Improving Lead Exposure Assessment Using Dried Blood Spots

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ABSTRACT

Lead (Pb) is a notable metal with a diverse range of industrial and societal applications. Unfortunately, Pb is also a toxicant of global concern given that it contaminates a range of natural and human ecosystems, affects multiple physiological systems, and its toxic effects can manifest at very low exposure concentrations. As such, Pb is regulated by many organizations such as Health Canada, the US Environmental Protection Agency (EPA), the American Occupational Safety and Health Administration (OSHA), and the World Health Organization (WHO).

Assessing exposures to Pb exposure is one of the best approaches in helping prevent Pb poisoning. Whole blood remains the preferred biomarker for biomonitoring Pb exposure in many population groups, however blood sampling has several limitations, including its invasive nature, the large volume of sample required, and the need for special conditions for its collection, transportation, and storage.

Dried Blood Spots (DBS) is a low-invasive blood sampling technique that has been applied as an alternative to traditional venipuncture methods for decades. While some studies have measured Pb in DBS, there remain outstanding limitations that have prevented the wider adoption of DBS for Pb exposure assessment.

The objective of this thesis was to identify the main limitations of DBS for Pb exposure assessment, and then to address these gaps by developing, validating, and applying novel methods and analysis approaches to improve the overall performance of this sampling technique.

We carried out a systematic review to gather information on the barriers and opportunities concerning the application of DBS for biomonitoring lead exposure. In doing so, data was synthesized and analyzed from 36 peer-reviewed publications, from which we identified three main challenges: a) the agreement between capillary and venous whole blood, b) the analytical sensitivity/low volume issues, and c) the differences of hematocrit in DBS samples. Based on this evidence, we designed and conducted a series of studies.

First, to evaluate the comparability between capillary and venous whole blood for the analysis of Pb, we obtained capillary and venous blood samples from volunteers from McGill University, and we evaluated: a) the differences in mean values between the two blood types using a t-test of paired samples (α= 0.05); b) the relationship of measurements between the two blood types using regression analysis; and c) the agreement between the two blood types using Bland-Altman analysis. We also evaluated concentrations of other relevant toxic and essential elements: Arsenic (As), Cooper (Cu), Cadmium (Cd), Iron (Fe), Manganese (Mn), and Selenium (Se). There were no significant differences in the mean concentrations of Pb between venous and capillary whole blood (α= 0.05) and we found a strong correlation (r^2= 0.96) between them. Besides, we noticed a good agreement between venous and capillary blood Pb measurements (mean of differences= 0.13 μg/L). We observed no statistically significant differences, positive correlations, and a good agreement for As and the essential elements, while we found an overestimation of capillary blood measurements compared to venous ones for Cd and Mn.

Second, to address challenges associated with the need for low sample volumes, faster preparation times, detection limits, and operational costs, we developed, validated, and

applied a novel method to assess lead exposure in DBS using a microanalytical technique called Total Reflection X-Ray Fluorescence. We first developed this method through the use of several blood reference materials with known concentrations of Pb, and then applied this method to two population groups: one of low Pb-exposed individuals (McGill members) and one of highly Pb-exposed individuals (workers from an electronic-waste site in Ghana). In terms of analytical performance, the limits of detection and quantification of the method were 0.28 and 0.69 μg/dL, respectively, which rival current approaches. The overall precision and accuracy of the method were 14.88 % (9.92-19.14 %) and 111.14 % (97.03-129.70 %), respectively. The McGill university members presented a mean (SD) of 0.78 (± 0.46) μg/dL, while the arithmetic mean (SD) was 3.78 (± 3.01) for the Ghanaian workers. In both population groups (and also combined), the Bland-Altman plots indicated a good agreement between Pb measurements in DBS samples between TXRF and ICP-MS, with a mean of differences of -0.02 μg/dL.

Third, to increase understanding of the hematocrit effect, we developed an AAS-based method to measure potassium concentrations in DBS, obtaining a LoD of 1.76 μg/L and a precision and accuracy values of 0.02-0.33% and 92.2-124.7%, respectively. Then, we developed a correction factor for hematocrit differences across DBS samples using these potassium measurements. We evaluated a range of hematocrit levels (0.25-0.60) and we evaluated the % of differences and the agreement between whole blood and DBS samples before and after applying the potassium-based correction. Following the same criteria in the second study, we decided to expand this analysis to other elements of interest, including metals and essential elements that are usually assessed in biomonitoring programs. Based on the agreement analysis, we observed that there was an improvement in the agreement of DBS and whole blood Pb measurements after applying the correction, with a reduction in the mean

of differences (bias) from 41.0 (95% CI: 0.26 to 0.55) to 0.17 μg/dL (95% CI: 0.01 to 0.33). Moreover, we evaluate the proportional error based on the regression line drawn in the Bland-Altman plot and we observed that the proportional bias was also reduced from a slope value of 0.55 to 0.007, showing a considerable improvement after the correction.

Collectively, this doctoral thesis demonstrates that the limitations of DBS for the assessment of Pb exposure can be improved, and thus, this sampling technique can be applied in vulnerable and general populations more extensible (including not only clinical settings but also field conditions). The methods developed here, can improve the application of DBS in biomonitoring programs and population studies, allowing for an increase in the study participants and the inclusion of remote populations.

RÉSUMÉ

Le plomb (Pb) est un métal remarquable avec une gamme diversifiée d'applications industrielles et sociétales. Malheureusement, le plomb est également un toxique de préoccupation mondiale étant donné qu'il contamine une gamme d'écosystèmes naturels et humains, affecte de multiples systèmes physiologiques et ses effets toxiques peuvent se manifester à de très faibles concentrations d'exposition. En tant que tel, le plomb est réglementé par de nombreuses organisations telles que Santé Canada, l'Agence américaine de protection de l'environnement (EPA), l'American Occupational Safety and Health Administration (OSHA) et l'Organisation mondiale de la santé (OMS).

L'évaluation des expositions à l'exposition au plomb est l'une des meilleures approches pour aider à prévenir l'empoisonnement au plomb. Le sang total reste le biomarqueur préféré pour la biosurveillance de l'exposition au plomb dans de nombreux groupes de population, mais l'échantillonnage sanguin présente plusieurs limites, notamment sa nature invasive, le grand volume d'échantillon requis et la nécessité de conditions spéciales pour sa collecte, son transport et son stockage.

Les taches de sang séché (DBS) sont une technique de prélèvement sanguin peu invasive qui est utilisée depuis des décennies comme alternative aux méthodes traditionnelles de ponction veineuse. Bien que certaines études aient mesuré le Pb dans le DBS, il reste des limites non résolues qui ont empêché l'adoption plus large du DBS pour l'évaluation de l'exposition au Pb.

L'objectif de cette thèse était d'identifier les principales limites du DBS pour l'évaluation de l'exposition au Pb, puis de combler ces lacunes en développant, validant et appliquant de

nouvelles méthodes et approches d'analyse pour améliorer les performances globales de cette technique d'échantillonnage.

Tout d'abord, nous avons effectué une revue systématique pour recueillir des informations sur les obstacles et les opportunités concernant l'application du DBS pour la biosurveillance de l'exposition au plomb. Ce faisant, les données ont été synthétisées et analysées à partir de 36 publications évaluées par des pairs, à partir desquelles nous avons identifié trois défis principaux : a) l'accord entre le sang total capillaire et veineux, b) les problèmes de sensibilité analytique/de faible volume, et c) les différences de l'hématocrite dans les échantillons DBS. Sur la base de ces preuves, nous avons conçu et mené une série d'études.

Premièrement, pour évaluer la comparabilité entre le sang total capillaire et veineux pour l'analyse du Pb, nous avons obtenu des échantillons de sang capillaire et veineux de volontaires de l'Université McGill, et nous avons évalué : a) les différences de valeurs moyennes entre les deux groupes sanguins en utilisant un t -test d'échantillons appariés (α= 0.05) ; b) la relation des mesures entre les deux groupes sanguins à l'aide d'une analyse de régression ; et c) l'accord entre les deux groupes sanguins à l'aide de l'analyse de Bland-Altman. Nous avons également évalué les concentrations d'autres éléments toxiques et essentiels pertinents : l'arsenic (As), le cuivre (Cu), le cadmium (Cd), le fer (Fe), le manganèse (Mn) et le sélénium (Se). Il n'y avait pas de différences significatives dans les concentrations moyennes de Pb entre le sang total veineux et capillaire (α= 0.05) et nous avons trouvé une forte corrélation (r^2= 0.96) entre elles. Par ailleurs, nous avons remarqué une bonne concordance entre les mesures de Pb dans le sang veineux et capillaire (moyenne des différences = 0.13 μg/L). Nous n'avons observé aucune différence statistiquement significative, des corrélations positives et un bon accord pour l'As et les éléments essentiels,

tandis que nous avons trouvé une surestimation des mesures du sang capillaire par rapport aux mesures veineuses pour le Cd et le Mn.

Deuxièmement, pour relever les défis associés au besoin de faibles volumes d'échantillons, de temps de préparation plus rapides, de limites de détection et de coûts opérationnels, nous avons développé, validé et appliqué une nouvelle méthode pour évaluer l'exposition au plomb dans le DBS à l'aide d'une technique microanalytique appelée Total Reflection X-Fluorescence des rayons. Nous avons d'abord développé cette méthode en utilisant plusieurs matériaux de référence sanguins avec des concentrations connues de Pb, puis nous avons appliqué cette méthode à deux groupes de population : l'un des individus faiblement exposés au Pb (membres de McGill) et l'autre des individus fortement exposés au Pb (travailleurs d'un site de déchets électroniques au Ghana). En termes de performances analytiques, les limites de détection et de quantification de la méthode étaient respectivement de 0.28 et 0.69 μg/dL, ce qui rivalise avec les approches actuelles. La précision et l'exactitude globales de la méthode étaient de 14,88 % (9.92-19.14 %) et de 111.14 % (97.03-129.70 %), respectivement. Les membres de l'université McGill ont présenté une moyenne (SD) de 0.78 (± 0.46) μg/dL, alors que la moyenne arithmétique (SD) était de 3.78 (± 3.01) pour les travailleurs ghanéens. Dans les deux groupes de population (et également combinés), les graphiques de Bland-Altman ont indiqué une bonne concordance entre les mesures de Pb dans les échantillons de DBS entre TXRF et ICP-MS, avec une moyenne de différences de -0.02 μg/dL.

Troisièmement, pour mieux comprendre l'effet de l'hématocrite, nous avons développé une méthode basée sur le AAS pour mesurer les concentrations de potassium dans le DBS,

obtenant une LoD de 1.76 μg/L et des valeurs de précision et d'exactitude de 0.02-0.33 % et 92.2-124.7 %, respectivement. Ensuite, nous avons développé un facteur de correction pour les différences d'hématocrite entre les échantillons DBS en utilisant ces mesures de potassium. Nous avons évalué une gamme de niveaux d'hématocrite (0.25-0.60) et nous avons évalué le % de différences et la concordance entre les échantillons de sang total et de DBS avant et après l'application de la correction à base de potassium. En suivant les mêmes critères dans la deuxième étude, nous avons décidé d'étendre cette analyse à d'autres éléments d'intérêt, y compris les métaux et les éléments essentiels qui sont habituellement évalués dans les programmes de biosurveillance. Nous avons observé une amélioration de la concordance des mesures de DBS et de Pb dans le sang total après application de la correction, avec une réduction de la moyenne des différences (biais) de 41.0 (IC à 95 % : 0.26 à 0.55) à 0.17 μg/dL (IC à 95 % : 0.01 à 0.33). De plus, nous évaluons l'erreur proportionnelle sur la base de la droite de régression tracée dans le graphique de Bland-Altman et nous avons observé que le biais proportionnel était également réduit d'une valeur de pente de 0.55 à 0.007, montrant une amélioration considérable après la correction.

