 mm x 6 mm in size, or approximately the equivalent of two 3mm diameter punches. Thus, twice the volume of the extraction solution was added to the punch and the rest of the sample preparation and analysis remained the same. As the samples selected for this project were stored at two temperatures and punched using two different tools, the samples in the different groups are compared to test for variation between the groups.

3.2.6 Data Analyses

First, the software used to control the instrument and quantify the elements was *Spectra 7* (Bruker AXS Inc.). Following instrumental quantification, the data on the selected elements were first analyzed using univariate descriptive statistics to understand basic features of the dataset, followed by coefficient of variation for precision and percent recoveries for accuracy. Measures of central tendency (mean, median) and associated variances (standard deviation, inter-quartile ranges) were calculated and compared against assay performance criteria. Samples we were unable to quantify, i.e the internal standard was not detected, appeared as extreme values. The generalized extreme studentized deviate (ESD) test is a statistical test used to detect multiple outliers in a univariate dataset assumed to be from a normally distributed population. This test was used in this dataset to detect outliers. T-tests and ANOVAs were run to test if elemental levels varied according to batch number (n=38), punch type (3mm diameter vs. rectangular punch), and storage temperature (ambient vs. -20°C). The p-value was set at α=0.05 for all tests. All analyses were conducted using Microsoft® Excel Version 15.4 and JMP®Pro 13.0.0.

3.3 Results and Discussion

3.3.1 Linearity and Range

The linearity of the method was assessed by measuring elemental concentrations in DBS created in the laboratory with whole blood SRM and sub-sampled, as outlined in *Section 3.2.2*. Selection of a matrix-matched calibration model is important for reliable quantification, especially for DBS where filter paper and specimen matrix are variables to consider in the analytical measurements (Marguí et al., 2017; Peters, Drummer, & Musshoff, 2007). Seven whole blood SRMs were selected from the *INSQP* to cover a biologically plausible range (Institute of Medicine, 2000, 2001; Iyengar & Woittiez, 1988). Three DBS sub-samples were punched from the edge of a blood spot and were analyzed as individual samples. The average of the three samples were calculated and compared to the known elemental concentration to develop a matrix-matched calibration curve and determine linearity. It is important to note that, although the DBS were created in the lab with a known volume, the volume of the sub-sampled punches are based on estimations from the literature and there remains uncertainty and variability across the individual punches.

For Cu, the resulting linear regression comparing concentrations analyzed on the TXRF to known SRM concentrations was $Y = 0.99X - 7.2$, with a coefficient of determination (R^2) of 0.98, shown in Figure 2 in the Appendix. The average recovery of the seven SRMs is 102.3 ± 5.9%. The average Cu concentration in an adult population according to Iyengar & Woittiez (1988), a comprehensive review of elements in multiple biological samples, is 970 ± 130 μg/L. This value is on the lower end of the matrix-matched calibration curve but captured within the curve. Three of the SRMs were selected to be run with each batch for quality control purposes and one SRM from the low, middle and high concentration of the curve were selected to capture the full range of values on the curve.

For Se, the resulting linear regression comparing concentrations analyzed on the TXRF to assigned SRM concentrations was $Y = 1.2X + 9.6$, with a R^2 of 0.975, shown in Figure 3 of the Appendix. This curve demonstrates a systematic under-estimation of the Se concentration in the DBS samples by ~20%. Thus, the values can be adjusted using the linear regression equation to account for this systematic error. After adjusting for the systematic error, the average recovery of the SRMs is 100.9% ± 8.6. Se population measurements reflect diet to some extent; although

homeostatially controlled, it is not as tightly regulated in the blood (Institute of Medicine, 2000). Iyengar & Woittiez (1988) determined the reference value for Se to be 105 μg/L with a range of 58 to 234 μg/L, based on 20 sets of results. The reference range for adults established by the review corresponds to the lower area of the curve and three of the SRMs representing a low, middle and high point of the standard curve were run with each batch.

For Zn, the resulting linear regression comparing concentrations analyzed on the TXRF to known SRM concentrations was Y = 1.05X – 585.5, with a coefficient of determination of 0.975, as shown in Figure 4 of the Appendix. The average recovery of the SRM is 102.3% ± 5.6. The high y-intercept could reflect contamination in the filter paper, a systematic error. Contamination of the filter paper will be discussed further in *Section 3.3.4*. The high y-intercept could alternatively be a statistical artifact as the independent variable is clustered in two areas of the curve. This is a limitation of the SRMs selected as SRMs were selected to reflect the range of values for all three elements in this study. Iyengar & Woittiez (1988) determined reference Zn concentrations to be 6500 ± 1100 μg/L in whole blood, captured in the lower end of the matrix-matched curve.

3.3.2 Precision

Precision was evaluated using two different calculations. First, the precision of the SRMs analyzed with each batch was pooled to evaluate inter-assay precision. SRMs from Batch 1 were damaged prior to analysis due to human error and the internal standard of SRMs from Batch 30 were not quantified, so those values were removed. Second, intra-assay precision was addressed by measuring two individual punches from one sample in the Michigan BioTrust Project. Relative percent difference (%RPD) was evaluated for the duplicates and measured as a percentage as a measure of intra-assay precision. In each batch, one replicate measurement was completed, except for the first batch. However, if one of the replicate samples was removed as an outlier following the ESD statistical test, the replicate could not be evaluated. The replicate sample from Batch 1 was damaged prior to analysis and not analyzed as a result. Batches that failed quality control could not be re-run for this project due to limited quantities of sample available and, thus, no possibility to repeat analyses.

The precision of the three selected SRMs for Cu, Se and Zn are outlined in Table 5 in the Appendix. For Cu, the inter-assay precision, evaluated as percent coefficient of variation of 36 individual SRM samples, ranged from 10.86% for SRM C to 16.80% for SRM B, as shown in Table 5. A plot of the %RPD of the duplicates in each batch for Cu concentration is visually represented in Figure 5 in the Appendix. For Cu, the %RPD ranged from 0.76% to 39.56%. Seven replicates of the total of thirty-eight were eliminated for one or both of their samples being considered an outlier. Five of the replicates had a %RPD greater than 30%.

For Se, the %CV ranged from 13.55% to 23.20% for all the SRMs selected. A plot of the %RPD of the duplicates in each batch for Se concentration is visually represented in Figure 6 in the Appendix. For Se, the %RPD ranged from 0.14% to 53.17%. Two replicates were eliminated for one or both of their samples being below the detection limit and no replicates were removed as outliers. Three of the replicates had a %RPD greater than 30%. The results in this study had greater variability than other results in the literature. Langer et al. (2010) had variability less than 10% for Cu and Se and inter-assay precision ranged between 4.0% and 8.5% in the study by Hsieh et al. (2011). However, Hsieh et al. (2011) measured four replicates of each sample in three analytical runs and Langer et al. (2010) measured triplicate spot samples in five analytical batches. The inter-assay variability presented in this study is individual SRM values from 38 analytical runs, which could explain the wider range. The intra-assay measurement of replicate samples is also higher than replicate measurements in the literature. However, this is the only study that measures sub-samples punches and intrinsic properties and challengies to elemental analysis, i.e hematocrit, unknown sample volume and contamination, could explain the high %RPD in some samples. Although sub-samples in the lab were punched systematically from the edge, the majority of samples were provided in a different punch size and it is unknown what part of the DBS they were sampled from. Langer et al. (2010), in comparison, measured half of a DBS, arguing that it would improve assay reproducibility that can be influenced by sample location on the filter paper and hematocrit level.

For Zn, the %CV ranged from 12.18% to 15.70%, as shown in Figure 7 in the Appendix. In Batch 34, SRM C was removed as it was a significant outlier with a recovery of 540.23%. Langer et al. (2010), Margui et al. (2017), Vacchina et al. (2014), and Hsieh et al. (2011) had variability greater than 10% for their Zn measurements, which was higher than variability in the other elements. A plot of the %RPD of the duplicates in each batch for Zn concentration is

visually represented in Figure 7 in the Appendix. Only one sample was eliminated for one of their replicates detected as an outlier. For Zn, the %RPD ranged from 0.44% to 70.5% with four of the replicates with a %RPD greater than 30%.

3.3.3 Accuracy

The accuracy of this method was assessed using the three selected SRMs that represent the range of concentrations in the matrix-matched calibration curve. The values for all three elements are found in Table 6 in the Appendix. For Cu, the mean measurements were 3103.7 ± 365.9 μg/L, which translates to an average recovery of 100.3% ± 11.8%. SRM B had mean TXRF concentrations at 3103.7 ± 365.9 μg/L and a mean recovery of 117.2% ± 19.6%. The last SRM, SRMC C, had a mean TXRF concentration at 3215.4 ± 349.3 μg/L and a mean recovery of 105.8% ± 11.5%. A box plot of the three individual SRMs in Figure 8 of the Appendix demonstrate the range of SRM recovery across the batches, ranging from the lowest recovery of 68.07% in SRM A to the highest recovery at 177% in SRM B. SRM B had the lowest concentration and highest variability, indicating a potential issue with contamination of the filter paper affecting the accuracy of Cu concentrations at lower concentrations. Margui et al. (2017) discussed filter paper contamination for Cu and concluded that they were unable to quantify Cu as a result.

For Se, the measurements were first adjusted using the matrix-matched calibration curve outlined in *Section 3.3.1*. The mean measurements were 264.4 ± 35.8 μg/L, 158.2 ± 36.7 μg/L, and 216.9 ± 33.1 μg/L, for SRM A, B, and C, respectively. The average recovery ranged from 90.98% ± 12.32% to 95.72% ± 14.62%. A box plot of the three individual SRMs in Figure 9 of the Appendix demonstrate the range of SRM recovery across the batches, ranging from the lowest recovery of 61.07% in SRM B to 128.25% in SRM C. Se SRM results had less variability than the Cu SRM results.

Last of all, Zn results ranged from 7297.46 ± 1145.67 μg/L to 11591.06 ± 1412.10 μg/L. The average recovery ranged from 104.88% ± 13.96% to 115.19% ± 18.08%. A box plot of the three individual SRMs demonstrate the range of SRM recovery across the batches, ranging from the lowest recovery of 69.65% in SRM A to 172.0% in SRM C and is located in Figure 10 in the

Appendix. The wide variability could also be explained by filter paper contamination, as described in Margui et al. (2017) and discussed further in *Section 3.3.4*.

3.3.4 Analysis of Blank Filter Paper and Method Detection Limit

In general, the main limiting factor in the quantification of elements in biological material is the inorganic matrix of the sample (Langer et al., 2010). However, for TXRF, this matrix effect is minimal as the extraction is dried as a thin film on the quartz disc. Due to the unique aspects of this sample collection method and subsequent analysis, a method detection limit is a highly relevant measure. Elemental levels from filter paper can either be used for subtracting, although this may cause a negative bias, or can be used to evaluate the overall extent of contamination in the filter paper and analytical process (Chaudhuri et al., 2009; Stove et al., 2012). In this study, the levels from the blank filter paper were not subtracted from the values. The method detection limit was calculated based on repeated measurements of blank filter paper areas adjacent to the blood spots. One 3 mm circular punch of blank filter paper was taken from approximately 10% of the blotted DBS cards analyzed in the Michigan BioTrust cohort and two blank filter papers were analyzed in every batch. The average of all the blank filter paper was calculated and the method detection limit was quantified using the 3σ criteria. For Cu, the average of all the blank filter papers was 818.04 ± 1413.91 μg/L. Two blank values from Batch 30 were already eliminated as their elemental values were not detected or measured by the instrument, evidenced by extremely low net count and background counts in the sample analysis. However, this mean and standard deviation measure is driven by two extremely high blank values at 6194.79 μg/L and 8262.44 μg/L. When these values are eliminated, the average is 629.49 ± 874.13 μg/L and, thus, the method detection limit for Cu is 2622.39 μg/L. The measurements of filter paper show a large standard deviation, indicating a wide variety in the values. This value calculated using this definition of method detection limit is higher than the maximum Cu concentration measured in the Michigan BioTrust Cohort. The method for measuring filter paper may not accurately reflect the instrument or method detection limit and needs to be re-considered to better reflect contamination in the filter paper and sample processing. Contamination of filter paper with Cu is discussed in Margui et al. (2017) and is considered a limitation in the ability to quantify Cu and Zn in samples.

Se is different from the other two elements as it was only detected in one sample of filter paper at a concentration of 13.36 μg/L. Thus, a method detection limit cannot be quantified for Se. As with the other two elements, the two blank filter paper samples in Batch 30 were not properly quantified and were removed from this analysis. The lack of elemental Se in the blank filter paper adjacent to the blood spots agrees with the literature as no other paper explored in the literature detected Se filter paper contamination.

For Zn, the average of all the blank filter paper was 2005.30 ± 1622.198 μg/L. Thus, the method detection limit for Zn is 4866.57 μg/L. Measurements from both blank filter paper samples in Batch 30 were eliminated. The method detection limit for Zn is high considering the mean concentration of Zn in the Michigan BioTrust Cohort is 4250.03 ± 1314.87 μg/L. However, the high mean and high standard deviation in relation to the mean is driven by high concentrations in select samples. This is likely a contamination issue in the filter paper, as discussed by various other studies (Cizdziel, 2007; Langer et al., 2010; Marguí et al., 2017).