Collectivement, cette thèse de doctorat démontre que les limites du DBS pour l'évaluation de l'exposition au Pb peuvent être améliorées, et ainsi, cette technique d'échantillonnage peut être appliquée dans des populations vulnérables et générales plus extensibles (non seulement les milieux cliniques mais aussi les conditions de terrain). Les méthodes développées ici peuvent améliorer l'application de la DBS dans les programmes de biosurveillance et les études de population, permettant une augmentation du nombre de participants à l'étude et l'inclusion de populations éloignées

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

This thesis fills important knowledge gaps and contributes to the advancement of knowledge as follows:

The exposure to lead (Pb) occurs on daily basis among all populations worldwide and it has negative effects in virtually all human systems even at low concentrations. The best strategy to cope with the health impacts of Pb exposure is monitoring the levels of Pb in the population, especially in those individuals who are vulnerable (e.g., children and occupationally exposed individuals). Whole blood has remained as the "gold standard" for the determination of Pb exposure in both surveillance programs and research studies. However, collecting whole blood poses several challenges including the need for especial settings and well-trained personnel, its accessibility, and its invasiveness.

Sampling blood is particularly challenging for vulnerable populations (e.g., children and elderly) and in remote or resource-limited regions, though these represent groups particularly at-risk for Pb exposure, so there is a need for alternative sampling techniques to overcome the limitation of venipuncture. One of the most popular alternatives to venous blood sampling, is the collection of capillary whole blood and alternative capillary blood-based samples such as dried blood spots (DBS). Unlike venipuncture, DBS sampling is simple, requires smaller blood volume, is less invasive and painful to subjects, in particular vulnerable groups (e.g., children, elderly). Despite the fact that DBS have been successfully applied for many years, there are some barriers that have been reported in the literature which have limited the wider adoption of DBS in biomonitoring programs. This thesis helps identify the main limitations of DBS in the determination of Pb exposure, and then develops, applies, and validates new methods to help overcome these potential barriers. The methods reported here are original, and they

contribute to advance the field of microanalytical techniques (particularly DBS) as well as the field of Pb exposure assessment.

The first manuscript (Chapter 2) is a systematic review of the existing literature of Pb exposure assessment studies using DBS from which main limitations of this sampling technique could be identified. To my knowledge, no systematic review on this subject matter has been conducted. In doing so, I was able to use quantitative and qualitative approaches to help identify critical next steps to improve the field of DBS for Pb exposure assessment. This chapter was used to help inform and refine the subsequent chapters (i.e., each subsequent chapter addresses a key analytical aspect often reported as a potential limitation). This manuscript was submitted for publication to the journal Environmental Toxicology and Pharmacology on July 8, 2021.

Chapter 3 is focused on the potential differences in Pb concentrations between capillary and venous blood. This study evaluated the association, differences, and most importantly, the agreement between the two sources of blood for the assessment of Pb (as well as other potentially toxic elements (As, Cd, and Mn) and essential elements (Cu, Fe, and Se)). Despite interest in the use of capillary blood as an alternative to venous blood, there is a lack of information in the literature on the comparability and agreement in the concentrations of toxic metals and essential elements between these two blood sources.

This study reports, for the first time, the degree of agreement between the two sources of blood for the assessment of relevant toxic elements in order to verify the reliability and quality of capillary blood as an alternative method in biomonitoring programs. The results indicated that measured concentrations of all elements were similar to values reported in

background populations. There were strong correlations (i.e., $p<0.001$) in measures taken for all these elements between capillary and venous blood and the Bland-Altman analysis demonstrated a relatively good agreement for blood As, Pb, Cu, and Fe. For blood Cd, Mn, and Se, the agreement between the measures taken in capillary and venous blood were less consistent, though more research is needed to determine if the method can be improved and/or if there are real differences. This research was submitted to the Journal of Trace Elements in Medicine and Biology on June 23, 2021, and it was presented in the ISEE 2018 conference on Environmental Epidemiology [Rodriguez Saldana, V., Basu, N., & Santa-Rios, A. (2018). Comparison and Agreement between Venous and Capillary Blood for the Analysis of Trace Elements. In ISEE Conference Abstracts (Vol. 2018, No. 1].

Chapter 4 comprises the development, validation, and application of a novel method to assess Pb exposure using two microanalytical techniques: DBS sampling and an analytical technique called Total Reflection X-Ray Fluorescence (TXRF). This study increases our comprehension of DBS as a potentially novel approach to characterize Pb exposures in both the general population (in a clinical environment) as well as a highly exposed population (in a resource limited and contaminated field site). The TXRF-based method we detail here helps overcome barriers associated with current strategies to measure Pb in DBS. In this study, TXRF was used for the first time in the analysis of DBS samples for the determination of Pb. The method developed here was applied with two groups of exposed individuals (i.e., a low-exposed group composed of McGill members and a group of highly exposed workers from an electronic waste site). The developed method was also compared with the "gold standard" for the analysis of Pb in DBS: Inductively Couple Plasma Mass Spectrometry (ICP-MS), obtaining comparable results and an acceptable agreement. This research was published in the journal Environmental Research [Rodríguez-Saldaña, V., Fobil, J., & Basu, N. (2021).

Lead (Pb) exposure assessment in dried blood spots using Total Reflection X-Ray Fluorescence (TXRF). Environmental Research, 198, 110444] and it was presented in the ISEE 2020 conference on Environmental Epidemiology [Rodriguez Saldana, V. and Basu, N. (2020, August). Lead (Pb) exposure assessment in dried blood spots using Total Reflection X-Ray Fluorescence (TXRF). In ISEE Conference Abstracts (Vol. 2020, No. 1)]

Last chapter (Chapter 5) focuses on the development of a method to measure potassium (K) in DBS samples, and to use this measure as a potential correction factor for the hematocrit differences in DBS samples. The "hematocrit effect" is one of the most reported limitations of DBS in the literature, and one of the biggest barriers to expand the use of DBS.

In this chapter, we reported a K-based method to correct for the hematocrit effect in the analysis of Pb, as well as other elements usually determined in biomonitoring programs (As, Mn, Cd, Cu, Fe, Se, and Zn). We evaluated a range of hematocrit levels (25 – 60%) of relevance to the general population as well as those with critically high and low hematocrit levels. Our results showed an improvement in the agreement of DBS and whole blood Pb measurements after applying the correction, with a reduction in the constant bias 41 (95% CI: 0.26 to 0.55) to 0.17 μg/dL (95% CI: 0.01 to 0.33), as well as a reduction in the proportional bias (based on the slope of regression line) from 0.55 to 0.007. This correction helps to extend the application of DBS sampling to other vulnerable populations (e.g., individuals with low hematocrit levels). This research will be submitted for publication to the journal Environmental Health in September 2021 and it was presented in the ISEE 2020 conference on Environmental Epidemiology [Rodriguez Saldana, V. and Basu, N. (2020, August). Correction of hematocrit for the analysis of lead (Pb) in dried blood spots using potassium measurements. In ISEE Conference Abstracts (Vol. 2020, No. 1)]

PREFACE AND CONTRIBUTION OF THE AUTHORS

This thesis is comprised of four original research chapters (Chapters 2 - 5), each of which have been prepared for submission, submitted, and/or published to an academic journal.

As the author of this thesis, and first author of each manuscript, I was responsible for the development of study objectives and hypotheses, literature review, lab work, data management, statistical analysis, interpretation of findings, and writing. Dr. Niladri Basu, as my primary advisor, provided guidance, feedback, and overall support throughout the thesis. For each manuscript, I developed the study design, methods, and rationale in collaboration with my co-authors.

Manuscript 1. The performance of Dried blood spots for the assessment of lead exposure: a narrative review with a systematic search. This manuscript was submitted to the Microchemical journal on September 12, 2021, and is now accepted for publication.

Authors: Verónica Rodríguez-Saldaña and Niladri Basu

Verónica Rodríguez-Saldaña: Conceptualization, methodology, formal analysis, investigation, resources, writing- original draft, writing-review and editing, and visualization.

Niladri Basu: Conceptualization, writing- review and editing, resources, and funding acquisition.

Manuscript 2. Comparability of venous and capillary whole blood in the assessment of metal(loid)s measured in human biomonitoring programs. This manuscript was accepted for publication and published online in the Journal Biological Trace Elements Research on September 21, 2021.

Authors: Verónica Rodríguez-Saldaña and Niladri Basu

Verónica Rodríguez-Saldaña: Conceptualization, methodology, formal analysis, investigation, resources, writing- original draft, writing-review and editing, and visualization.

Niladri Basu: Conceptualization, writing- review and editing, resources, and funding acquisition.

Manuscript 3. Lead (Pb) exposure assessment in dried blood spots using Total Reflection X-Ray Fluorescence (TXRF). This manuscript was published in the journal Environmental Research:

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Authors: Verónica Rodríguez-Saldaña, Julius Fobil, Niladri Basu

Verónica Rodríguez-Saldaña: Conceptualization, methodology, formal analysis, investigation, resources, writing- original draft, writing-review and editing, and visualization.

Julius Fobil: Resources, writing-review, and editing

Niladri Basu: Conceptualization, writing- review and editing, resources, and funding acquisition.

Manuscript 4. Correction of hematocrit for the analysis of metals and essential elements in dried blood spots using potassium measurements. This manuscript is planned to be submitted for publication to the Journal of Pharmaceutical and Biomedical Analysis in November 2021.

Authors: Verónica Rodríguez-Saldaña, Andrea Santa-Rios, and Niladri Basu

Verónica Rodríguez-Saldaña: Conceptualization, methodology, formal analysis, investigation, resources, writing- original draft, writing-review and editing, and visualization.

Andrea Santa-Rios: Sample collection assistance and writing-review and editing

Niladri Basu: Conceptualization, writing- review and editing, resources, and funding acquisition.

LIST OF ABBREVIATIONS

° C	Degree Celsius
µg/dL	Micrograms per deciliter
µg/L	Micrograms per liter
AAS	Atomic Absorption Spectroscopy
As	Arsenic
BLL	Blood Lead Levels
Cd	Cadmium
CDC	Centers for Disease Control and Prevention
CHMS	Canadian Health Measurements Survey
CV	Coefficient of Variation
DBS	Dried blood spots
E-waste	Electronic waste
dw	Dried weight
g	grams
Ga	Gallium
GM	Geometric Mean
Hct	Hematocrit
HCl	Hydrochloric acid
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
K^+	Potassium
KeV	Kiloelectron volt

LoD	Limits of Detection
LoQ	Limits of Quantification
MIBK	Methyl isobutyl ketone
mg	Milligrams
Mn	Manganese
NHANES	National Health and Nutrition Examination survey
Ni	Nickel
Pb	Lead
ppb	Parts per billion
ppm	Parts per million
QC	Quality control
RSD	Relative Standard Deviation
SD	Standard Deviation
Sr	Strontium
SRM	Standard Reference Material
TXRF	Total Reflection X-Ray Fluorescence
ww	Wet weight
XRF	X-Ray Fluorescence

1. **CHAPTER 1.** Introduction and literature review

1.1 General introduction

Lead (Pb) is an element of great concern to environmental and human health worldwide due to its intrinsic hazard potential and widespread use and distribution. This element appears on several priority lists of toxic substances by many regulatory groups owing to three main characteristics: a) it has multiple applications, and it has been widely used by humans for centuries (De Keersmaecker, 2018); b) it is a biocumulative metal that affects multiple body organs (WHO, 2018); and c) it does not have a "safe level" of exposure with adverse outcomes reported at very low exposure levels (WHO, 2018; CDC, 2020). Assessing and monitoring Pb exposure is key to identify sources, determine if there is an exposure, take decisions based on the level of exposure, and confirm that the blood Pb levels are declining after removing the sources of Pb.

Lead exposure has been assessed for decades as part of several biomonitoring programs of different countries. The "gold standard" to perform this assessment is collecting venous blood samples intravenously, followed by a chemical analysis using spectrometric techniques. However, this approach has been challenged by several limitations, mainly the invasive nature of venipuncture, as well as other logistical issues such as special conditions of samples collection, transportation, and storage. Consequently, alternative approaches have emerged to cope with these challenges.

Dried blood spots (DBS) is an alternative sampling technique that involves the use of capillary blood collected from a finger or heel prick onto standardized filter paper (USAID, 2012). This approach may help to overcome the barriers related to traditional venipuncture

sampling since the collection of capillary blood is less invasive, highly trained personnel are not required, and samples can be stored and transported without special conditions of temperature (Li and Lee, 2014; Sharma *et al*., 2014). Despite this promise, there have been challenges in measuring analyte concentrations in DBS owing to small sample volumes, concentrations, the potential contamination of samples, and other factors.

The overall objective of this work is to improve the use of DBS sampling in Pb exposure assessment by increasing our understanding of the main limitations of this alternative method (Chapter 2) and address these limitations by developing novel methods and applying analyses that cope with a) the differences of capillary and venous blood in the analysis of Pb (Chapter 3), b) the sample volume limitations of DBS (Chapter 4), and the hematocrit effect in the analysis of Pb in DBS by implementing a correction method to minimize it (Chapter 5).

1.1.1. Specific aims

Chapter 2: Investigate the limitations in the use of DBS as an alternative method for Pb exposure assessment among general and vulnerable populations.

- Conduct a comprehensive review of peer-reviewed scientific literature in databases on the current challenges and opportunities of DBS in the determination of Pb exposure.
- Extract and synthesize the information obtained from the searches to capture the variables of interest.
- Perform a narrative analysis (and a quantitative analysis when feasible) to identify the limitations and advantages of DBS to assess Pb exposure and provide recommendations based on the evidence.

Chapter 3: Determine the comparability between venous and capillary blood for the determination of Pb levels (and other elements of interest) in human whole blood samples, with special attention on the agreement between the two sources of blood.

- Evaluate the association between venous and capillary blood in the analysis of Pb and other relevant toxic elements.
- Determine the differences between the measurements of the elements of interest in venous and capillary whole blood.
- Perform an agreement analysis between venous and capillary blood for the assessment of Pb and other relevant elements.

Chapter 4: Development, validation, and application of a novel method to assess Pb exposure using DBS.

- Develop an accurate and precise method to determine Pb concentrations in DBS samples by Total Reflection X-Ray Fluorescence (TXRF).
    - Compare two different methods of samples digestion.
    - Investigate the most suitable element to be used as an internal standard.
    - Evaluate the improvement in the accuracy by implementing a Fe extraction procedure.
- Determine validation parameters to judge the quality, reliability, and consistency of the analytical results.
    - Calculate the limits of detection and quantification of the analytical method.
    - Determine the linearity and working range of the developed method.
    - Investigate the accuracy and precision of the method using entire DBS and sub-sampled DBS.
- Apply the developed method in the analysis of DBS samples from two different population groups: students from McGill University (low-exposed individuals) and workers from an electronic waste site in Ghana (high-exposed individuals).
    - Calculate descriptive statistics and the mean blood Pb levels of each group.
    - Compare the results between sources of blood: venous and capillary blood samples.
    - Assess the agreement between the analytical technique applied in this study (TXRF) and the “gold standard” technique (ICP-MS).

Chapter 5: Evaluate the application of potassium measurements to correct the "hematocrit effect" in the analysis of Pb concentrations.

- Estimate the hematocrit content of whole blood used to create DBS by utilizing potassium concentrations.
- Determine and apply a correction factor to correct the Pb concentrations of an independent set of DBS samples.
- Evaluate the differences between whole blood and DBS samples before and after correction.

To accomplish these objectives, we carried out several tests and statistical analyses as specified as follows:

Chapter 2: In this chapter, descriptive statistics including geometric and arithmetic means, percentages, minimum and maximum values were used to describe specific characteristics from the records included in the systematic review. For example, the mean and range of Pb concentrations reported, the % of record per geographic region, the minimum and maximum values of the limits of detection per analytical technique used, etc. All statistical analyses were carried out using Microsoft Excel 2019.

Chapter 3: The accuracy (% recovery) and precision (relative standard deviation, % RSD) of the method was determined through the analysis of the blood reference materials and replicate analysis of samples, respectively. I determined the differences in mean values between capillary and venous whole blood using a t-test of paired samples ($\alpha = 0.05$), the relationship of measurements between the two matrices using a correlative analysis, and their agreement using Bland-Altman plot analysis. From the Bland-Altman plots, data analysis focused on constant (mean of differences between the two measures) and proportional (based on the slope

of a plotted regression line of the two measures) biases, the line of equality (the zero line, where no differences between the two measures would be expected), and the lower and upper limits of agreement (LoA) (where 95% of the differences of the two matrices exist). All statistical analyses were carried out using Microsoft Excel 2019 and R version 3.6.3, with figures created using the latter program.

Chapter 4: Several parameters are needed to assure the optimal performance of the method. The linearity and the working concentration range of the method were determined by constructing a calibration curve using DBS created with the five different whole blood reference materials (Pb levels spanning from 1 to 50 µg/dL). The calibration curve was generated by linear regression (using least squares) and linearity was assessed by Fisher´s test. The LoD_{method} (mean concentration of blanks + 3x SD) and LoQ_{method} (mean concentration of blanks + 10x SD) were calculated from Pb measurements in blank filter paper treated with sample preparation reagents. Accuracy was determined by measuring Pb in DBS created from blood reference materials. Precision was determined by calculating the percent relative standard deviation % RSD, based on replicate analysis (n=9 separate cards) of the reference materials. For the method development stage, comparisons between the different sample preparation conditions were carried out using unifactorial ANOVA (normality of data was previously determined). In addition, I used ANOVAs to assess if the Pb concentrations varied across batch runs. Finally, Bland-Altman plots were constructed to evaluate the agreement between a range of comparisons (e.g., Pb in DBS versus whole blood; measurements in TXRF versus ICP-MS) as per criteria listed above under Chapter 3. Statistical analysis was conducted using Microsoft Excel 2017 and all figures were generated using R version 3.6.3.

Chapter 5: Accuracy and precision of the AAS and ICP-MS methods applied were assessed by calculating the recovery % and the coefficients of variation (CV %) of replicate samples using whole blood reference materials. A linear calibration curve was created with 5 hematocrit levels. Unweighted linear regression (assumptions previously assessed) analysis was performed to compare the K^+ and hematocrit levels and to obtain the correction equation from the whole blood/DBS ratio plotted against K^+ levels. Based on this regressions, I obtained equations to calculate new DBS corrected concentrations from a derived algorithm (*new element concentration in DBS= original element concentration in DBS* ((-slope * K^+ + intercept)*). The effectivity of the correction was determined by plotting the % of differences between whole blood and DBS samples across the relevant range of hematocrit levels before and after the correction. Bland-Altman plots were used to assess the effect of the correction on the agreement between the levels of the elements of interest in DBS samples compared to the measurements of the same elements in paired whole blood samples.

All data were evaluated using the software MedCalc version 18.10.2 (MedCalc Software, Mariakerke, Belgium) and Microsoft Excel 2017. All figures were generated using R version 3.6.3.

1.2 Literature review

1.2.1 Pb in the environment

1.2.1.1 The sources and fate of Pb

Pb is a non-essential metal that has been used widely by humans since 6,500 B.C. (De Keersmaecker, 2018). Pb is naturally present in the crust of the earth, usually found in soils, sediments, water, and vegetation. Although Pb is ubiquitous in the environment, several studies

including classical and recent ones have shown that the Pb levels in the atmosphere, sediments, and oceans have increased due to anthropogenic activities, (Schaule and Patterson, 1981; Shirahata *et al*., 1980; WHO, 2018) principally mining and smelting activities, followed by refining processes, manufacturing, and recycling (WHO, 2018).

The accumulation, removal, and dispersion of Pb through environmental components such as water and sediments, depends on different physicochemical factors including pH, dissolved oxygen, hardness, salinity, and the presence of organic matter or organic chelating agents (ASTDR, 2017; Rahman and Singh, 2019). The two main sources of Pb human exposure occur through environmental sources and occupational activities.

1.2.1.2 Pb exposure and Toxicokinetics

We are all exposed to Pb to a certain extent through contaminated ambient air, drinking water, soil, and dust. Of all the numerous industrial activities linked to Pb exposure, mining and smelting are particularly relevant as they increase the Pb levels around their operation sites (ATSDR, 2017). Children are the main vulnerable group to Pb exposure (WHO, 2018) due to physiological (e.g., metabolism and body weight) and behavioral factors (e.g., hand to mouth activity). Other vulnerable groups include elders, populations from low-income countries, and occupationally exposed individuals.

The most common pathways of Pb exposure among populations are ingestion and inhalation. Ingestion accounts for 50% of the Pb absorbed into the body in children (and 10% in adults), while the rest is excreted (ASTDR, 2010). Inhalation accounts for about 30-40% of Pb particles retained in the respiratory system (WHO, 2000). Dermal absorption is a less significant route

of exposure compared to ingestion and inhalation because it is only relevant in the organic Pb exposure and is usually rare (ASTDR, 1997; EPA, 2006).

After gastrointestinal and pulmonary exposure, Pb is transferred to the bloodstream where it binds to erythrocytes (99%) (Cake, 1996). The half-life of Pb in the blood is about 30 days (Rabinowitz, 1991). Eventually, Pb is absorbed in bones (O´Flaherty, 1993; ASTDR, 2017), with a half-life of 10-30 years (Rabinowitz, 1991). The skeleton is the main reservoir for Pb, accounting for 70-90% of the total body burden of Pb depending on age, followed by the kidney, the liver, the heart, and the brain, accounting for about 6% of the total body burden of Pb (Barry, 1975). Finally, Pb is eliminated from the blood into the urine (76%) via the kidney, with smaller fractions eliminated in bile (16%), via the gastrointestinal tract (Zenz *et al*., 1994). Pb interferes with many biological functions (**Fig. 1-1**), through multiple mechanisms, but the main qualities underlying these mechanisms are the ability of Pb to bind to sulfhydryl and amide groups, which are generally components of enzymes (Fullmer, 1985; Flora *et al*., 2012), and its competition with essential cations (Sanders *et al*., 2009; **Fig. 1-3**).