Some studies have discussed the high elemental concentration in filter papers as a key limitation in the ability to quantitatively analyze elements in DBS (Chaudhuri et al., 2009; Langer et al., 2010; Marguí et al., 2017; Stove et al., 2012). Margui et al. (2017) measured the elements using ICP-MS and found significant peaks of Na, Ca, Cu, and Zn in the blank filter paper. They concluded that these elements cannot be quantified at physiologic concentrations due to this contamination, which reflects our findings. Additional studies are needed to develop strategies and techniqes to overcome or account for the filter paper contamination in order to be able to quantify the affected elements in DBS.

3.3.5 Assay Application: Michigan BioTrust for Health Project

The three selected essential elements were measured in DBS samples obtained from 675 newborns from the Michigan BioTrust for Health Project. The elemental values were obtained in 38 batch runs and analyzed for normality, the presence of outliers and univariate statistics, shown in Table 7 in the Appendix. The filter paper samples were analyzed as part of quality control indicators and the blank values were not subtracted from the values for the infant samples. Some samples were excluded as extreme outliers following the ESD statistical test. These samples were likely not properly quantified and are not physiologically plausible. For example, if the

internal standard is not detected in a particular sample, the elements of interest cannot be properly quantified and the value will be extreme as the quantification is based on the relative concentration to the pre-determined internal standard concentration. For Cu, a total of 643 samples out of the 675 samples were included in the analysis and 32 samples were excluded as extreme outliers using the ESD statistical test. For Se, 13 samples were excluded as their concentration was below the instrument's detection limit and 3 samples were excluded as outliers. Last of all, 17 samples were excluded for Zn as extreme outliers and a total of 658 samples were analyzed.

3.3.5.1 Copper

The distribution of Cu concentrations (in μg/L) in the sample population follow a normal distribution as expected for essential micronutrients. The mean and standard deviation of Cu in the Michigan BioTrust cohort is 1018.12 ± 269.94 μg/L. This is comparable to reference values of Cu in Iyengar & Woittiez (1988), a comprehensive review of elements in multiple biological samples. They found Cu levels in whole blood in an adult population were 970 ± 130 μg/L, based on 16 sets of results. Custodio et al. (2005) found Cu values ranged from 3000 to 13000 μg/L in maternal blood and umbilical cord whole blood, significantly higher than the findings in Iyengar & Woittiez (1988) and this study. Krachler et al. 1998 estalished that serum Cu was lower in newborns than in adults and increased significantly with age. They established a reference range of 590-1390 μg/L for Cu in serum, which overlaps with the findings of our cohort. However, this study measured serum Cu, which may be different than Cu in whole blood.

As the samples were analyzed in 38 different batches, the batches were compared in order to determine if the batches are analytically equivalent as each batch was selected to represent the diversity in the cohort. It is important to note the confounding effect of different individuals in the batches yet this analysis is critical in understanding overall patterns across the batches. As shown in Figure 11 in the Appendix, there is variability within the batches and some variability across batches for Cu concentrations. A one-way ANOVA statistical test was used to evaluate if the batches were statistically different. Three of the batches exceed the α-level set at 0.05, with two batches exceeding the upper limit and one batch exceeding the lower limit. Batch 30 is significantly below the lower limit, reflecting the issues in quality control measures discussed in the previous sections.

The potential variation between the two different types of punches was also evaluated. The first 200 samples were punched using a 3mm circular punch from the perimeter of the DBS in the lab following the same protocol used for the SRM samples and outlined in *Section 3.2.2*. The remaining samples were provided from the Michigan BioTrust for Health as rectangular punches of 2mm x 6 mm in size. Therefore, their location on the DBS is unknown and could be from the center or edge, increasing the potential variability. The means of the two different punches were compared using a Student's t-test to measure a difference between the groups and there was no statistically significant difference found for Cu concentrations ($p = 0.99$). However, as shown in the box plots in Figure 13 in the Appendix, there is greater variability among samples with rectangular punches, which is supported by the literature discussing the importance of punch location. O'Mara et al. (2011) has shown that there may be significant differences in analyte distribution between the perimeter and center of the blood spot, stating that analyte concentration in perimeter punches were greater (O'Mara et al., 2011). Specific to elemental concentrations, findings from Perkins & Basu (2018) support O'Mara et al. (2011) and have shown that total mercury concentrations of punches removed from the perimeter were significantly higher (Perkins & Basu, 2018).

Last of all, it is important to compare the long-term storage method for potential differences in the values. The newborn DBS samples were collected from 2003 to 2015 and were analyzed in the lab in 2016, indicating a maximum potential storage time of 13 years. However, the storage time of each individual sample is unknown and the samples were stored in the lab under the same condition they were previously stored at. A Student's t-test was used to compare the two groups, shown in Figure 14 in the Appendix. The DBS cards that were kept stored at an ambient temperature ($n=380$) prior to analysis had an equivalent amount of Cu levels to the 295 cards that were kept frozen at -20°C ($p=0.066$), shown in Figure 14 in the Appendix. This demonstrates that analytes remain stable over an extend period of time, several years in this study, at both ambient temperature and frozen.

3.4.5.2 Selenium

Se concentrations (in μg/L) in the sample population also follow a normal distribution. In this population, Se concentrations were 240.97±58.18 μg/L. Iyengar & Woittiez (1988) determined the reference value for Se to be 105 μg/L with a range of 58 to 234 μg/L, based on

20 sets of results. The values in this cohort were at the maximum end of the reference values. However, there are two matters to consider. First of all, Se measurements reflect an individual's diet and additional factors, such as age, ethnicity and geography have a larger impact on population values and introduce greater variability in the population (Iyengar & Woittiez, 1988). Thus, it is important to note that the reference values are based on an adult population whereas the Michigan BioTrust cohort is composed of only newborns. Se was only measured in the serum of newborns in other studies. Galinier et al. (2005) established a reference range of 47.4±7.9 μg/L in the umbilical cord serum of neonates (n=241). This is significantly below the measurements in whole blood in a population as it is measured in serum.

The batches were compared in order to determine if the batches are analytically equivalent for Se concentration. As shown in Figure 15 in the Appendix, there is variability within the batches and variability across batches. Five of the batches exceed the α-level set at 0.05, with two batches exceeding the upper limit and three batches exceeding the lower limit. Batch 30 is again shown to be significantly below the lower limit, similar to the Cu results. The potential variation between the two different types of punches was also evaluated differences in Se concentration, shown in Figure 17. There is a statistically significant difference between the two groups ($p = 0.0143$), which needs to be explored further. One possible explanation is the lack of filter contamination unique to Se has not confounded the results and there is a difference between the two punches that needs to be taken into consideration when interpreting the results. Last of all, the effect of long-term storage method was also evaluated with regards to Se concentration, shown in Figure 18. There is no significant difference between samples stored at ambient temperature or frozen prior to analysis ($p = 0.77$).

3.4.5.3 Zinc

The distribution of Zn concentrations (in μg/L) in the sample population follows a normal distribution and the average Zn values in the Michign BioTrust cohort were 4250.03±1314. μg/L. However, Iyengar & Woittiez (1988) determined Zn concentrations to be 6500 ± 1100 μg/L in whole blood. This discrepancy is consistent with findings in the literature. Custodio et al. (2005) established Zn concentrations between 10,000 and 40,000 μg/L in maternal whole blood at delivery and umbilical cord blood. They also found Zn concentrations were higher in maternal

samples than corresponding umbilical cord blood samples, supporting the lower Zn concentrations in the Michigan BioTrust Cohort when compared to reference ranges in the adult population.

The batches were compared in order to determine if the batches are analytically equivalent for Zn concentrations, shown in Figure 19 in the Appendix. There was greater variability in the Zn concentrations than for Cu and Se. Seven of the batches exceed the α-level set at 0.05, with four batches exceeding the upper limit and three batches exceeding the lower limit. Batch 30 is again shown to be significantly below the lower limit, similar to the Cu results.

The potential variation between the two different types of punches was also evaluated for differences in Zn concentration, shown in Figure 21 in the Appendix. There is a statistically significant difference between the two groups ($p < 0.001$), similar to the findings for Se. As with the other elements, the effect of long-term storage method was also evaluated with regards to Zn concentration and shown in Figure 22 in the Appendix. There is a significant difference between samples stored at ambient temperature or frozen prior to analysis ($p < 0.001$), with frozen samples having significantly lower concentrations than those at ambient temperature. However, as the majority of frozen samples were also the samples with a rectangular punch, it is difficult to identify whether the difference between groups is due to the punch used or the storage method. In addition, the difference between the storage methods should be explored within the context of contamination of the filter paper and lot-to-lot variability in the filter paper.

3.3.6 Study Limitations and Future Directions

There are notable study limitations that warrant attention. One of the greatest challenges in the analysis of DBS is the unknown volume in a punch. Punches allow for a consistent area to be analyzed and various studies have attempted to quantify the volume in a given punch but the sample volume remains unknown due to sample collection methods and physiological variations among human. For example, an individual's hematocrit level affects the spread of blood on the filter paper and, thus, the volume of blood in a given punch. Hematocrit has a wide range of normal values that can change based on gender, age, environment, health status and treatment state (O'Mara et al., 2011). Furthermore, the hematocrit range for infants under two years of age is from 28% to 55%, compared to 41% to 50% in adult males and 36% to 44% in adult females (Wong & James, 2014). As a result, it is very difficult to calculate an accurate concentration and

the concentrations calculated in this study are an estimation based on an assumption in the literature that a 3mm diameter punch contains a 3.1 μL volume (Li & Lee, 2014). This explains some of the population variability and the increase in variability in the group of rectangular punches, as the larger punch area leads to an increase in potential error. Results from O'Mara et al. (2011) indicate that a portion of the bias (>15%) exists due to the effect of hematocrit and non-homogenous distribution of analytes across the spot. They demonstrated that factors of compound, card type and hematocrit significantly affected quantitative concentrations obtained from DBS samples. To minimize this bias, systematic methods of processing and analyzing the samples were developed. The error can be reduced with a fixed-diameter punch, as used in the processing of SRMs and DBS punched in the lab, and systematic methodology. For example, samples were consistently punched close to the edge to minimize potential bias. Although the systemtic methodology was applied to all SRMs and samples prepared in the lab, n=475 samples were provided pre-punched from the Michigan BioTrust Project and it is impossible to ascertain the location of the DBS they were punched from and other potential variables. In addition, another potential source of error is the inaccurate delivery of spot volume to the filter paper and it is impossible to ascertain if the punches were from a homogenous and complete spot.

Furthermore, this method was developed using SRMs and it is important to recognize that whole blood SRM has been processed and does not have all of the physiologic characteristics of human whole blood. Human whole blood is significantly more heterogeneous, affecting its spread on the filter paper and, thus, the quantification of analytes. It is important to use SRM DBS for evaluation of accuracy and precision, quality control measures, and developing a matrix-matched calibration curve for analyzing the residual newborn samples. However, there are limits within the range of available SRM values from the INSPQ; for example, zinc is clustered in two concentrations, and there are differences between SRMs and human whole blood that may affect the elemental quantification of DBS.

One of the challenges specific to elemental analysis is the potential contamination of the filter paper. Contamination in the filter paper may arise during the manufacturing of the cards, blood collection, storage, transportation as well as sample preparation. Several studies have found high variability in contamination within a card and across cards (Chaudhuri et al., 2009; Hsieh et al., 2011; Langer et al., 2010; Marguí et al., 2017). The spikes and variability in contamination could explain some of the outliers in this analysis but create a barrier to accurate

elemental quantification of DBS. Additionally, in this project, two samples of blank filter paper were run in each batch and pooled to measure the method detection limit of each element. However, it was not feasible to run a sample of blank filter paper from each newborn in the cohort due to time restrictions and limited sample availability. In addition, Chaudhuri et al. (2009) simultaneously analyzed blank filter paper punches and the DBS punch from an individual and measured homogeneity of different lots of blank filter paper in a study focused on heavy metal quantification in DBS. They found that lead concentratons are not homogenously distributed on or throughout the filter paper, limiting the practicality of analyzing adjacent blank filter paper for each sample (Chaudhuri et al., 2009). This finding was supported by Cizdziel (2007). One limitation to this study is the lack of blank discs, a disc without filter paper or DBS sample, analyzed in each batch. Thus, contamination throughout the sampe preparation and analytical process cannot be determined.

Normalization may be a possible future improvement and tool to overcome contamination in elemental analysis as well as account for both variations in hematocrit and unknown sample volume (Stove et al., 2012). Normalization can be done with one or multiple elements that have a narrow physiological distribution and is absent in the blank filter paper (Stove et al. 2012, Langer et al. 2010). Langer et al. (2010) suggests the use of potassium (K) as it is found at low levels in unspotted filter paper and could be used to normalize volume differences. Additional elements, such as magnesium (Mg) and calcium (Ca), have a narrow range in blood and could also be used to normalize values. However, Langer et al. (2010) suggests they may be inappropriate due to high concentrations and variability in blank filter paper samples. Although these elements can be quantified with the current TXRF method, the SRMs used in this study are not certified for Ca, K, or Mg and, thus, accuracy and precision currently cannot be calculated to explore this method.