Figure 1-1. The health outcomes of Pb poisoning

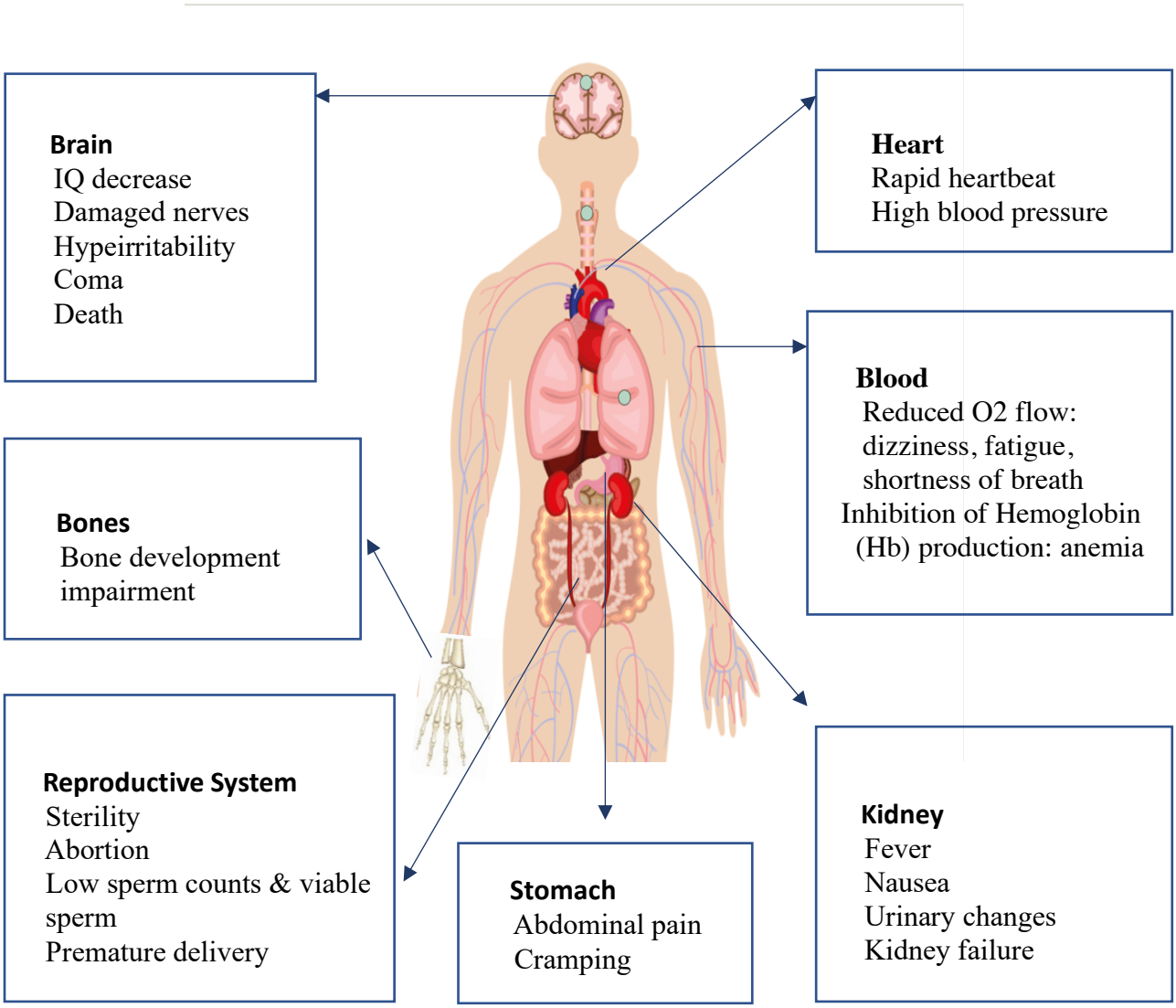

Figure created by author using the BioRender platform

Figure 1-2. Mechanisms of Pb toxicity

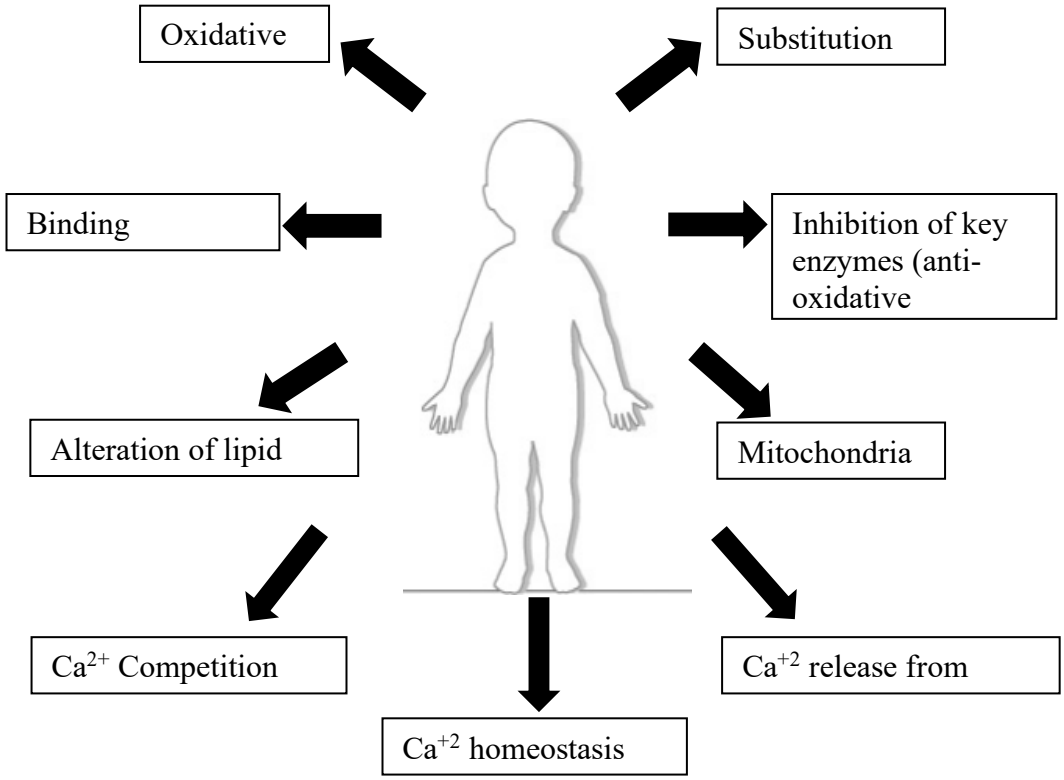

Figure created by author using Microsoft Word

1.2.2 Pb exposure assessment

1.2.2.1 Whole blood as a biomarker

Biomonitoring Pb exposure in a population is a complex task. Identifying a suitable biomarker is important for implementing prevention programs, medical management, and supporting public health decisions (Barbosa, 2005; Sanders *et al*., 2009; **Table 1-1**).

Whole blood remains the most used biomarker of Pb exposure (Skerfving and Bergdahl, 2007), it is an attractive biomarker to assess both short-term and long-term exposure to Pb, but it poses several challenges including invasiveness, the need for clinical settings, and well-trained personnel, accessibility, special requirements of material, and special conditions for transportation and storage (Fleming and Mills, 2007).

Table 1-1 Advantages (▭) and limitations (▭) of Pb biomarkers

Biomarker	Analytical considerations	Sampling	Transportation/Storage	References
Whole blood	• Low detection limits can be reach • Variations below 5% • Intercalibration between laboratories is widespread in Pb determination • Reference blood samples available	• Venipuncture offers the opportunity of avoiding contamination	• There are guidelines for blood sampling and storage	Skerfving and Bergdahl, 2007 Sanders *et al*., 2009
	• Blood is a complex matrix • High organic matter content	• Invasive • Trained personnel are needed	• Cold-chains are required • Storage at low temperature	Fleming and Mills, 2007 Barbosa, 2005
Bone/Teeth	• Pb concentrations in bone can be very high (detection limits are not a concern) • Bone Pb is useful to estimate total body burden of Pb	• Non-invasive, *in vivo* methods available	• Teeth are very stable for preservation proposes	Sanders *et al*., 2009
	• Sensitivity might vary according to the bone type • Calibration is more complicated • Precision can be challenging • Detection limits depends on the soft tissue of the subject	• Subject movement interferes with the measurement • There may be differences between tooth types	• Requires portable equipment • Cost increases	Sanders *et al*., 2009 Flora, 2014 Barbosa, 2005
Urine	• Used in occupational studies • Long-term monitoring	• Non- invasive	• Low cost	Barbosa, 2005 Wongsasuluk *et al*., 2021
	• Low reproducibility (biological variations) • Lower Pb concentrations • Better detection limits are required	• Variations according to kidney infiltration rates		Barbosa, 2005 Sanders *et al*., 2009
Hair	• Long term monitoring	• Non-invasive • Minimal cost	• Easy transportation and storage	Barbosa, 2005

	• Preanalytical concerns about sampling and contamination control			Wongsasuluk *et al*., 2021
	• High variability (age, sex, hair color) • Is not commonly used as biomarker	• It is challenging to distinguish between exogenous and endogenous Pb • Reference ranges	• A washing step is required but no reliable method exist to remove exogenous Pb	Barbosa, 2005

1.2.2.2 Traditional methods to determine Pb in whole blood

From the 1940s through to the mid-1960s, Pb analysis was commonly performed in whole blood by colorimetry/spectrophotometry using dithizone (diphenylthiocarbazone). Around 10 mL of whole blood was necessary, and the oxidation was completed with strong acids (e.g., HNO_3, $HClO_4$, H_2SO_4) or dry ashing, followed by complexation/extraction into a chloroform solution of dithizone (Blaxter and Allcroft, 1950: Vinter, 1964). The main limitations of this approach were the large sample volume required (more than 10 mL), the need for complete oxidation, the complexity of the method, and the long analysis times.

In 1970, Flame Atomic Absorption Spectroscopy (FAAS) and Anodic Stripping Voltammetry (ASV) were proposed, and they were probed as valuable alternatives for Pb analysis in blood samples. The samples were commonly digested using acids or prepared by the "exchange" method, in which samples were treated with a solution containing $(CH_3COO)_2Ca$ to dissociate Pb from blood proteins. The main disadvantages of these techniques were the lack of sensitivity and the lack of precision, respectively (Khan, 1969; Searle et al., 1973). Between the decade 1970 and 1980 Furnace AAS was improved and consolidated as a reliable analytical technique for the determination of Pb in whole blood (L'vov, 1970). However, even though this technique is much more sensitive than the flame version of AAS and it no longer requires deproteinization

of the blood before injecting the sample into the furnace, it is not a multi-elemental technique, the analysis time is still long, and it usually requires matrix modifiers during the analysis.

Finally, Inductively Couple Plasma Mass Spectrometry (ICP-MS) was introduced late in the decade of 1980 and early 1990 (Campbell and Delves, 1989: Paschal et al., 1995). This is a multi-elemental and highly sensitive technique that can reach limits of detection in the order of ppb and ppt depending on the sample matrix. However, this equipment is less tolerant to samples with high organic content and high percentages of dissolved solids. Moreover, the high operations costs and potential interferences have been other limitations to discuss further.

1.2.2.3 Considerations in Pb exposure assessment

Nowadays, the "gold standard" to assess Pb exposure is the analysis of Pb concentrations in whole blood samples, usually applying spectrometric techniques (e.g., ICP-MS) as the analytical procedure. Concentrations of Pb in whole blood are generally in the range of ppb (ASTDR, 2017; Cake, 1996), for example, the 10-95th percentile values among the general Canadian population aged 3-79 are 0.4-2.7 µg/dL (CHMS Cycle 4, 2014-2015).

There are several considerations in the analysis of Pb in whole blood:

- ***Sample volume.*** The amount of whole blood that is typically collected for ICP-MS analysis is between 200-500 µL (Mcdade, 2013). Sample preparation includes the addition of reagents that represents a dilution of the whole blood sample. This factor must be considered to calculate the final concentration. The volume of the sample represents one of the main limitations for the analysis of some types of blood such as capillary blood since the volume collected is typically on the order of microliters.

- ***Sampling and storage.*** For the analysis of Pb, some precautions are required when handling blood samples, including preventing contamination of samples. It has been documented that inadequate collection procedures or the use of contaminated materials can lead to falsely elevated Pb concentrations (CDC, 1997; Mester and Sturgeon, 2003).
- ***Ambient factors*.** There is evidence that environmental factors, such as temperature and humidity upon storage of samples may affect the concentrations of Pb (Chaudhuri, 2009), therefore, the stability test is a parameter required to fully validate a study (Lee and Li, 2014).
- ***Costs.*** Several analytical techniques are used to analyze Pb in blood samples such as ICP-MS or ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry), however, these instruments are very expensive. The purchase price of these instruments is ~ $220K and $120K (CAD), respectively, and the cost per sample analysis can range from $20 to $90 (Agilent, 2005). They also require water chillers, a digestion microwave (recommended), and lab adjustments (e.g., fume hoods). All these costs may interfere with the routine analyses.

1.2.2.3 Alternatives to traditional blood Pb measurements

Recently, there have been several technological improvements in the way Pb is measured in whole blood samples. The analytical technology has improved not only in implementing new devices and equipment, but it also includes the enhancement of already existent instruments. Some of the instruments that have been released recently include portable specialized analyzers such as Pb care® and XRF Pb testing gun (Bruker, Germany) and other improved previously existent techniques such as Laser Ablation ICP-MS (LA-ICP-MS), single particle ICP-MS (SP-ICP-MS), and Total Reflection X-Ray Fluorescence (TXRF).

The development of novel methods is essential as part of biomonitoring programs that look for new ideas to make the analysis of Pb in blood samples faster (in the preparation of samples and also to obtain the results), less complex, safer, and by utilizing less resources.

Such novel instruments or methods would be expected to measure Pb at a concentration of <5 µg/dL, at a lower cost per test than current lab-based methods, allow for Pb exposure screening in clinical settings, remote locations, in-the-field surveys, among others (CDC, 2019), and also allow for the reanalysis of samples with elevated blood Pb results immediately.

1.2.2.4 Alternatives to traditional blood draw methods

The need for alternative methods to classical venipuncture is increasing. For example, it is estimated that remote patient monitoring of blood is a market that will reach $1 billion in 2020 (Forbes, 2015).

Capillary blood is an alternative to venipuncture, and there are two widely applied methods for its collection: Dried Blood Spots (DBS) and Volumetric Absorptive Microsampling (VAMS). DBS sampling has been successfully used for about 40 years in multiple validated studies (Olshan, 2007; Spooner *et al*., 2009; Nys *et al*., 2017; Li and Lee, 2014). On the other hand, VAMS is a new approach that has been claimed to be as reliable as traditional methods since it allows for collecting a precise amount of blood. However, there is evidence of a high variability upon storage of VAMS samples at room temperature (Houbart, 2015; Miao, 2015; Parker, 2015), and it has been recognized that it needs more evaluation through validation

studies. This is of tremendous importance since many healthcare technologies lack scientific evidence that supports, they were validated in the lab before their use by health practitioners.

1.2.3 Dried Blood Spots

1.2.3.1 The benefits and limitations of DBS

Dried blood spots (DBS) is a sampling technique that involves applying capillary whole blood onto filter paper, providing an easy way to collect, transport, and store samples for several analytical purposes (Li and Lee, 2014).

This technique has been used since 1960 mainly for the screening of inborn metabolic disorders (Li and Lee, 2014), and over the years other uses for DBS have emerged since then.(Smit *et al*., 2014; Van Loo *et al*., 2017; Li and Lee, 2014). The advantages of this sampling method are numerous, and they can be summarized in **Table 1-2**. Despite these advantages there remain notable challenges that DBS must overcome to meet international standards requirements (i.e., ASTM consensus standards) and stakeholder adoption.

Table 1-2. Comparisons of DBS and whole blood samples

Biomarker	Analytical considerations	Sampling	Transportation/Storage	References
Whole blood	• Serum and plasma can also be measured • Most of the information of Pb exposure is based on whole blood as biomarker • Known volume	• There are guidelines published for sampling of blood	• There are a lot of information available on samples stability and storage conditions	Skerfving and Bergdahl, 2007 Ettinger *et al*., 2019 (CDC's Lead Poisoning Prevention Program)
	• Blood is a complex matrix • High organic matter content	• Invasive • Trained personnel are needed • Sample collection facilities are mandatory • Collection is not simple	• Cold chains are required • Storage at low temperature • Transportation and storage settings increase the overall analysis cost	Mcdade, 2013 Mester and Sturgeon, 2003 Chaudhuri, 2009
Dried Blood Spots	• More samples can be collected from each subject	• Non-invasive • Painless • Collection is simple • Low-cost sampling technique • Low blood volume is required	• Transportation is simple • Cold-chains and special settings are not required • Less space requirements • Low-cost	Li and Lee, 2014 Nys *et al*., 2017 Van Loo *et al*., 2017
	• Inherent complexity of blood matrix • No methods available • Low/Unknown volume	• Subject hydration and body temperature may affect the collection	• Scarce information on analyte stability in DBS	Li and Lee, 2014

Advantages
Limitations

Some barriers to the wider adoption of DBS are the potential heterogeneity of blood spread on a spot mainly due to the differences in their hematocrit content, also known as the "hematocrit effect" (Chaudhuri, 2009; Pedersen, 2017). Other notable challenges include low sample volumes (Lim, 2018), the potential of contamination of samples (Funk *et al*., 2015, Lim, 2018), and the potential changes in analyte stability due to storage conditions (Adam *et al*., 2013).

There are protocols to overcome some of these challenges such as the DBS handling procedures and guidelines to minimize the risk of contamination and avoiding the analyte stability to be affected (Li and Lee, 2014). However, the issues such as the hematocrit effect and the low volume of samples require more research effort to guarantee the precision and accuracy of newly developed methods for DBS analysis and allow them to be formally adopted by regulatory agencies and other stakeholders.

1.2.3.2 DBS and the analytical/detection aspects in the assessment of Pb exposure

The first studies that analyzed Pb in DBS were performed in the 1990s using AAS-based methods (Verebey *et al*., 1991; Wang and Demshar, 1992; Yee and Holtrop, 1997). These studies were developed using samples from newborns and children. Some of the methods used in these studies showed acceptable accuracy (~80-125%, ICH guidelines), but other validation parameters (i.e., limits of detection) were not fully addressed.

The introduction of ICP-MS allowed the improvement of the Pb analysis, particularly the detection limits. Since ~2005, other studies have analyzed DBS samples by ICP-MS (Cizdziel, 2007; Chaudhuri, 2009; Archer, 2012; Lehner *et al*., 2013; Pedersen *et al*., 2017),

showing better limits of detection (ppb-ppt range), accuracy, (~95-121%) and precision (<15%), compared to previously applied techniques. Despite this, ICP-MS still has limitations including the relatively large volume of sample required, the high purchase and operational costs, its low tolerance for dissolved solids, the complex sample preparation, the potential analytical interferences, and the large dimensions of the equipment. These inconveniences may dissipate the advantages of using DBS such as low-cost and practicality.

1.2.3.3 TXRF to overcome the sample volume issues in DBS analysis

TXRF is an analytical technique that emerged in ~1980 and consists of a monochromatic X-ray beam that is directed onto the sample at a small angle (<0.1°), below the critical angle Θc for total reflection. TXRF allows for the analysis of micro samples (≤ 10 µL are sufficient to perform the analysis) (Stosnach, 2007; Dhara and Misra, 2011; Towett *et al*., 2013), which makes it more logistically feasible for the analysis of biological samples (e.g., DBS). Moreover, excitation by the totally reflected beam lessens the scattering of the beam in the sample matrix, resulting in decreased matrix effects. This is key for the analysis of samples with complex matrices (e.g., blood).

The benefits of this technique can be summarized as a) Detection limits in the ppb range, b) fewer interferences, c) fast preparation of the samples, d) low sample volume required, and e) low operational costs (Bilo *et al*., 2014). There are few studies that involve the analysis of DBS samples by TXRF (Pawly *et al*., 2019; Czuban *et al*., 2015). However, these studies are focused on essential elements (mainly selenium), to be applied in the characterization of essential elements as part of newborn screening programs.

The advantages of TXRF make it a good alternative to traditional techniques like AAS or ICP-MS to the analysis of DBS and to be used as a surveillance tool for Pb exposure assessment (**Table 1-3**).

Table 1-3. Advantages (▭) and limitations (▭) of TXRF and ICP-MS

Technique	Analytical considerations	Cost	Space and other requirements	References
TXRF	• Matrix effects are negligible (sample is placed as a thin layer) • Better limits of detection than XRF (ppb) • Low volume of sample required • Samples of different matrix can be analyzed • Preparation of samples is faster compared to other spectrometry techniques • Analysis time is relatively short (100-1000 s)	• Low operations costs • Low acquisition cost	• Gases are not required • A chiller is not required • Is a portable device	Bilo *et al*., 2014 Stosnach, 2007 Towett *et al*., 2013
	• Interferences and low sensitivity for low-Z elements due to the resolution • There is less information about TXRF methods than other spectrometry techniques • Sample preparation is a critical step		• The alignment of the optical components after moving the equipment can take up to 3 hours*	Stosnach, 2007 *Recommendations from Bruker
ICP-MS	• The best limits of detection (ppt-ppb) • Some spectrometric and non-spectrometric interferences can be removed		• Can work with autosampler using the same vials	Cizdziel, 2007

	• There are a lot of information about ICP-MS methods • Can be coupled with sample introduction devices			Chaudhuri, 2009 *Own professional experience
	• The plasma is highly affected by the organic matter content in samples • Low tolerance to dissolved solids • Long preparation times • Only liquid samples	• High cost of acquisition and operation • Constant maintenance can be problematic in routine analysis	• Equipment is too heavy • The dimensions of the equipment are high • Requires Ar to generate the plasma • Different gases are required to remove interferences	Bilo *et al*., 2014 Agilent, 2005 *Own professional experience

1.2.3.4 The hematocrit effect in DBS analysis

The hematocrit effect (also known as hematocrit bias) is one of the biggest challenges in the analysis and the development of novel methods applying DBS (Velghe *et al*., 2019). The hematocrit content affects the viscosity of the whole blood and thus, how the blood spreads on the cellulose-based filter cards. A high hematocrit content might result in reduced DBS area and increased volume in the fixed diameter sub-samples (e.g., punches) that are taken from the DBS samples. The differences in the hematocrit percentage among DBS samples in the analysis of a particular analyte can lead to errors in the determination of its concentration (De Kesel *et al*., 2014; Velghe *et al*., 2019). Even though there is no universal or standardized approach to cope with the hematocrit effect DBS analysis, there are several strategies that have emerged in recent years to overcome this issue:

- ***The analysis of whole spots.*** One of the most practical ways to deal with the hematocrit effect is by simply analyzing the whole circle in the DBS samples that was volumetrically created, instead of using fixed-size portions of them or "punches". This approach involves filling the spots with a defined volume of blood or spotting the blood onto pre-punched discs. The disadvantage of this procedure is the need for special equipment and materials, and potentially clinical settings. Even though there are several devices that have been developed to facilitate the collection of a defined volume of blood such as micro-sampling devices and precision capillaries, the direct application from the fingertip may be preferred due to its practicality and feasibility in some types of studies (e.g., studies performed in remote areas).
- ***Variations of filter cards.*** Other alternatives to reduce the hematocrit effect include developing filter cards with different substrates or different configurations to spread the blood onto the surface of the cards. Some of them containing an absorbent paper and desiccant covered with a small opening to allow entry for blood in the surface (e.g. HemaSpot-HF ™), DBS cards equipped with inlet ports and microchannels to allow a fixed volume of blood to enter the device and dry evenly (e.g. Capitainer-B device), and cards that generate a fixed amount of plasma after the whole blood is filtrated in the card (dried plasma cards, e.g. Noviplex LCC). While all of these approaches may help to alleviate the problem, they also involve utilizing specialized materials and equipment, which represents limitations logistically and economically speaking. Moreover, using dried plasma may not be possible for some applications since the concentrations of certain analytes could be very low or even below the analytical limits of detection.
- ***Estimating the hematocrit of a DBS.*** One of the most recently explored solutions is estimating the hematocrit content of DBS samples. There are several proxies of hematocrit

that have been investigated. One of the first approaches was the use of reflectance (Miller, 2013). After exploring several wavelengths, the authors found that reflectance at 980 nm was highly correlated with the hematocrit level. However, some of the limitations of this study included that reflectance is highly dependent on the spotted blood volume and it may be influenced by the age of the sample too since the color of DBS tends to change over time. Another creative method involved forming a hemoglobin complex by adding a reagent containing sodium lauryl sulfate to a DBS extract, therefore, hemoglobin can be easily quantified by UV-VIS spectrophotometry (Richardson, 2018). This method was fully validated and applied to analyze samples from 59 patients. However, the hematocrit is not directly estimated, the sample preparation is labor-intensive, the effect on the hematocrit prediction from the use of different organic solvents was not evaluated, and finally, sample analysis is overall is time-consuming.

One of the latest approaches first introduced by Capiau (Capiau *et al*., 2013) was focused on the use of potassium as a marker of hematocrit. This method was fully validated in a hematocrit range representative of most of the population (19-63%) and successfully applied in the prediction of hematocrit with real samples of patients (n=111). In follow-up studies, this method has been further evaluated (Rufail, 2017) and applied to correct for the hematocrit effect in the analysis of several analytes, including caffeine, paraxanthine, and creatinine (De Kesel *et al*., 2015; den Burgen *et al*., 2015). Probably the main barrier of this method is that, unlike the reflectance-based method, this is a destructive technique. Yet, the method can be performed with only two 3mm punches from one DBS. This approach shows potential to be applied for the correction of the hematocrit differences in DBS samples, and thus, the improvement in the analysis of other analytes, including toxic metals.

1.2.3.5 Emerging technologies to potentially overcome the limitations of traditional methods

While some novel analytical techniques, such as TXRF, offer benefits to potentially help overcome analytical constraints in the analysis of whole blood, in this section I want to recognize recent emerging technologies that also have potential to become advantageous alternative options in the analysis of Pb and metals in general compared to traditional methods.

Environmental sensors. These devices are useful for the assessment of exposure information at different scales, including macro and microscale, as well as nanoscale exposures (Haruyama, 2003). Macroscale sensors include devices such as laser-based and infrared-based sensors, and microscale sensors include personal dosimeters. The latter devices offer many advantages usually associated with microanalytical methods, such as increased sensitivity, reduced quantity of reagents, and increase sample throughput. Finally, nanoscale devices are used at the point of human contact, this category includes "lab-on-a-chip" sensing devices (Hood *et al*., 2004).

GIS technology. This technology has been recently used to layer electronic data that is linked temporally or spatially by applying mathematical models. The displayed information is developed by combining data sets, for example, of land-use, climate, population, and environmental data (Nuckols *et al*., 2004). Nowadays, the number of studies that apply GIS technology in epidemiological studies to determine environmental exposures is growing fast.

Toxicogenomics. The field of Toxicogenomics has been growing considerably in the last few years. These methods are applied in laboratory settings to develop gene signatures that may serve as biomarkers of exposure, early biologic response, and susceptibility (Wu *et al*., 2016). The Toxicogenomics-based methods have been used for classifying exposures to a variety of chemicals and drugs and contaminants, including metals, and they are useful for classifying health outcomes. This approach also allows to move from traditional methods to determine

exposures (one contaminant in one medium at a time) to a more holistic exposure scenario with multiple environmental contaminants and exploring several biologic responses.

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CONNECTING PARAGRAPH

The literature on Pb exposure assessment and DBS is vast and deep. There are a wide variety of aspects around the application of DBS to determine Pb levels, yet, articulating the main limitations of DBS sampling is not always clear and the consensus on them is not set.

Chapter 2 is focused on reporting qualitative and quantitative findings from a narrative review with a systematic search on the major drawbacks and knowledge gaps of Pb levels assessment in human populations using DBS. This review presents the selection, screening, and inclusion process and of relevant studies that highlight the state of understanding and limitations of DBS sampling applied to Pb levels determination, and it serves as a guide for the development and organization of the following chapters.

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2. **CHAPTER 2.** The performance of dried blood spots for the assessment of lead exposure: a narrative review with a systematic search

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2.1 Abstract

There is interest in the use of dried blood spots (DBS) to monitor lead (Pb) exposure yet concerns raised about the methods used have yet to be rigorously researched. Here, a systematic review and narrative synthesis of the literature was performed to increase understanding of the current state of methods, their performance against QC benchmarks, and common areas of concern across studies. Through keyword searches in databases (years 1972-2021), 208 articles were found of which 36 were deemed eligible. The included studies hailed from 11 countries, and most used Whatman 903 (31%) or Schleicher and Schuell (33%) filter cards (31%). The studies included a range of DBS subsample sizes with 1/4" (24%) and 3/16" (15%) being most common. Most earlier studies used GF-AAS (39%), and since the 2000s there has been a shift towards ICPMS (33.3%) and more recently XRF. The mean limit of detection across studies using GF-AAS was 1.0μg/dL versus 0.6μg/dL for ICPMS. In synthesizing the results, more research is needed on the limit of detections achieved by the various methods, potential differences in Pb concentrations between capillary and venous blood, the influence of the hematocrit effect, and the potential contamination of samples with Pb.

2.2 Introduction

Lead (Pb) is widely well-recognized as being a chemical of major public health concern (CDC, 2019; Health Canada, 2019; WHO, 2020). To evaluate and manage risks associated with Pb exposure, biomonitoring Pb in human populations has been key in helping to characterize exposures, identify and prevent Pb poisoning, increase understanding and awareness of its health effects (even at low concentrations), and support the implementation of bans and regulations (Ettinger, 2019). With growing international interest in biomonitoring Pb exposure, several challenges have emerged. Foremost is that Pb is biomonitored through the analysis of whole blood samples obtained through venipuncture. The ethical, practical, technical, and financial concerns associated with venipuncture have been highlighted elsewhere (Taksande *et al*., 2005; Spagrud *et al*., 2008), and thus there is interest to develop and apply alternative sampling methods.

Dried blood spot (DBS) is a microsampling technique that has been successful used in diverse applications since it was first developed in 1963 (Guthrie and Susi, 1963). A DBS-based approach requires an individual's finger (or heel) to be pricked with a lancet following which a few drops of blood are captured onto filter cards (USAID, 2012). As such, this approach offers a fast, cost-effective, and minimally invasive sampling procedure as compared to traditional venipuncture (Li and Lee, 2014; Lei and Prow, 2019). DBS samples are routinely collected from newborns for different analytical purposes such as inherited metabolic disorders and surveys of infectious diseases (Olney *et al*., 2006).

There is increasing interest in the use of DBS-based methods to characterize Pb exposure. For example, since the 1990s newborn screening programs in Canada, U.S., and Italy have

assessed Pb exposure using DBS (Wang and Demshar, 1992; Chaudhuri *et al*., 2009; Michniewicz *et al*., 2015). In addition, relatively large DBS-based Pb exposure studies have been performed in children, pregnant women, and adults with sample sizes ranging from 526 to 1,546 individuals (Schonfeld *et al*. 1994; Rello *et al*. 2015). Collectively, these studies demonstrate the potential of DBS as a valid method to biomonitor Pb, however there are notable limitations with this approach as acknowledged by the aforementioned studies including the potential of Pb -contaminated samples (Chaudhuri *et al*., 2009) and the variability in the Pb concentration due to the so-called hematocrit effect (Michniewicz *et al*., 2015). These challenges, and others, hinder the wider adoption of DBS-based methods for biomonitoring Pb exposure. Accordingly, the current study was conducted to help increase our understanding of the challenges associated with the use of DBS for biomonitoring Pb exposure through a systematic search of the literature. By carefully analyzing the relevant studies in the field, and benchmarking them against quality control and performance expectations, the goal here was to identify areas of particular concern along with opportunities for future research activities in an evidence-based manner.

2.3 Materials and methods

2.3.1 Protocol

This study followed the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) criteria. The protocol is included in the supporting information (**S2**).

2.3.2 Databases and searches

We performed a systematic search in three databases: PubMed, Scopus and Web of Science. Since DBS were developed at the beginning of the 1960's, we used a date interval between

1960 to March 26, 2021, to perform our searches. We organized our search to gather information from three categories: 1) Pb exposure (keywords: 'Lead poisoning' OR 'Pb' OR 'Lead exposure' OR 'lead poisoning' [MeSH Terms]), 2) DBS (keywords: 'Dried blood spots' OR 'DBS' OR 'Dried bloodspots' OR 'filter paper'), and 3) assessment (keywords: 'assess*' OR 'monitoring' OR 'analysis' OR 'quantif*' OR 'determin*' OR 'measure*' OR 'evaluat*').

In our initial screening activity, we excluded papers that focused on animal studies, case report studies and molecular studies. The results from the searches were uploaded to the software program Rayyan, where duplicated articles were removed, and the titles and abstracts of the remaining articles were manually evaluated. The inclusion criteria included peer-reviewed studies that: 1) were carried out in humans, 2) dealt with the application of DBS for the assessment of Pb exposure, and 3) were published in English.

2.2.3 Data extraction and quality appraisal

The information retrieved from each paper included title, author(s), year of publication, IRB approval, geographic location, sample size, central values (mean, median, etc.) of blood Pb levels (BLL), DBS collection methods, type of filter paper card, use of Standard Reference Material (SRM), quality controls parameters (accuracy, precision, limits of detection and quantification, % of samples < LoD), and type of DBS studied (e.g., punches) and volume (**Supplementary table S2-1**). The data was extracted and evaluated by two reviewers independently and disagreements were resolved through discussion and consensus.