3.4 Conclusion

This study develops and applies a novel method to address the quantification of micronutrients among newborns, a vulnerable population with limited available nutritional data. The method developed using TXRF was applied to samples from 675 newborns from the Michigan BioTrust Project in Michigan. The selected method helps overcome some challenges associated with other analytical instruments, such as sample volume, matrix effect and the cost of analysis. This paper contributes to a limited body of literature outlining laboratory techniques for the simultaneous multi-element analysis of essential elements in DBS and is one of the few methods applicable to residual newborn DBS. However, additional validation is required to overcome the limitations and variability of the analytical procedure. This method could be applied to screening projects and evaluation of population intervention efforts but it is currently not precise enough to be applied to quantify concentrations of individual samples. Archived samples from newborn screening programs, previously collecting DBS as well as newly collected samples from other populations can be analyzed using this technique. Furthermore, communities in resource-limited settings which may be challenged with sample collection costs and logistics may benefit from a low-cost and effective method of nutritional assessment. Objective data collection efforts may be particularly effective in mobilizing policy, informing interventions and evaluating program effectiveness around important nutritional issues.

CHAPTER 4. DISCUSSION

4.1 Overview

This method used an emerging instrument in the field of analytical chemistry to develop a novel method of elemental quantification of DBS. DBS are cost-effective and facilitate the collection of biological samples and integration of objective values into nutritional research and policy decisions. In addition to the various benefits of DBS and ability to measure residual samples, there are benefits to using the TXRF for elemental quantification. The method developed in this research project is novel and overcomes some of the challenges present in the literature. First of all, the use of the TXRF minimizes the matrix effect that is a predominant issue in ICP-MS and other analytical instruments (Langer et al., 2010; Towett et al., 2015). In comparison to other current methods, this method also uses sub-sampled punches of filter paper widely used in newborn screening programs, allowing the method to be easily applied to analyze residual samples from newborn screening programs. Although this has resulted in increased variability in the analysis, the method is more accessible and has the potential to be widely implemented in research and health surveys for semi-quantitative data or screening programs. Most importantly, this method was applied to a cohort of 675 newborns from the Michigan BioTrust Project, demonstrating its applicability. Langer et al. (2010) applied their method to a cohort of 150 newborns from Minnesota but all the other studies used SRMs or select adult volunteers to develop and validate their method. To my knowledge, this is currently the largest application of DBS elemental quantification in the literature and the population values reflect the expected values of a newborn cohort. Despite the many benefits of DBS and the study design as discussed throughout this thesis, there are several challenges that need to be reiterated.

4.2 Challenges to Quantification of Elements in DBS

There are many challenges to the quantification of DBS. First of all, and fundamental to the development of this project, analytical techniques are generally designed to measure liquid samples and there is a need for unique techniques to analyze DBS samples. The method development needs to meet certain criteria with adequate recovery values and precision values to ensure repeatability and reproducibility. One challenge outlined by Margui et al. 2017 is the availability of reference material with similar matrix compositions for calibration. This study uses seven SRMs certified by the INSPQ that cover the range of physiologically relevant

elemental concentrations. This was essential to developing matrix-matched calibration curves and demonstrating linearity, as outlined in *Section 3.3.1* of this thesis. Furthermore, for quality assurance purposes, three of the SRMs were carefully selected to cover a low, middle and high concentration of each element and run with every batch of samples from the Michigan BioTrust for Health cohort.

Another challenge in the field of DBS is the matrix effect of analyzing blood and potentially the filter paper as well. Thus, the use of matrix-matched calibration curves is critical when analyzing DBS (Marguí et al., 2017). The accuracy precision of methods using ICP-MS is dependent on reducing the matrix effect, a challenge with DBS (Langer et al., 2010). The TXRF provides a key advantage as matrix effects are considered negligible since the sample is dried as a thin sheet on the sample carrier.

Despite the importance of developing external calibration curves with a range of standard material, it is important to recognize that whole blood SRM has been processed and does not have all of the characteristics of human whole blood. Human whole blood is significantly more heterogeneous, affecting its spread on the filter paper and, thus, the quantification of analytes. SRM DBS are critical to the evaluation of accuracy and precision, quality control measures, and developing a matrix-matched calibration curve for analyzing the residual newborn samples. Cizdziel (2007) noted the freeze-dried nature of the Seronorm, their selected SRM, had different spreading characteristics on the filter paper from fresh whole blood, which has intact erythrocytes and other constituents. This is critical to note in multi-element analyses where elements bound to proteins may interact with the filter paper matrix differently than other elements (Cizdziel, 2007). There are also additional limitations in the range of elemental concentrations in available SRMs from the INSPQ, for example zinc is clustered in two concentrations.

Additional challenges to quantifying elements in DBS is the influence of hematocrit levels, which affects the spread of the sample, and, fundamentally, the unknown sample volume. One study by Capiau et al. (2013) demonstrates a direct correlation between hematocrit level and potassium (K) content in the blood. They suggest using K levels to predict hematocrit in a DBS, which can then be used to calculate the sample volume in a given punch or area (Capiau, Stove, Lambert, & Stove, 2013).

4.3 Contamination of the Filter Paper

As discussed, one of the key limitations, arguably the most important limitation in elemental analysis, is the background element contamination in the filter paper that is discussed extensively in the literature. Margui et al. (2017) found that quantitative determination of Cu and Zn was not possible due to instrumental blanks. Langer et al. (2010) found filter paper median levels were highly variable within and between lots for most elements and state that the primary obstacle to quantitative elemental analysis is element contributions from filter paper. The minimization of the contribution of elemental contamination from the filter paper is critical to quantification. In some publications, such as Hsieh et al. (2011) and Vacchina et al. (2014), a different type of filter paper was used to resolve the issue or the filter paper was processed to remove elemental contamination. Other publications suggest normalization as a method of reducing the influence of contamination on DBS elemental values.

A solution is to normalize the samples using one or multiple elements with a narrow physiological distribution and is absent in the blank filter paper (Stove et al. 2012, Langer et al. 2010). This tool can overcome contamination in elemental analysis and account for both variations in hematocrit and unknown sample volume (Stove et al., 2012). Langer et al. (2010) suggests the use of potassium (K) as it is found at low levels in unspotted filter paper and could be used to normalize volume differences. Additional elements, such as magnesium (Mg) and calcium (Ca), have a narrow range in blood and could also be used to normalize values. However, Langer et al. (2010) suggests they may be inappropriate due to high concentrations and variability in blank filter paper samples. Although these elements can be quantified with the current TXRF method, the SRMs used in this study are not certified for Ca, K, or Mg and, thus, accuracy and precision currently cannot be calculated to explore this method.

CHAPTER 5. CONCLUSION

5.1 Key Findings and Contributions

The development of minimally invasive methods for sample collection facilitates the implementation of biomarkers into research programs and is an essential component in health assessments and evaluation of health interventions. These results indicate that elemental analysis of DBS using TXRF provide a semi-quantitative method for measuring select essential elements in sub-sampled DBS. This paper contributes to a very limited body of literature outlining laboratory techniques for the simultaneous multi-element analysis of essential elements in DBS and is one of the few methods applicable to residual newborn DBS. These results can help establish a cost-effective screening method of collecting and measuring essential elements in nutritional research, overcoming many of the challenges associated with venipuncture and current analytical methods, potentially leading to informed public health policies and interventions. This method allows for the analysis of sub-samples of DBS, providing acess to limited samples archived from newborn screening programs or other global demographic surveys. Furthermore, the ability to draw larger and more diverse representative samples due to decreased burden on participants, reduced costs and increased ease of sample collection leads to an increase in the generalizatibility of research findings and may identify subgroups of individuals that require immediate action or additional monitoring. Overall, these results provide an alternative and cost-effective method to analyze DBS to better evaluate, monitor and manage newborn nutrition and population health. This method overcomes many of the challenges associated with venipuncture and current analytical methods available, potentially leading to informed health policies and inventions.

5.2 Future Directions

As discussed extensively, there are currently many gaps and challenges in our ability to fully quantify elements in DBS. First of all, there is a need to account for variation in hematocrity and the unknown sample volume as this have significant effects on the quatificaton of analytes. Normalization may be a possible future improvement and tool to overcome contamination in elemental analysis and account for both the variation in hematocrit and unknown sample volume (Stove et al., 2012). This has been discussed as a solution in various papers but has not been applied to current analytical methods as far as we know. The use of elements such as Ca, K and Mg, which are physiologically regulated, provide opportunities to account for variation. However, additional methods need to be validated to reliably measure these elements in addition to analytes of interest. Furthermore, future research should address the homogeneity for each individual elements as elements bound to protein may interact with the filter paper matrix differently than those not bound to protein or elements in SRMs (Cizdziel, 2007).

In addition, the elemental variability in the filter paper has been a topic of discussion in various publications and needs to be further investigated. Some publications, such as Hsieh et al. (2011) and Vacchina et al. (2014), use a different type of filter paper to circumvent the issue of elemental contamination in their analysis. Although this has led to favorable results and is an effective protocol in collecting samples for prospective studies, it limits the ability to use residual samples from newborn screening programs or other global demographic surveys. The variability in the fitler paper and potential methods for accounting for contamination need to be further investigated.

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APPENDICES

Figure 1. Dose-Response Relation for Vitamin and Mineral Nutrients

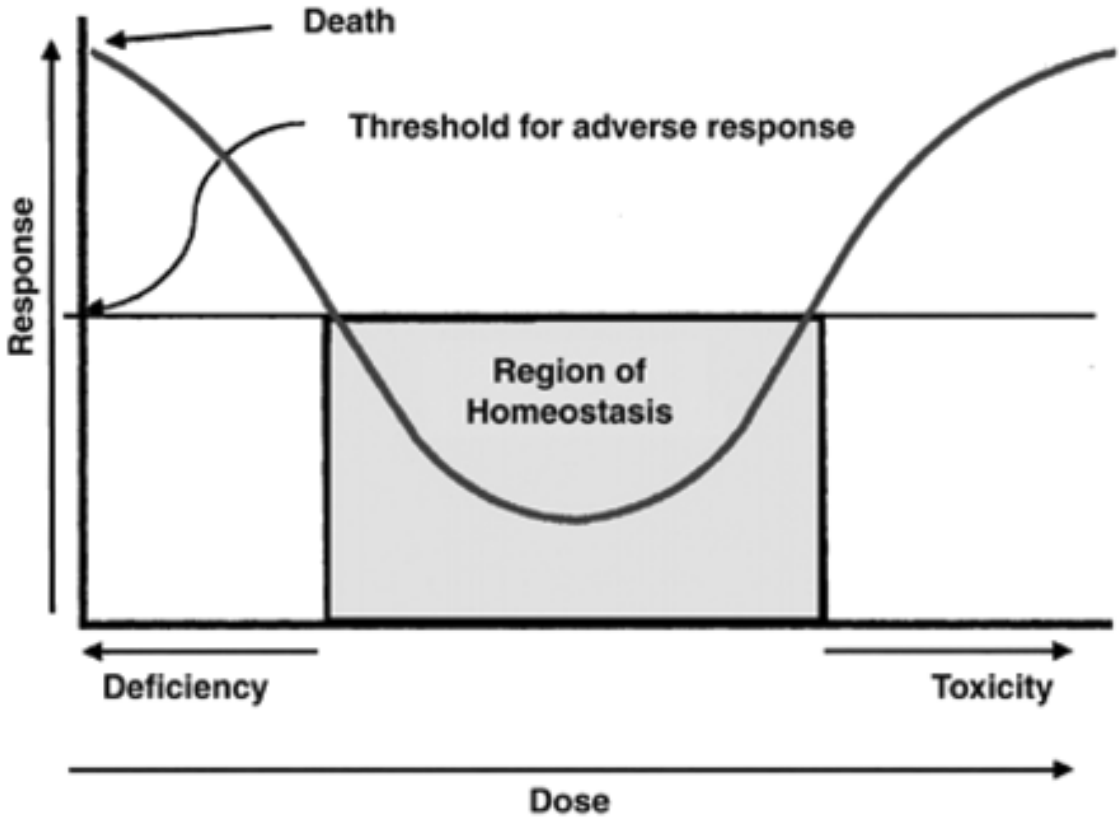

Figure 1. Adapted from Hayes (2008). Demonstrates the dose-response relation for essential vitamin and mineral nutrients. The U-shaped response has a region of homeostasis between thresholds of adverse effects from both deficiency and toxicity.