We gauged the quality and the completeness of the information (e.g., reporting the essential information to replicate the studies) included in the studies. We used a modified checklist of

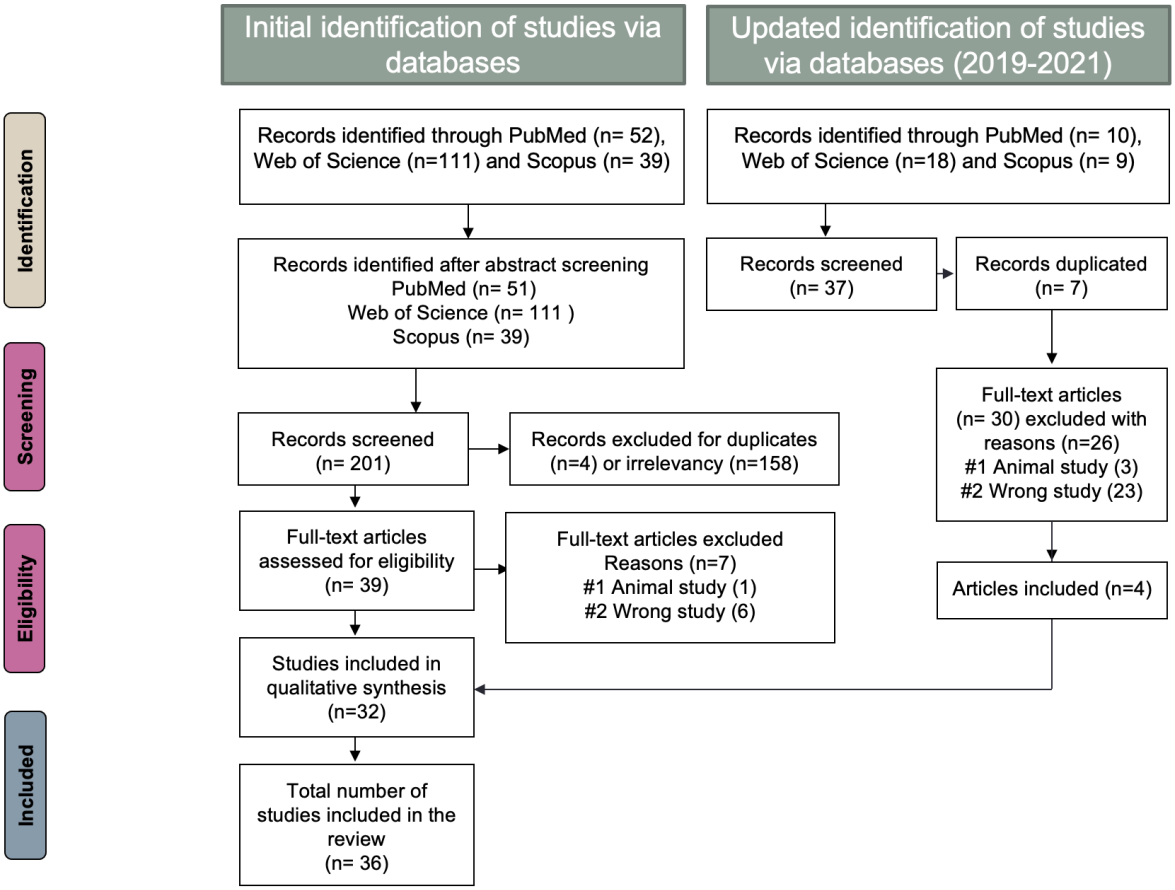
the standards for reporting diagnostic accuracy studies (STARD statement; Bossuyt *et al*., 2003) that applied to studies on DBS and exposure assessment. The checklist contained questions along each section (e.g., introduction, methods, etc). The complete checklist along with the responses are presented in the **Supplementary table S2-2**.

2.4 Results and discussion

2.4.1 Search outcomes

The initial search identified 201 titles from the three electronic databases (**Fig. 2-1**). After inspecting the titles and abstracts for duplicates and eligibility, we identified 39 articles to be read in full. We excluded 7 of these articles with reasons (presented in the PRISMA chart). We performed an updated search from 2019 to 2021 and included 4 additional studies. In total, 36 studies were finally included in this review.

Figure 2-1. PRISMA chart summary of the literature searches and article selection process.



2.4.2 Characteristics of included studies

The studies selected for inclusion here were published from 1972 to 2021. The sample size reported in these studies ranged from n=2 to 2300. Of all the studies, 41.7% of them provided a statement reporting IRB approval, 16.6% are validation studies that used only reference materials, and the rest of them did not report IRB approval.

The studies included in this review were carried out in eleven countries, with a majority of them from the United States (52.7%), followed by United Kingdom (8.3 %), Spain (8.3%), Italy (5.5%), Taiwan (5.5%), Canada (5.5%), and Tanzania (5.5%), while Denmark (2.7%), France (2.7%), Nigeria (2.7%), and China (2.7%) each had one study captured here (**Table 2-1**).

Table 2-1. Studies per geographic location (as % of total publications) and their main characteristics

Geographic location	% of studies in this review (n)	Sample size, mean (range)	Study conditions	Target population
United States	52.7 (18)	258 (8-2300)	> 50 % under clinical conditions	> 50 % on newborns and children
United Kingdom	8.3 (3)	64 (50-77)	100% under clinical conditions	50% on newborns and 50 % on children
Spain	8.5 (3)	184 (7-823)	100% under clinical conditions	100% on patients. Newborns, adults, and one study included pregnant women
Italy	5.5 (2)	75 (20-130)	100% under clinical conditions	100% on newborns screening tests
Tanzania	5.5 (2)	299 (160-439)	100% under field conditions	100% on pregnant women from artisanal and gold mining areas
Canada	5.5 (2)	253 (81-425)	Mostly clinical conditions. One study compared with field conditions	50% on newborns and 50 % on adults (low-exposed and high-exposed)
Taiwan	5.5 (2)	Not applicable (only SRMs)	100% under clinical conditions	Certified reference materials and matrix-matched standards
France	2.7 (1)	18	100% under clinical conditions	%100 on adults
Denmark	2.7 (1)	20	100% under clinical conditions	100% on adults

Nigeria	2.7 (1)	218	100% under field conditions	100% on children
China	2.7 (1)	159	100% under clinical conditions	100% on children

2.4.3 DBS sampling and analysis

Regarding the type of filter card used for collecting blood samples, most studies used Whatman no. 903 filter cards (30.6%) or Schleicher and Schuell (33.3%). The other studies reported using PVDF filter membranes, P.K.U cards, or just filter membranes/special filter paper (16.6%). The remaining studies (19.4%) did not specify any filter paper brand or type.

From all the studies included, 23.5% used 1/4"DBS subsamples (or 6.35 mm in diameter) to perform the analysis, followed by 14.7% of the studies that used 3/16" DBS subsamples (4.76 mm in diameter). The rest of the studies reported diverse sizes of DBS subsamples, including 1/8" (3.17 mm), 1/2 " (12.7 mm), and 14 mm diameter punches (32.4%), while 29.4% of the studies used non-destructive methods or did not report the DBS size used.

Finally, 38.2% of the studies controlled the volume of blood on the DBS sub-samples, 32.4 % of the studies calculated the blood volume (mainly using gravimetric methods) in the DBS subsamples, 17.6% of them assumed the subsamples volumes based on previous studies, and 11.8 % of them used analytical methods that did not required the volume (only the mass) or did not clearly report the volume used.

The analytical techniques used in the studies were primarily Atomic Absorption Spectroscopy (AAS, including the two variants: flame (F-AAS) and graphite furnace (GF-

AAS)) or Inductively Couple Plasma Mass Spectrometry (ICP-MS, including the variant of laser ablation: LA-ICP-MS) (**Table 2-2**). Most of the studies conducted in the 1990s used AAS (F-AAS and GF-AAS). Of the 19 studies that reported using AAS, only four of them were carried out after the year 2000 (Pfitzner et al., 2000; Di Martino *et al*., 2004; Rello *et al*., 2015; Michniewicz *et al*., 2015). After the year 2000, most studies used ICP-MS. Finally, in recent years, two studies used X-ray Fluorescence-based techniques (TXRF and EDXRF) (Rodríguez-Saldaña *et al*., 2020; Specht *et al*., 2021).

In more than half the studies (55.9%), the reported limit of detection (LoD) was less than 2 μg/dL in 55.9%, while 11.7% of the studies mentioned a LoD greater than 2 μg/dL. Nearly one third (32.5%) of the studies did not report a LoD or did not clearly specify their LoD value (e.g., Funk *et al*., 2013 reported a LoD in the "ppt range"). Most studies (79.4%) reported that 100% of their samples were above the LoD or did not mention detection problems. The rest of the studies (20.6%) reported undetected samples (<LoD), with a detection frequency range from 0.7 to 34%.

Table 2-2. Instruments used in the selected studies (% of studies), with time range and associated LoD.

Instrument	**% Studies (n)**	**Years (range)**	**LoD (range, μg/dL)**
GF-AAS	41.6 (15)	1972-2015	0.22 -1.03
F-AAS	8.3 (3)	1974-1991	Not specified (< 2.0)
HR-CS-AAS	2.7 (1)	2015	0.1
ICP-MS	30.5 (11)	2007-2021	0.008 - 3.72
LA-ICP-MS	11.1 (4)	2007-2017	0.9 - 1.19
TXRF	2.7 (1)	2020	0.28
EDXRF	2.7 (1)	2021	1.70

2.4.4 Main limitations of DBS

In our review of the included studies, we identified four main limitations for the analysis of Pb in DBS: LoD, potential differences in Pb concentrations between capillary and venous blood, hematocrit effect, and the potential contamination of samples. Below we discuss each of these limitations as part of our narrative synthesis of the existing literature.

Analytical sensitivity/LoD

As presented earlier in the '*DBS sampling and analysis'* section, the LoD for Pb measurements reported in the included studies ranged from 0.008 μg/dL to 3.7 μg/dL. The BLL of several populations around the world have been decreasing in recent years (CHMS, 2017; CDC, 2019) and thus, new techniques with improved LoD are needed to achieve a reasonable detection frequency. The mean LoD was 1.0 μg/dL from earlier studies (~1970s-1990s) that largely used AAS. With the increasing use of ICP-MS in the 2000s, the mean LoD of this instrumentation was nearly 50% lower than AAS-based methods at 0.6 μg/dL. In addition to the LoD, the sample throughput of ICP-MS (~ 1-2 minutes per sample, Thermo Elemental, 2002) is higher than that of GF-AAS (~3-4 minutes/sample), and this can increase sample throughput and help reduce costs. In terms of the newest X-ray-based methods (TXRF and EDXRF), the reported LoDs (0.28-1.70 μg/dL) are comparable to ICP-MS (Rodríguez-Saldaña *et al*., 2020; Specht *et al*., 2021). Such X-ray-based methods may represent a third generation approach as they have comparable sensitivity and specificity for Pb measures as ICP-MS, but with relatively lower background noise and elemental interferences, lower operational and maintenance costs, and samples can usually be processed in a few hours versus several hours to days.

The calculated LoD for DBS Pb from ICP-MS (and XRF-based) studies is still higher than what is being reported as part of national biomonitoring programs though the latter focus on BLL measures taken on whole blood (e.g., CHMS Cycle 4 LoD was 0.16 μg/dL; CHMS, 2017). Such a difference is not surprising given that DBS samples have low sample volumes and potential matrix effects due to the filter paper (versus whole blood samples that tend to provide several milliliters of sample for analysis). Regardless, the LoD reported in newer studies using ICP-MS seems sufficient when compared to BLL in the general population. For example, the 10% value for Canadians (aged 6-79 years, both sexes) from the CHMS is 0.43 μg/dL (CHMS, 2017) which is relatively close to the mean of LoD values we calculate here (0.6 μg/dL).