Figure 2. Linear Regression of Assigned Cu SRM Concentrations to TXRF Concentrations

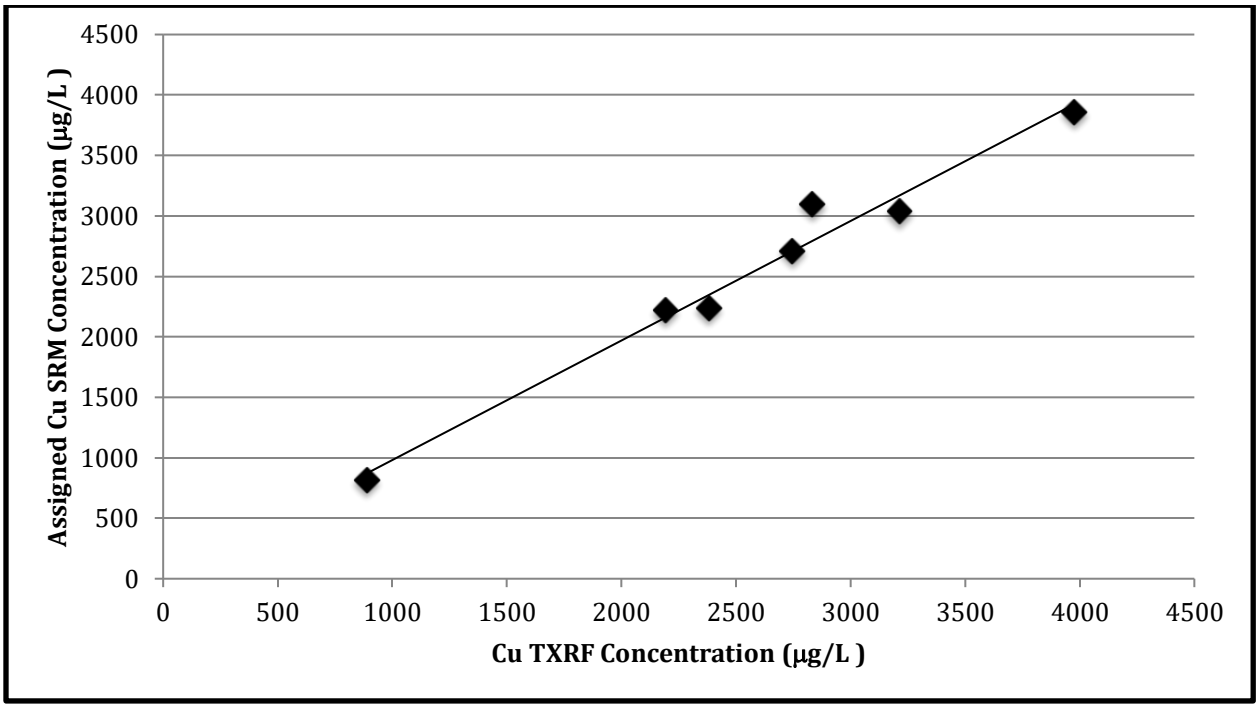

Figure 2. Linear Regression of Cu TXRF Concentration (µg/L) to Assigned SRM Cu Concentration (µg/L). Each point is an average of 3 independent samples (n=3). $y=0.99x - 7.2$, $R^2 = 0.98$, Average Recovery of all measurements is 102.3% ± 5.9.

Figure 3. Linear Regression of Assigned Se SRM Concentrations to TXRF Concentrations

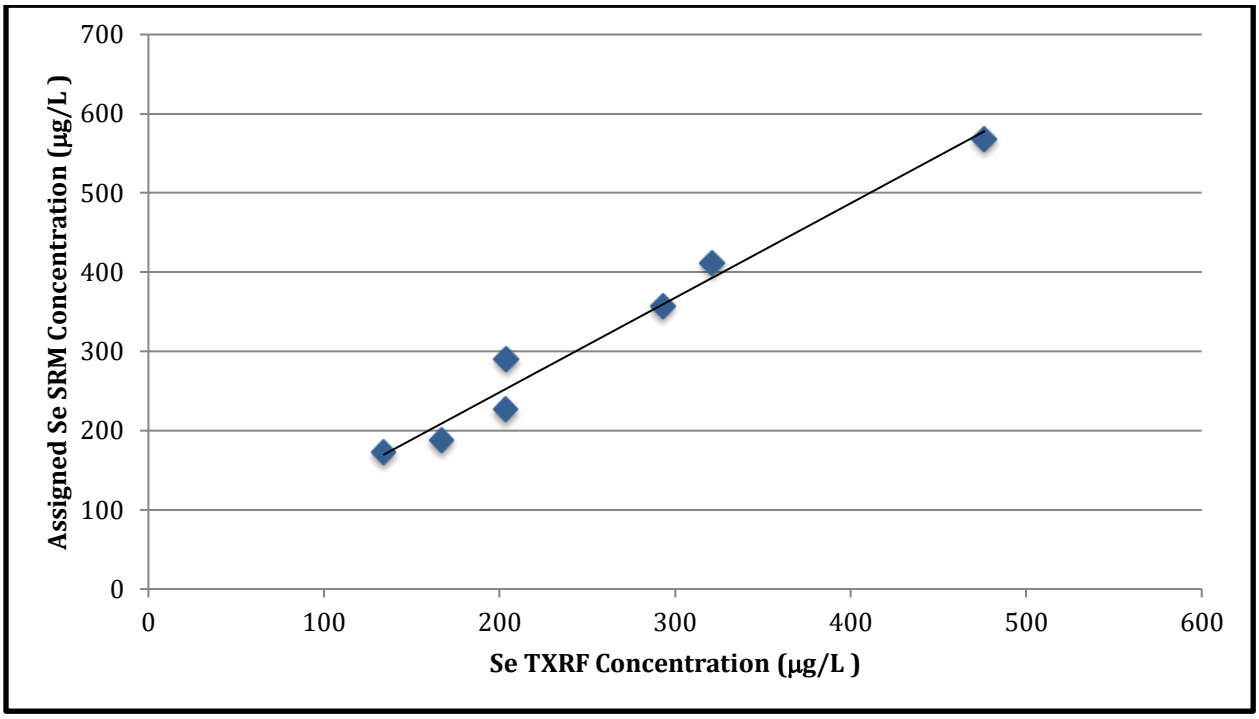

Figure 3. Linear Regression of Se TXRF Concentration (µg/L) to Assigned SRM Se Concentration (µg/L). Each point is an average of 3 independent samples (n=3). $y=1.2x + 9.6$, $R^2 = 0.975$, Average Recovery of all measurements is 100.9% ± 8.6.

Figure 4. Linear Regression of Assigned Zn SRM Concentrations to TXRF Concentrations

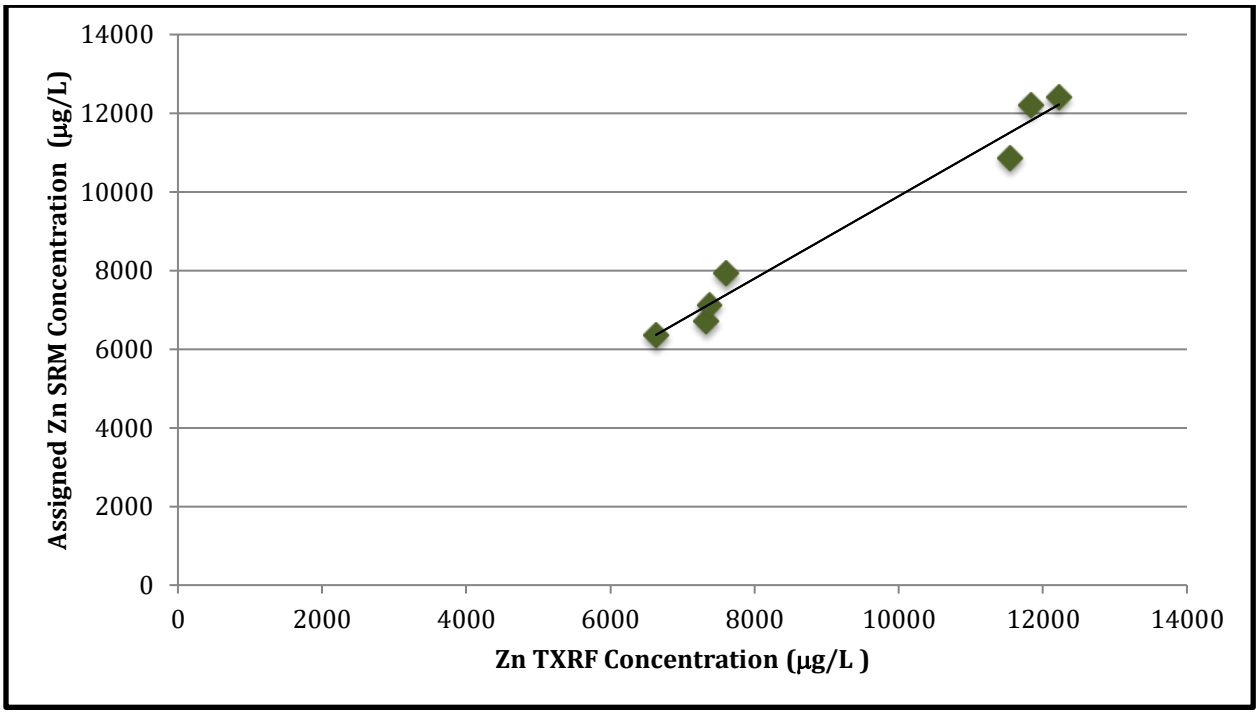

Figure 4. Linear Regression of Zn TXRF Concentration (µg/L) to Assigned SRM Zn Concentration (µg/L). Each point is an average of 3 independent samples (n=3). $y=1.05x - 585.5$, $R^2 = 0.975$, Average Recovery: 102.3% ± 5.6.

Figure 5. Intra-Assay Precision of Copper

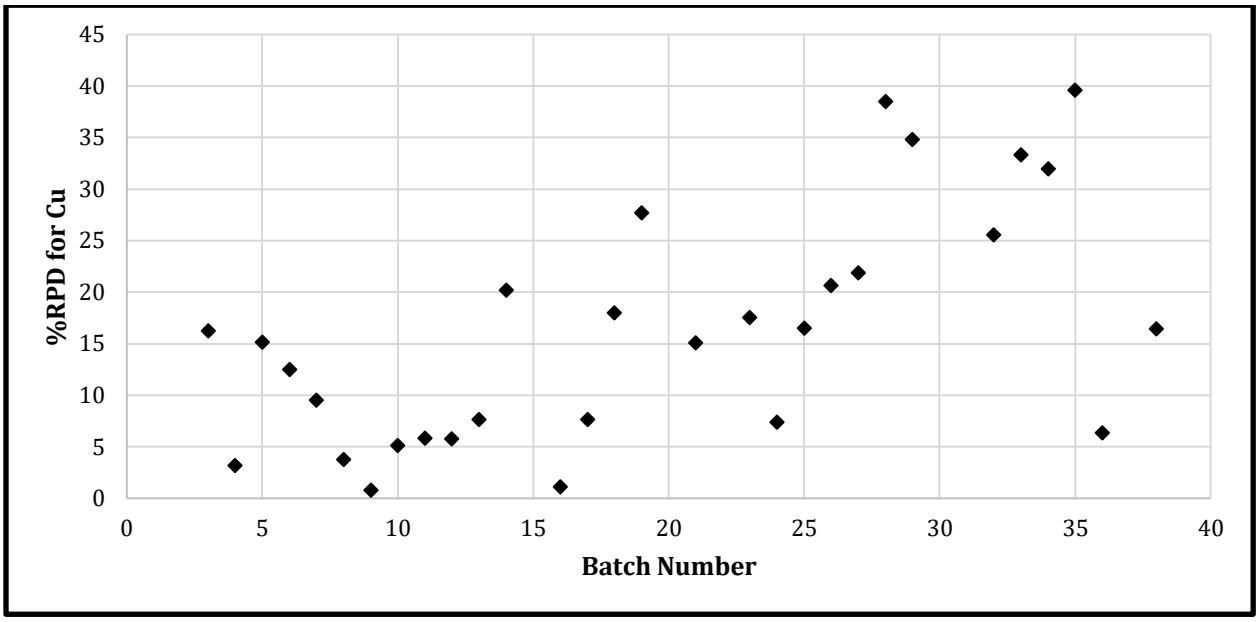

Figure 5. Plot of %RPD for Copper (Cu) concentration of duplicates in a given batch (n=30) as a measure of intra-assay precision. The %RPD values ranged from 0.76% to 39.56%.

Figure 6. Intra-Assay Precision of Selenium

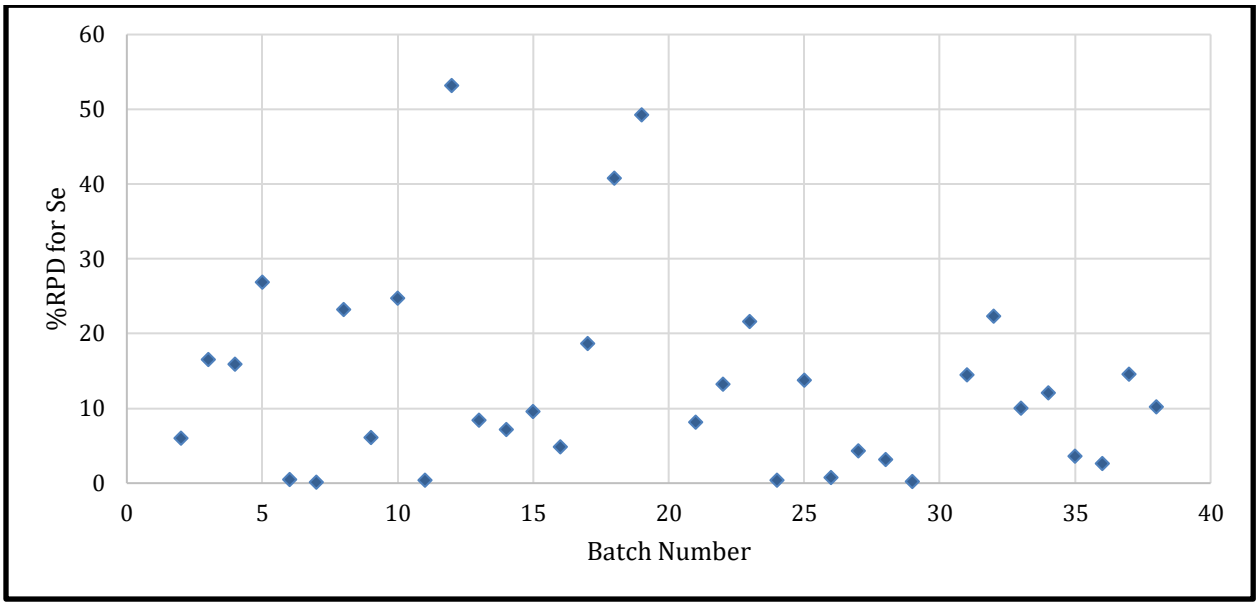

Figure 6. Plot of %RPD for Selenium (Se) concentration for duplicates in a given batch (n=35) as a measure of intra-assay precision. The RPD% values ranged from 0.14% to 53.17%.

Figure 7. Intra-Assay Precision of Zinc

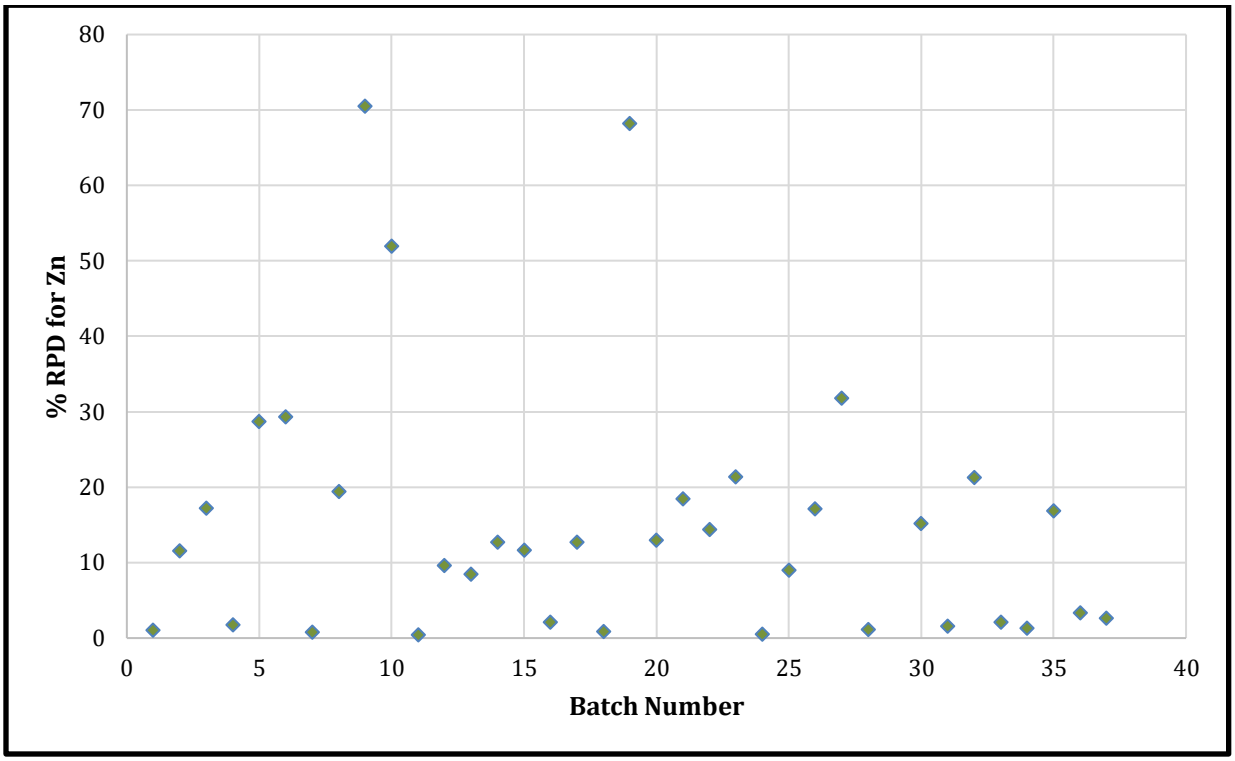

Figure 7. Plot of %RSD for Zinc (Zn) concentration for duplicates in a given batch (n=36) as a measure of intra-assay precision. The %RPD values ranged from 0.44% to 70.5%.

Figure 8. Box Plots of Accuracy by SRM of Copper

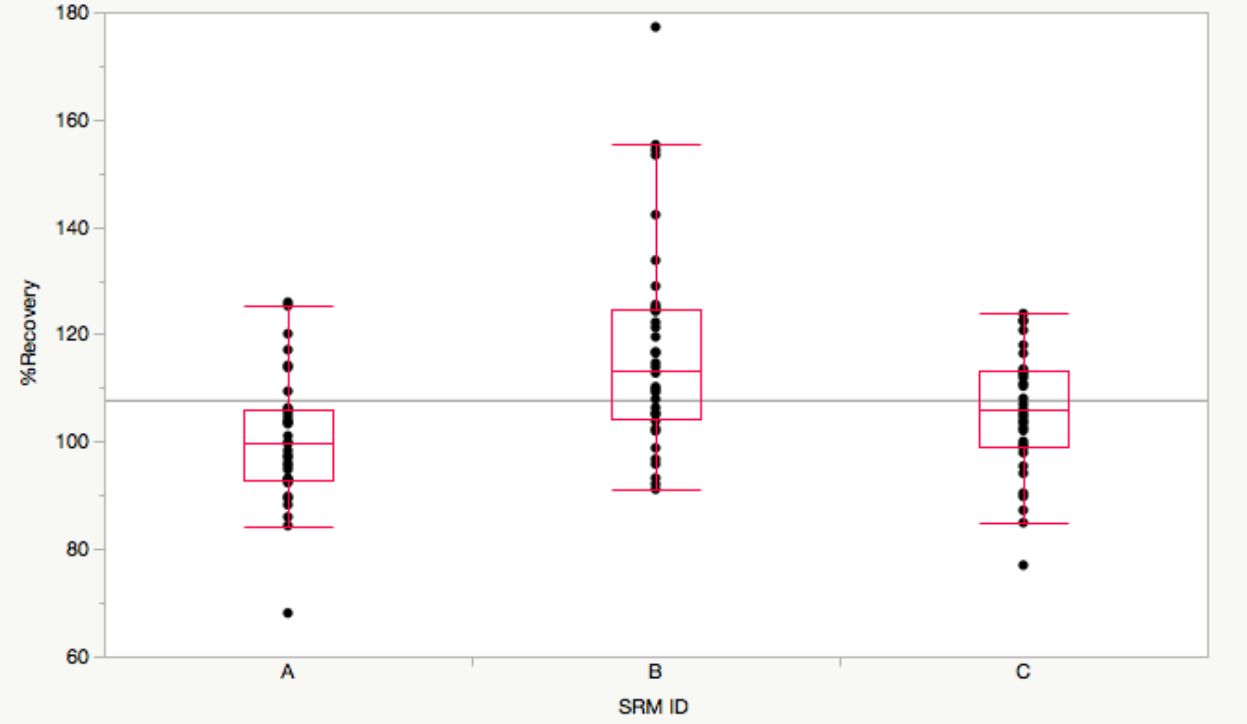

Figure 8. Box Plots of the %Recovery, a measure of accuracy, for Copper (Cu) in the three different SRMs across the different batches (n=37). The %Recovery ranged from 68.1% in SRM A to 177% in SRM B.

Figure 9. Box Plots of Accuracy by SRM of Selenium

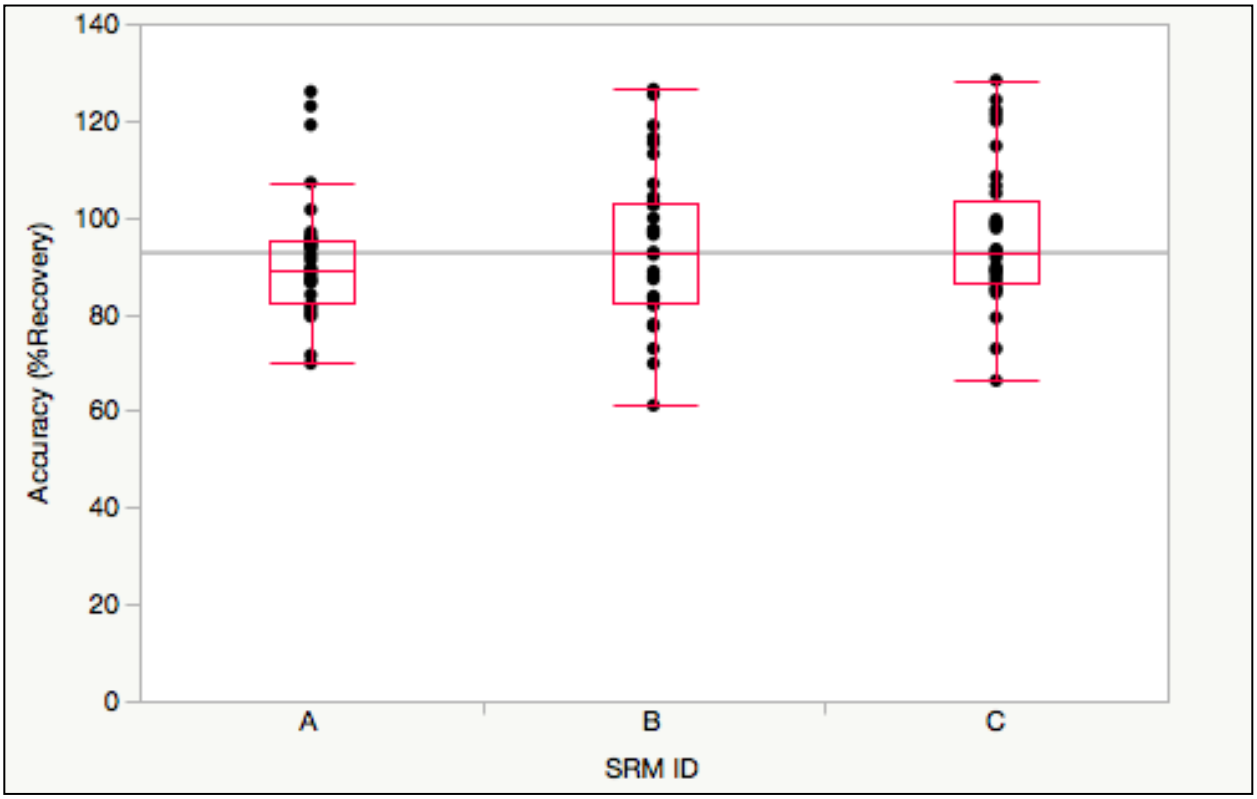

Figure 9. Box Plots of the % Recovery, a measure of accuracy, for Selenium (Se) in the three different SRMs across the different batches (n=37). The %Recovery ranged from 61.07% in SRM B to 128.25% in SRM C.

Figure 10. Box Plots of Accuracy by SRM of Zinc

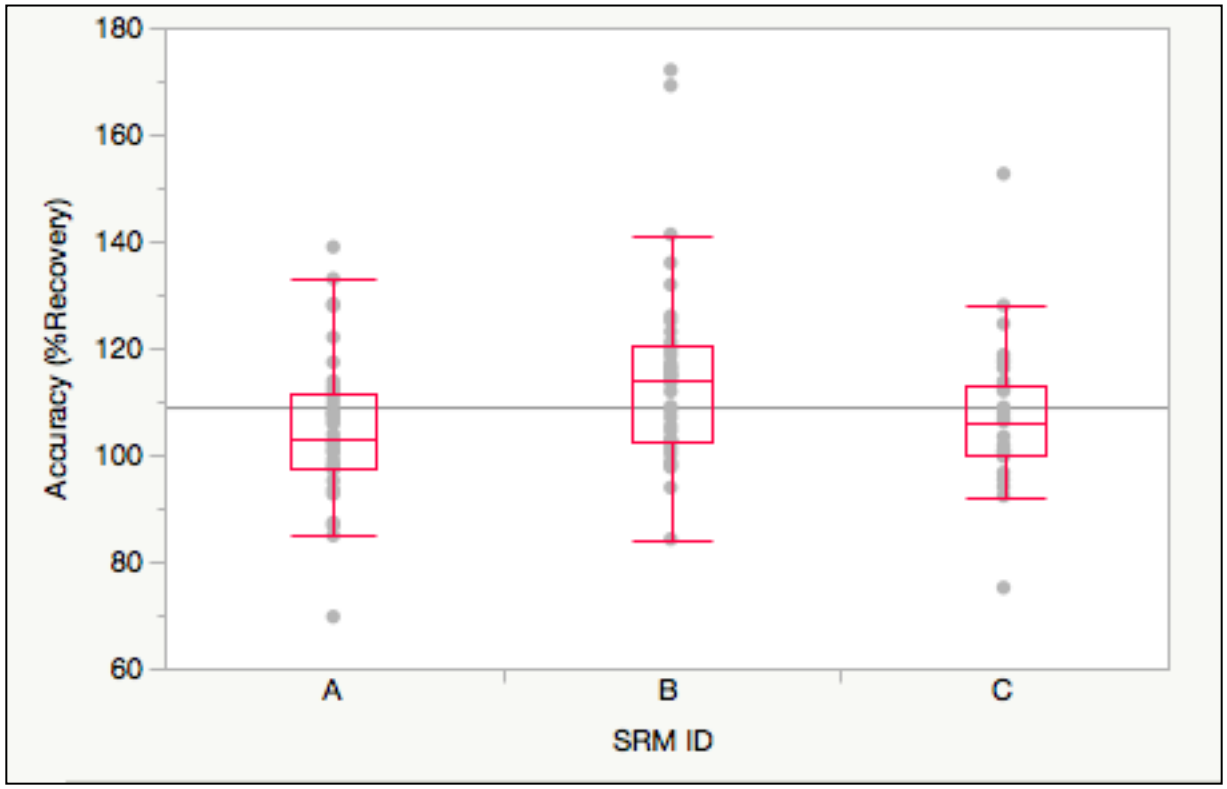

Figure 10. Box Plots of the % Recovery, a measure of accuracy, for Zinc (Zn) in the three different SRMs across the different batches (n=37). The %Recovery ranged from 69.65% in SRM A to 172.0% in SRM C.

Figure 11. Distribution of Copper by Batch in the Michigan BioTrust Cohort

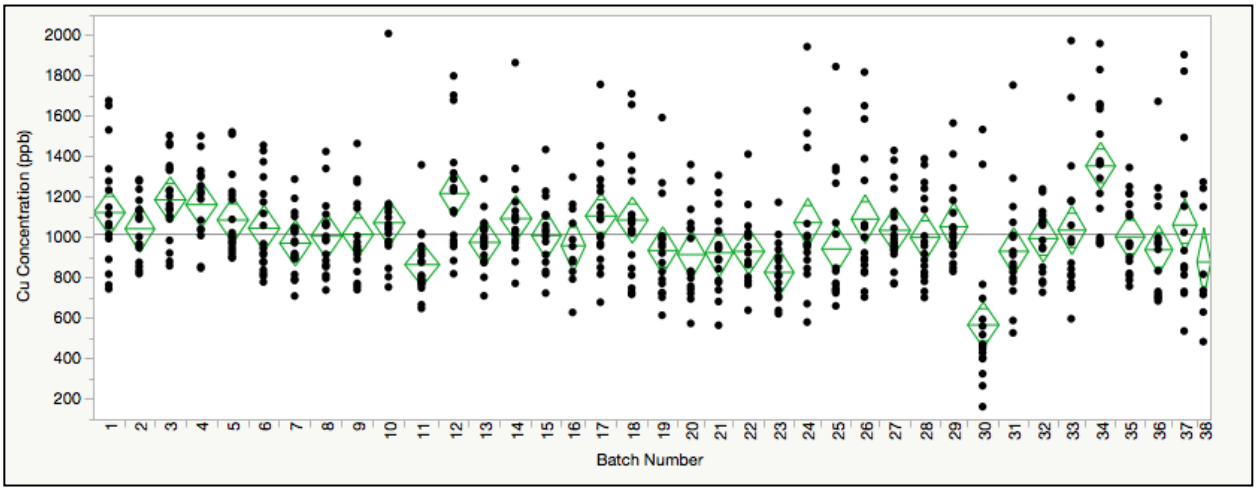

Figure 11. Comparison of Cu concentration across batches in the Michigan BioTrust for Health Cohort (n=38).

Figure 12. One-Way ANOVA Comparison of Copper in the Michigan BioTrust Cohort

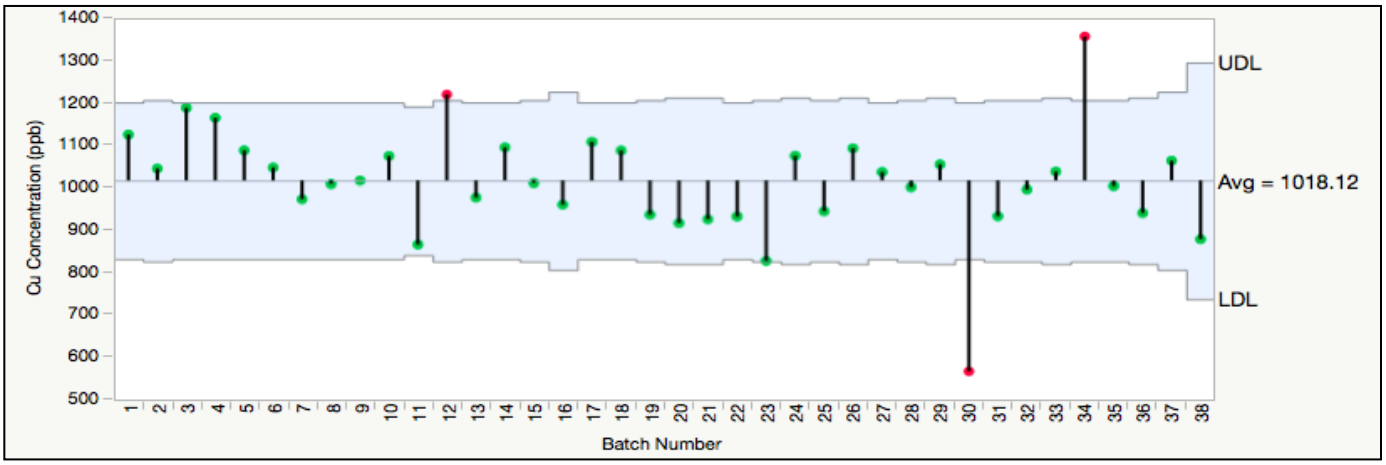

Figure 13. One-way ANOVA comparison across the batches analyzed for Cu in the Michigan BioTrust for Health Cohort (p-value < 0.05, 3 batches exceed the limits at α=0.05). The batch numbers line up with Figure 11 for comparison.

Figure 13. Comparison of Copper Concentrations in Two Different Punches

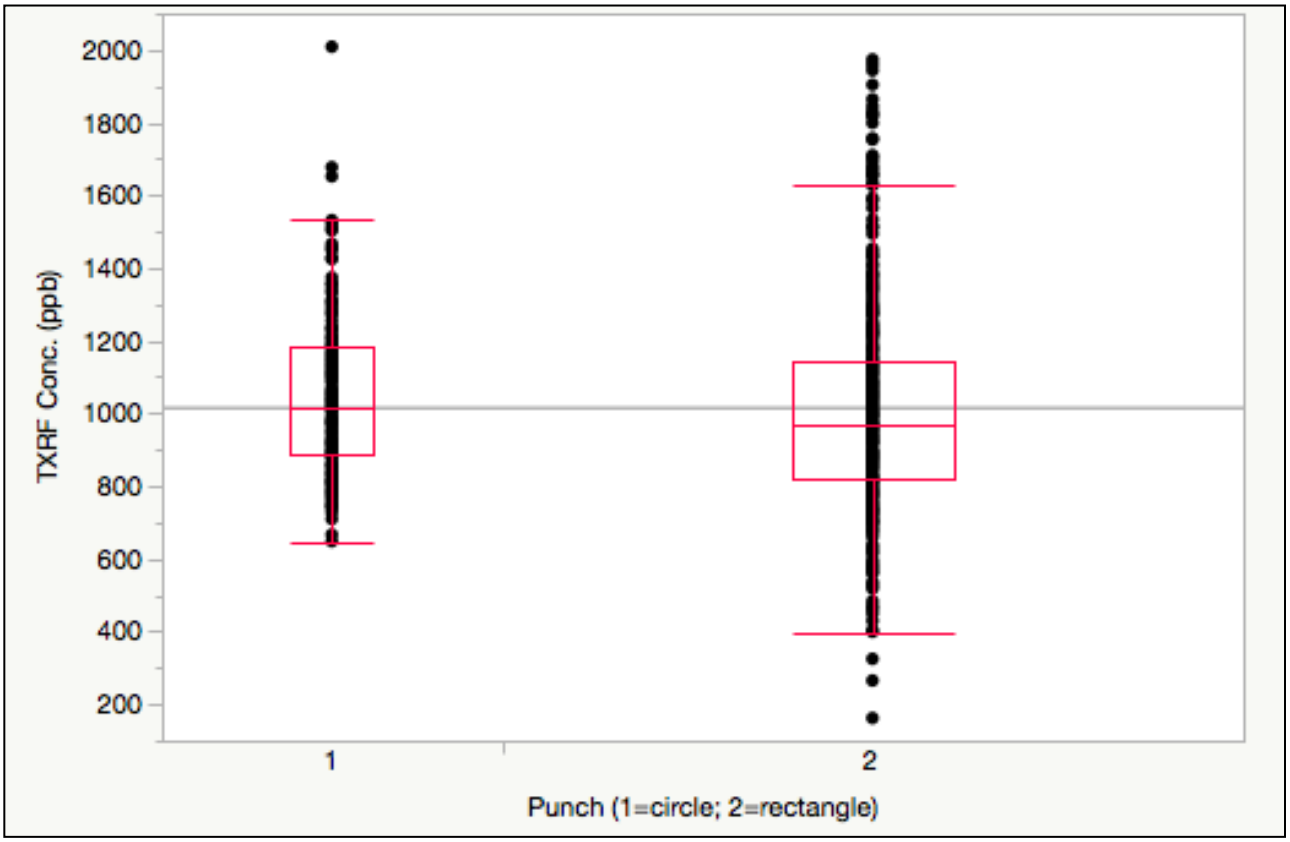

Figure 13. Comparison of circular punch (3mm diameter) to the rectangular punch (2mm x 6 mm) provided by the Michigan BioTrust Project using a t-test (p-value = 0.099).

Figure 14. Comparison of Copper Concentrations of Two Storage Methods

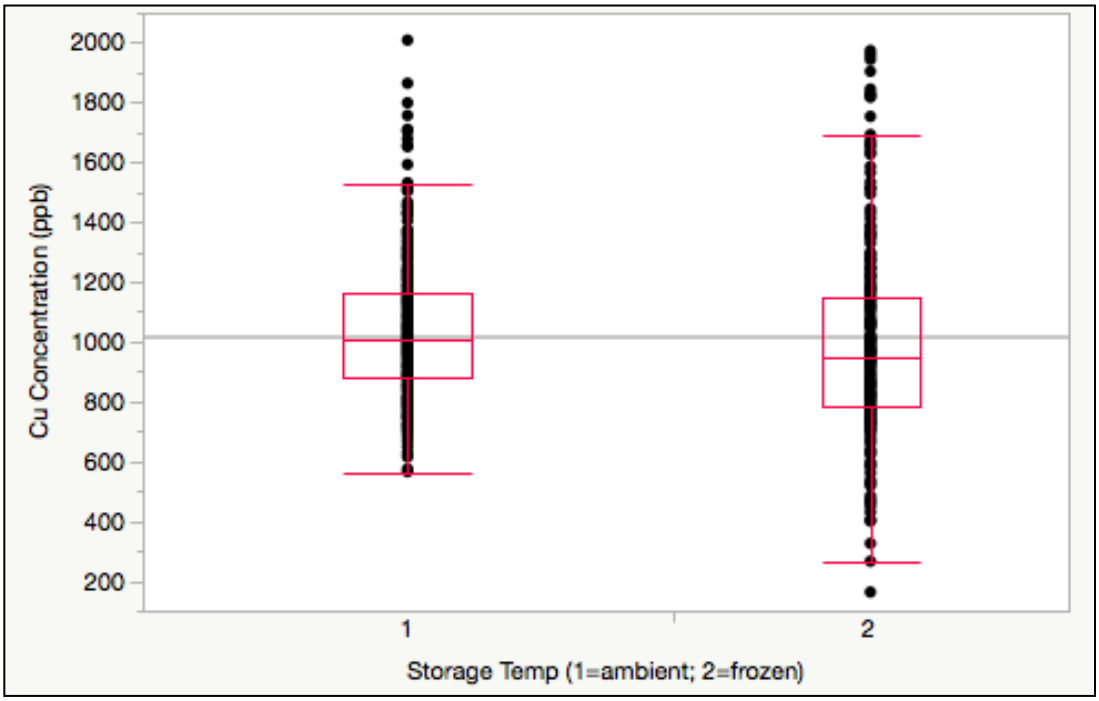

Figure 14. Comparison of ambient to frozen long-term storage temperatures in the Michigan BioTrust Project using a t-test (p-value = 0.066)

Figure 15. Distribution of Selenium by Batch in the Michigan BioTrust Cohort

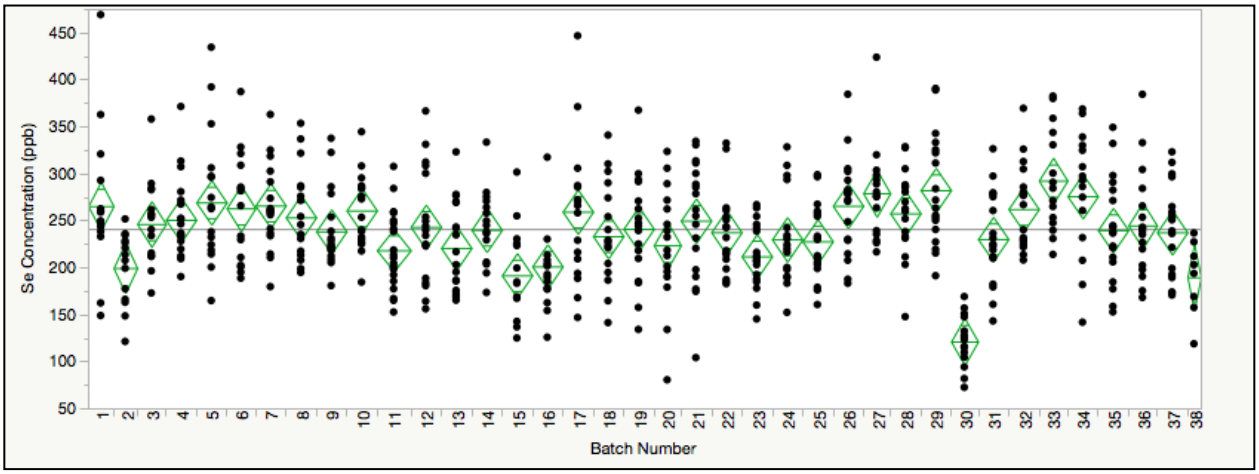

Figure 15. Comparison of Se concentration across batches in the Michigan BioTrust for Health Cohort (n=38).

Figure 16. One-Way ANOVA Comparison of Selenium in the Michigan BioTrust Cohort

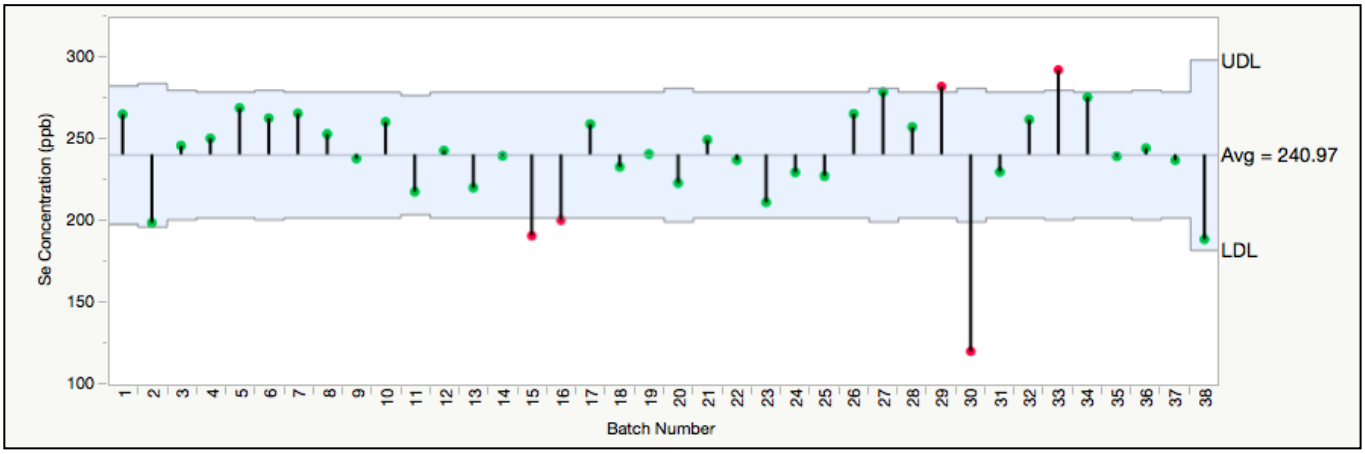

Figure 16. One-way ANOVA comparison across the batches analyzed for Se in the Michigan BioTrust for Health Cohort (p-value < 0.05, 5 batches exceed the limits at α=0.05). The batch numbers line up with Figure 15 for comparison.

Figure 17. Comparison of Selenium Concentrations in Two Different Punches

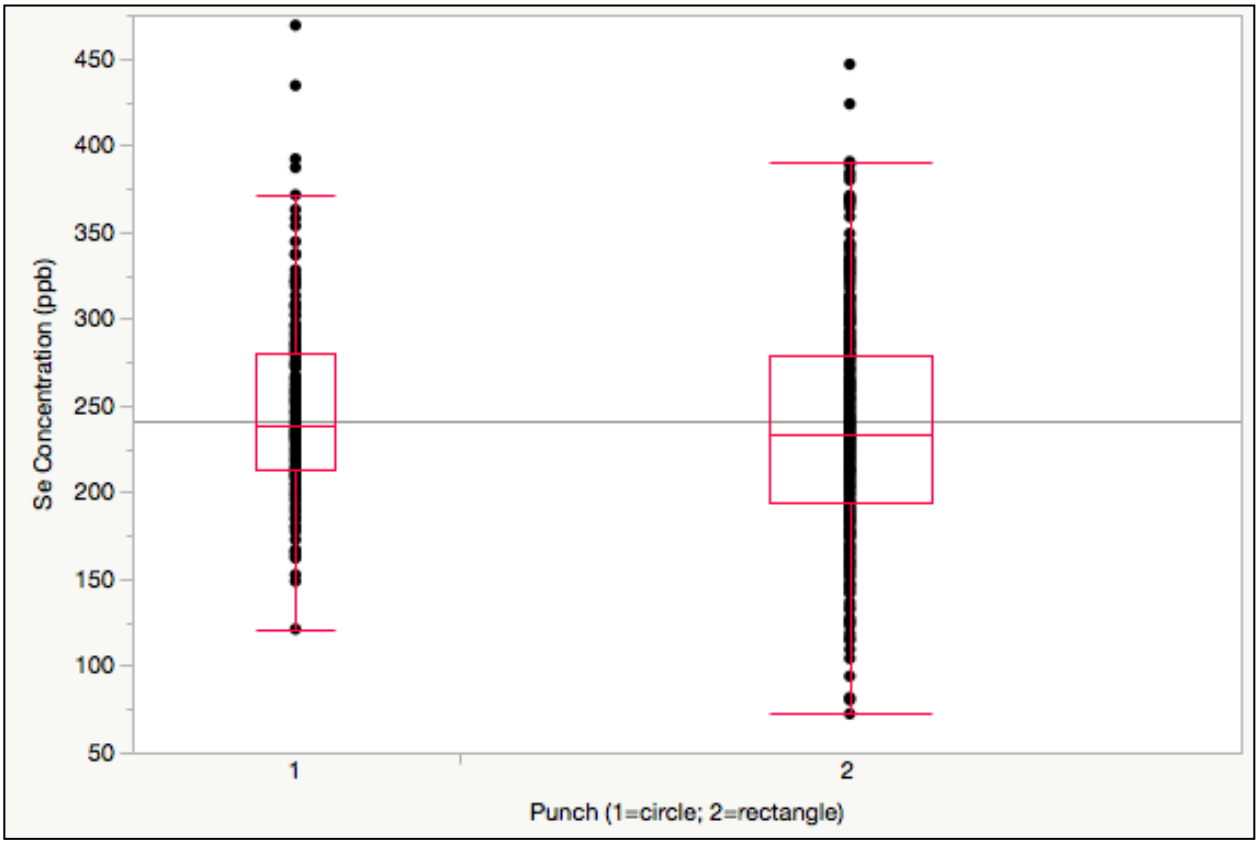

Figure 17. Comparison of circular punch (3mm diameter) to the rectangular punch (2mm x 6 mm) provided by the Michigan BioTrust Project using a t-test (p-value = 0.0143)

Figure 18. Comparison of Selenium Concentrations of Two Storage Methods

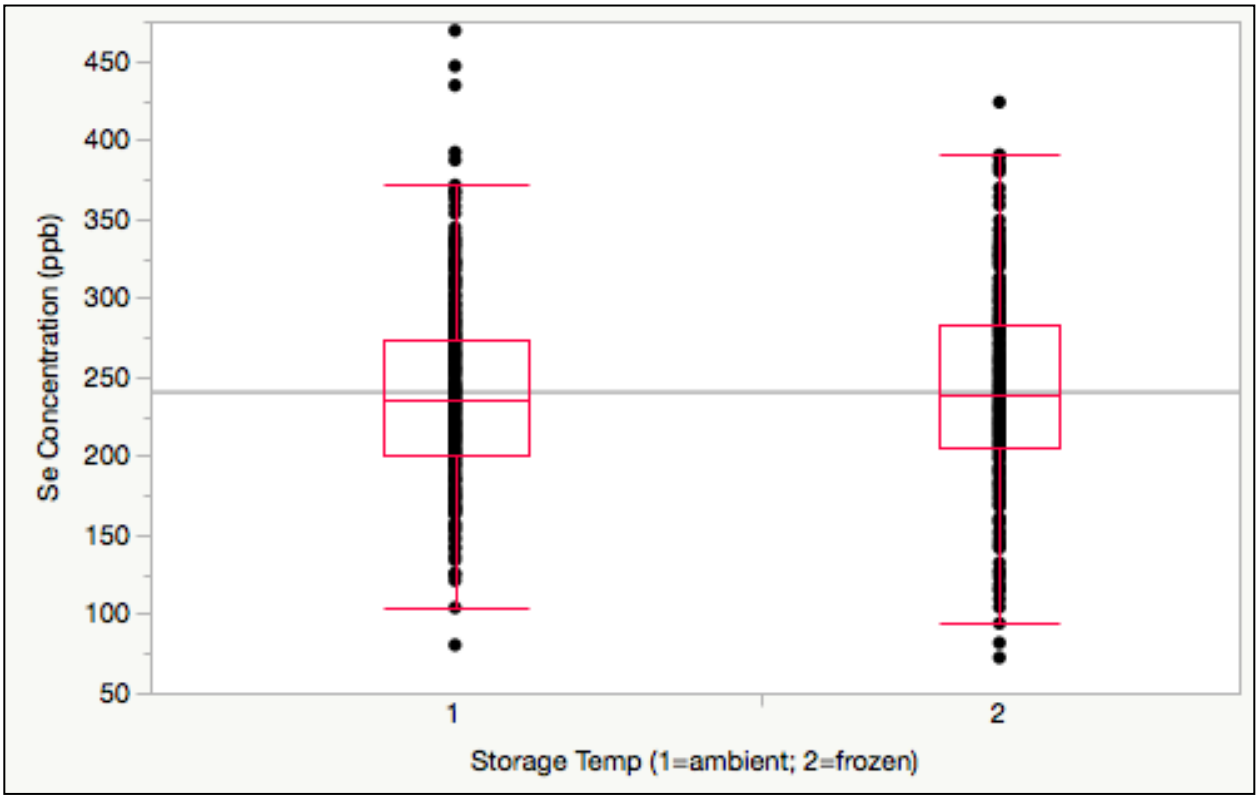

Figure 18. Comparison of ambient to frozen long-term storage temperatures in the Michigan BioTrust Project using a t-test (p-value = 0.77).

Figure 19. Distribution of Zinc by Batch in the Michigan BioTrust Cohort

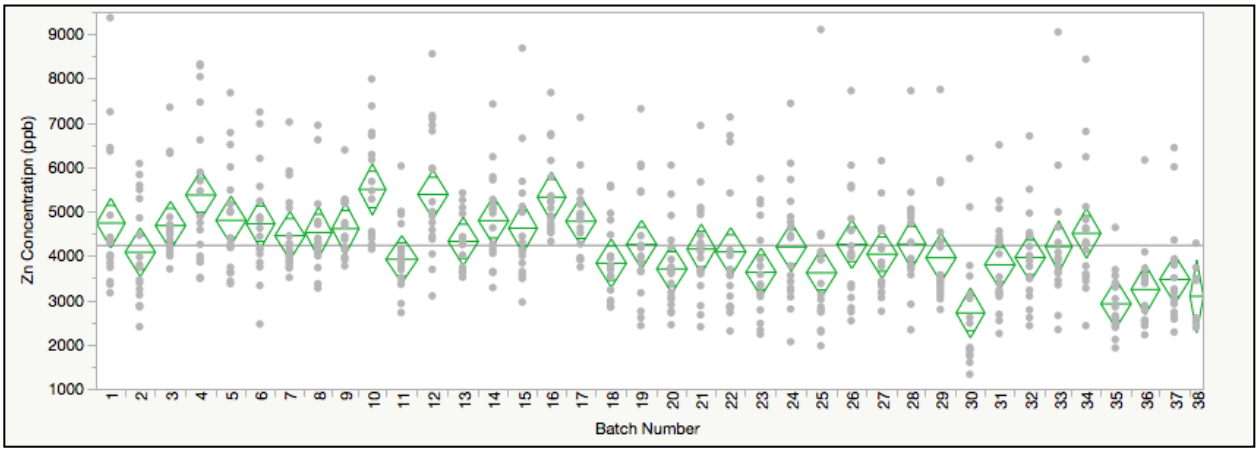

Figure 19. Comparison of Zn concentration across batches in the Michigan BioTrust for Health Cohort (n=38).

Figure 20. One-Way ANOVA Comparison of Copper in the Michigan BioTrust Cohort

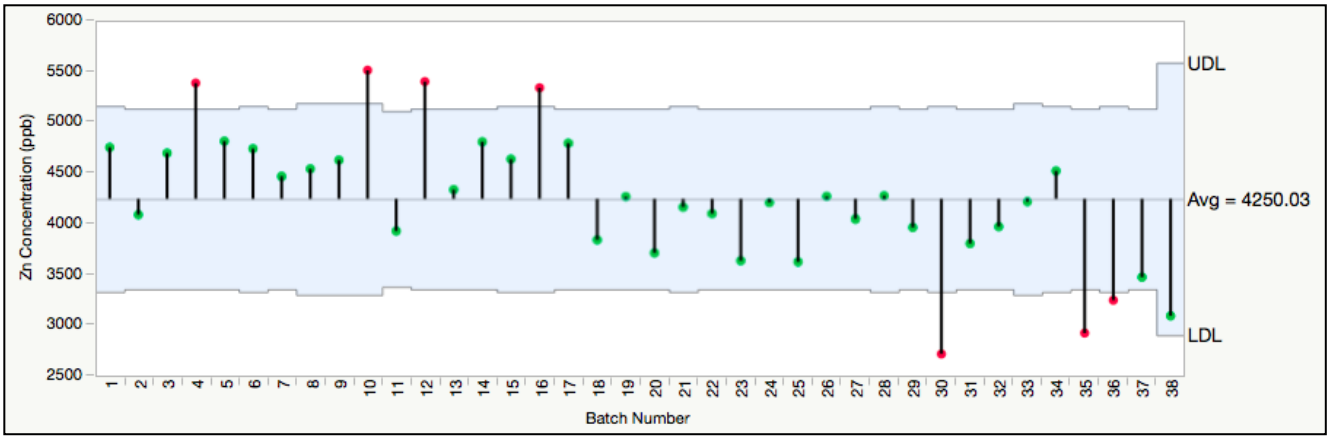

Figure 20. One-way ANOVA comparison across the batches analyzed in the Michigan BioTrust for Health Cohort (p-value < 0.05, 7 batches exceed the limits at α=0.05). The batch numbers line up with Figure 19 for comparison.

Figure 21. Comparison of Zinc Concentrations in Two Different Punches

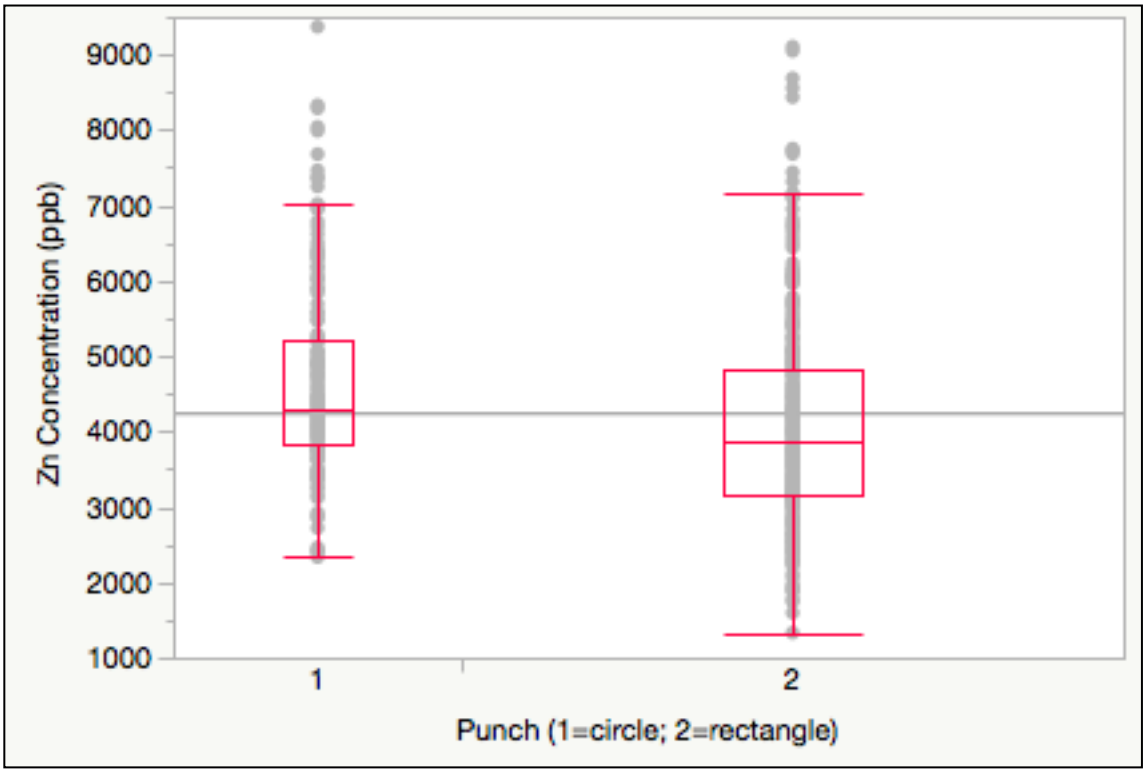

Figure 21. Comparison of circular punch (3mm diameter) to the rectangular punch (2mm x 6 mm) provided by the Michigan BioTrust Project using a t-test (p-value < 0.001)

Figure 22. Comparison of Zinc Concentrations of Two Storage Methods

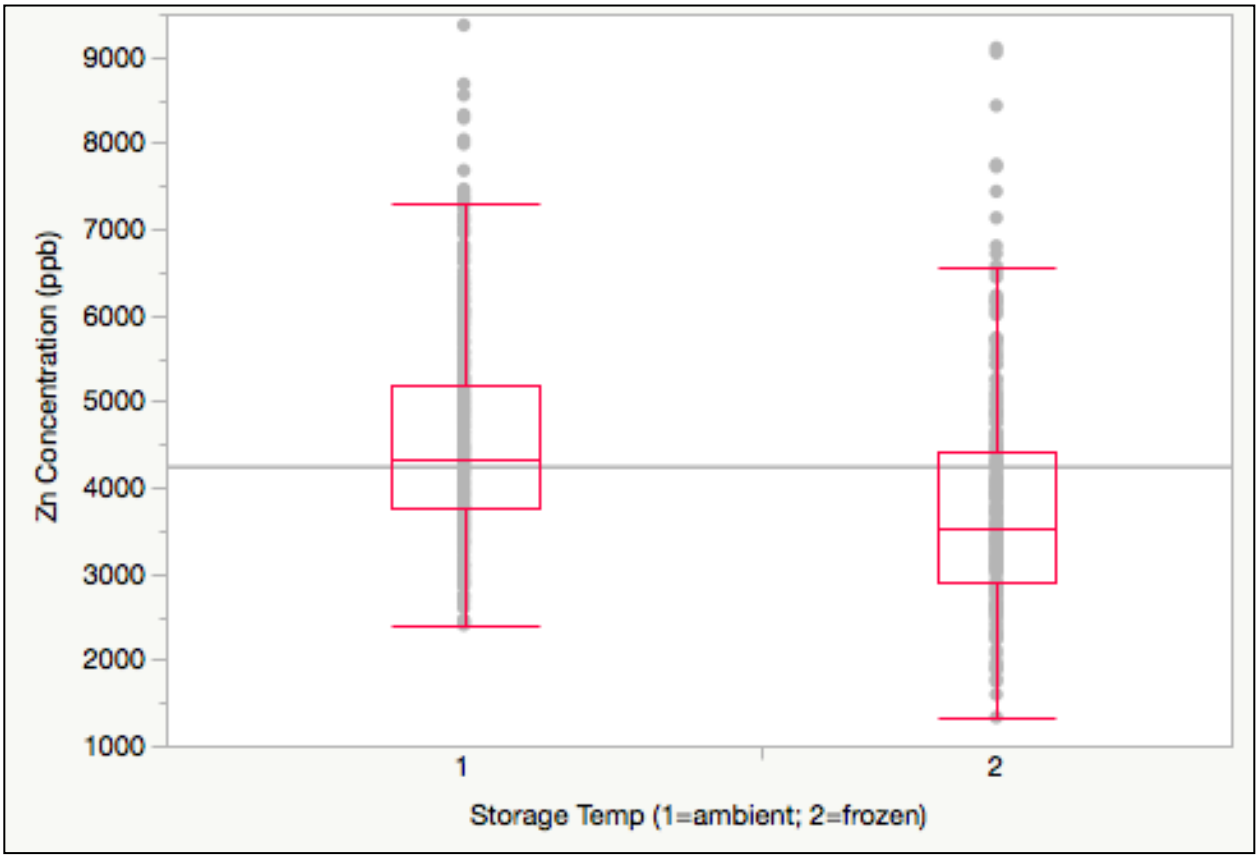

Figure 22. Comparison of ambient to frozen long-term storage temperatures for Zn concentrations in the Michigan BioTrust Project using a t-test (p-value = 0.77).

Table 1. Summary of Literature Measuring Multiple Elements in Dried Blood Spots (DBS)

Author	Year of Study	Population	Essential Elements*	Analytical Instrument
Cizdziel	2007	SRM	Cu, Zn	Laser ablation ICP time-of-flight- mass spectrometry (LA-ICP-TOF-MS)
Langer et al.	2010	SRM, newborns from Minnesota (n=150)	Cu, Zn	Sector-field ICP-MS (SF-ICP-MS)
Hsieh et al.	2011	SRM	Cu, Zn	Laser ablation coupled with ICP-MS (LA-ICP-MS)
Vacchina et al.	2014	SRM, healthy adults (n=2)	Cu, Se, Zn	ICP-MS
Margui et al.	2017	SRM, healthy adults (n=4)	Cu, Se, Zn	Energy dispersive X-ray fluorescence spectrometry (EDXRF)
Lombeck et al.	1989	Mothers and newborns from Greece and China	Se	Atomic Absorption Spectrophotometer

**This column does not include all the elements analyzed in the study, only the elements relevant to this research, specifically Cu, Se, and Zn..*

Table 2. Technical specification of the TXRF spectrometer S2 PICOFOX

Technical Specification	Properties
X-ray tube	Mo target, microfocus, 50 kV, 0.75 mA
Element range	Al to Y, Pd to U
Optics	Multilayer monochromator, 17.5 keV
Detector	Si drift chamber, 30 mm^2; <160 eV resolution at Mn Kα
Sample station	Cassette changer for 25 samples
Size, weight	600 x 300 x 450 mm, 37 kg
Power consumption	150 W

Table 2. adapted from Bruker Nano GmbH (2011); Stosnach & Mages (2009)

.

Table 3. Variables that were tested for Assay Optimization

Variables	Test Conditions
Acid Extraction	Concentrated HCl
	Conconcentrated HNO3
Incubation	Room Temperature Overnight
	Microwave Digest
	IsoTemp at 55°C for 1 hour
	IsoTemp at 55°C for 1.5 hours
Centrifuge	15 min, 12000 rpm
	12 min, 15000 rpm
Internal Standard	Gallium (various levels)
	Multi-element
PVA	1% Polyvinyl Alcohol (PVA)
	0.5% Polyvinyl Alcohol (PVA
	No Polyvinyl Alcohol (PVA
Serva	Serva
	No Serva
Drying Method	Room temperature
	Hot plate
	Vacuum Desiccator
	Lab oven

Table 4 Specifications of Human Whole Blood Reference Material from the *Institut National de Santé Publique du Québec* (INSPQ)

Whole Blood SRM Identification	Cu Concentration (μg/L)	Se Concentration (μg/L)	Zn Concentration (μg/L)
1313 (SRM A)	2236.82	411.44	5812.28
1314	3094.69	290.61	7910.98
1404	3857.24	568.59	7126.42
1505 (SRM B)	813.39	172.16	6335.32
1506 (SRM C)	3037.50	226.65	10853.08
10B	2220.0	357.0	12400.0
9B	2710.0	188.0	12200.0
Mean ± SD	2567.1 ± 956.5	316.4 ± 141.6	8948.3 ± 2804.0

Designations: 1314 = SRM A, 1505 = SRM B, 1506 = SRM C

Table 5. Precision Measurements of Copper, Selenium and Zinc SRM

	SRM	Mean (μg/L)	Standard Deviation (μg/L)	%Coefficient of Variation (CV)
Cu	SRM A	3103.66	365.89	11.78
	SRM B	953.38	160.20	16.80
	SRM C	3215.41	349.27	10.86
Se	SRM A	264.37	53.73	13.55
	SRM B	158.23	36.71	23.20
	SRM C	216.91	33.13	15.27
Zn	SRM A	8297.06	1104.13	13.31
	SRM B	7297.46	1145.67	15.70
	SRM C	11591.06	1412.10	12.18

Table 6. Accuracy Measurements of Copper, Selenium and Zinc SRM

	SRM	Assigned SRM Concentration (μg/L)	TXRF Concentration (μg/L)	Recovery (%)
Copper (Cu)	SRM A	3094.9	3103.7 ± 365.9	100.3 ± 11.8
	SRM B	813.4	953.4 ± 160.2	117.2 ± 19.6
	SRM C	3037.7	3215.4 ± 349.3	105.8 ± 11.5
Selenium (Se)	SRM A	290.6	264.4 ± 35.9	90.9 ± 12.2
	SRM B	172.1	158.2 ± 36.7	91.9 ± 21.3
	SRM C	226.6	216.9 ± 33.1	95.7 ± 14.6
Zinc (Zn)	SRM A	7910.9	8297.1 ± 1104.1	104.9 ± 13.9
	SRM B	6335.3	7297.5 ± 1145.7	115.2 ± 18.1
	SRM C	10853.1	11591.1 ± 1412.1	106.8 ± 13.0

Table 7. Descriptive Statistics of the Michigan BioTrust for Health Cohort

Statistics	Copper (μg/L)	Selenium (μg/L)	Zinc (μg/L)
Sample Size	643	659	658
Mean	1018.1	240.9	4250.0
Standard Deviation	269.9	58.2	1314.9
Minimum	161.9	72.2	1328.1
1st Quartile	832.8	202.8	3375.0
Median	984.7	236.1	4041.1
3rd Quartile	1154.5	279.5	4919.4
Maximum	2008.3	468.9	9367.4