Capillary and venous blood differences

DBS are derived from capillary blood, which may have some differences compared to venous blood. There are few studies that have evaluated differences in Pb levels between capillary and venous blood. In the 1990s, three laboratories were funded by US CDC to develop validation studies on the suitability of DBS sampling in the assessment of Pb exposure (Parsons *et al*., 1997; Schlenker *et al*., 1994; Schonfeld *et al*., 1994) (Appendix 3). These studies found encouraging results and reported a good correlation between Pb measurements in capillary DBS and venous whole blood. Specifically, Schlenker (1994) reported a correlation coefficient of >0.96 in a study of 295 children from Wisconsin, Parsons *et al*. (1997) reported a correlation coefficient of 0.98 in their study of 533 children from New York City, and Schonfeld *et al*. (1994) reported a correlation coefficient 0.97 in their study of 172 children from Connecticut. However, to our knowledge the differences and agreement between the two sources of blood was not further explored in these studies without which we cannot understand the variance and bias across the exposure range. In subsequent years, other

studies have also reported a strong positive correlation between the two types of blood with correlation coefficients exceeding 0.8. Specifically, Yee and Holtrop (1997) reported a correlation coefficient of 0.96 in a study of 163 samples from Michigan, and Nyanza *et al*., 2019 found a similar correlation (r^2= 0.99) in a study of 40 pregnant women sampled from an artisanal and small-scale gold mining community in Tanzania. In a study by our research group exploring the potential of a TXRF-based approach to analyzing Pb in DBS (Rodríguez-Saldaña *et al*., 2020), a strong correlation was found between measures taken in capillary and venous blood in a low Pb-exposed group of university community members in Canada (r^2=0.82; mean BLL was 0.78 μg/dL) as well as a highly exposed group of electronic waste workers in Ghana (r^2=0.91; mean BLL was 3.8 μg/dL). This latter study also used Bland Altman analyses to assess the agreement between the two measures and whether there were any systematic biases. In doing so, no constant bias or proportional bias were found between the two measures (in each population group separately and also combined), and the range of the limit of agreement was 1.8 μg/dL. Moving forward there is a need for more studies that move beyond correlative analysis to increase understanding of variance and bias across the exposure range.

Contamination issues

The potential contamination of DBS samples in Pb analysis has been one of the most debated in the literature. The findings (and conclusions) by the authors is mixed with 30.7% of the studies pointing out that contamination of samples had an impact in the accuracy and precision of their measurements (Cooke *et al*., 1974; Moore *et al*., 1977; Morgan *et al*., 1979; Schonfeld *et al*., 1994; Cizdziel, 2007; Chaudhuri *et al*., 2009; Funk *et al*., 2013; Michniewicz *et al*., 2015), with even more studies (69.2 %) concluding that contamination of samples was minimal and did not affect the reliability of their results (Joselow and Bodgen,

1972; Piomelli *et al*., 1980; Verebey *et al*., 1991; Wang and Demshar, 1992; Parsons, 1993; Yee *et al*., 1995; Yee and Holtrop, 1997; Stanton, 1999; Pfitzner *et al*., 2000; Shen *et al*., 2003; Resano *et al*., 2007; Hsieh *et al*., 2009; Hsieh *et al*., 2011; Funk *et al*., 2015; Rello *et al*., 2015; Moreda-Piñeiro *et al*., 2017; Nyanza *et al*., 2019; Rodríguez-Saldaña *et al*., 2020). In general, these studies that did not report an issue with Pb contamination are ones that purposefully included steps in their methods (e.g., thorough cleaning protocols, analyzing many blank filter cards, working in a clean room environment) to minimize the possibility of contamination.

The studies point out three considerations with respect to potential contamination by Pb: 1) the filter paper itself; 2) technology and methods used to perform the studies; and 3) the protocols followed during the collection and processing of DBS samples. Authors have pointed out that the filter paper itself could contribute to contamination issues (Cizdziel, 2007; Chaudhuri *et al*., 2009). While a few brands of filter paper tend to dominate this area, there are many manufactured lot numbers. The majority of studies reviewed did not well characterize the background level of Pb in their DBS cards (Appendix 3). In a previous study of ours, we found an average of 0.02 ng of Pb in each DBS circle on a blank card, and when considering that a circle is estimated to hold 60 μL whole blood the estimated BLL would be 0.03 μg/dL which is very low (Rodríguez-Saldaña *et al*., 2020). However, others have reported contamination issues related to the filter cards themselves (Chaudhuri *et al*., 2009; Funk *et al*., 2013). We note that in a subsequent study by Funk et al. (2015) that the application of a pre-cleaning treatment successfully removed contamination from their filter cards. Besides the card itself, others have found trace amounts of Pb in soap bottles (Cooke *et al*., 1974) or have suspected contamination related to handling or storage (Moore *et al*., 1977; Morgan *et al*., 1979; Michniewicz *et al*., 2015), thus necessitating that researchers ensure that

their work environment and materials are clean. Earlier we discussed advances in the analytical instruments and impacts this may have on detection limits, which in turn could influence if contamination is detected.

Hematocrit effect

The hematocrit effect is widely discussed in the literature as a potential problem in the analysis of any analyte in a DBS card (De Kesel *et al*., 2013; Daousani *et al*., 2019; Velghe *et al*., 2019). Hematocrit influences blood viscosity and thus how blood distributes across the filter paper cards. As Pb binds preferentially to erythrocytes, the uneven distribution of blood and its components on the card can affect the validity of the measurement. In some studies, a whole DBS may be sampled though in these cases the volume may not be known (i.e., when blood is dropped onto the card versus being volume-pipetted). In other cases, a punch of a DBS is taken, and in these cases the volume of blood (as well as its composition) is not easily resolved.

In the current review, around 15% of the studies included discussed the hematocrit effect (Yee and Holtrop, 1997; El-Hajjar, 2007; Resano *et al*., 2007; Peck *et al*., 2009), with reported hematocrit levels ranging from 25 to 55%. The main conclusions from these studies were that: a) excluding the periphery of DBS circles could help to avoid measurement errors, b) DBS samples prepared at the normal range of hematocrit does not differ from paired venous blood samples, and c) correcting the hematocrit effect in the DBS measurements is a useful way to maintain the reliability of the method.

From our review only one study evaluated the influence of hemoglobin (which gives similar information as hematocrit) on the concentrations of Pb in DBS samples (Carter, 1978). The

hemoglobin range considered in this study was 4.6-16.1 g/dL. Carter (1978) found that samples with low hemoglobin levels spread more than those with high concentrations and proposed that Pb values obtained from blood samples with low hemoglobin levels should be adjusted to improve reliability.

2.5 Concluding remarks

For several decades there has been an interest in the use of DBS to biomonitor Pb exposure. Individual researchers have raised potential concerns about the various approaches used, and taken together these have likely contributed to hindering the wider adoption of DBS-based methods. As such, the current study was conducted to increase our understanding of the current state of methods used to analyze Pb in DBS, the performance of these methods against quality control benchmarks, and specific areas of concern that are common across studies. By synthesizing such information, we are able to point out fruitful areas of future research. Specifically, more research is needed on the LoDs achieved by the various methods, potential differences in Pb concentrations between capillary and venous blood, the influence of the hematocrit effect (and ways to account for it), and the potential contamination of samples with Pb.

2.6 Author contributions

This manuscript was written by all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally.

2.7 Conflict of interest declaration

The authors declare no conflict of interest

2.8 Acknowledgments

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Supporting information

S2- Review protocol

The performance of dried blood spots for the assessment of lead exposure: a narrative review with a systematic search

Verónica Rodríguez-Saldana, Niladri Basu

Review question

What are the main limitations in the application of DBS sampling in the assessment of Pb exposure?

Searches

Studies will be identified via electronic searches of PubMed, Scopus, and Web of Science, including the Medical Subject Headings (MeSH): "Lead", "Lead Poisoning" and the headings "Pb" ,"Lead exposure", "dried blood spots", "DBS", "bloodspots", "filter paper", between December 2018 and March 2021 (updated)

Types of study to be included

1. Must present BLL data derived from venous, capillary, or umbilical cord DBS samples.
2. Must include BLL data from human populations residing in [any country].
3. Utilized data must have been collected after 1960.
4. Must be published in English.
5. Validation studies will be also included.

Condition or domain being studied

Blood lead levels (BLL)

Participants/population

All human BLL data from DBS samples will be included

Intervention(s), exposure(s)

No interventions are being reviewed. All exposures resulting in detected BLLs will be included.

Comparator(s)/control

Not applicable

Context

Main outcome(s)

Lead exposure assessment

Additional outcome(s)

Not applicable

Data extraction (selection and coding)

Two reviewers will independently search databases with the defined search terms

Due to the broad nature of the search terms, the search will yield a large number of irrelevant articles

In cases where searches return 50 or fewer results, titles will be manually reviewed
Titles that indicate that the study is relevant to the present effort will be exported to a .csv file. When searches return more than 50 results, all titles will be downloaded to a .csv file for manual review

Keywords will be used to identify any titles missed in the manual review

Abstracts will be reviewed for relevance. If the abstract indicates that the article is not relevant, it will be excluded. In case the abstract indicates that the article is relevant, the reviewer will endeavor to extract the following information: **title**; **author**; **year**; **geographic location**; **population characteristics** (sex; age; occupational/non occupational; number), **IRB approval**, **BLL** statistics (means, median, SD/ SE, range), **QC parameters (**limits of detection, precision, accuracy, % non-detected samples, maximum value**)**; **sample volume**; **DBS studied** (e.g., punches).

Each step in the review will be conducted independently by each reviewer with discrepancies resolved through discussion

Risk of bias (quality) assessment

Each reviewer will assess each study using checklist of the standards for reporting diagnostic accuracy studies (STARD statement). Discrepancies will be resolved through discussion

Strategy for data synthesis

We will provide a narrative description of the limitations identified (consistently reported) in the use of DBS for the assessment of Pb exposure. We do not anticipate that the data will adequately support a robust quantitative assessment, however, quantitative data will be provided to understand the characteristics of the studies and to enrich the discussion on whether the reported analytical aspects among the studies represent or not a limitation for DBS analysis.

At a minimum we expect to be able to qualitatively indicate which limitations may appear as the most significant in lead exposure assessment using DBS.

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Type and method of review
Systematic review

Anticipated or actual start date
20 December 2018

Anticipated completion date
26 March 2021

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Conflict of Interest
The authors declare no conflict of interest

Language
English

Country
Canada

State of review
Review completed

Subject index terms
Environment; Environmental exposure; Human exposure; Exposure assessment; Lead; Lead poisoning; Biomonitoring; Dried blood spots

Stage	Started	Completed
Preliminary searches	Yes	Yes
Piloting of the study selection process	Yes	Yes
Formal screening of search results against eligibility criteria	Yes	Yes
Data extraction	Yes	Yes
Risk of bias (quality) assessment	Yes	Yes
Data analysis	Yes	Yes

Versions

June, 2021

Supplementary table S2-1. Compilation of studies on Pb exposure assessment using dried blood spots and the information retrieved

Study location	Reference:
Texas, U.S.	Archer et al., 2012
Utah and three other states in the Rocky Mountains region of U.S. (collection over a period of 3 years)	Chaudhuri, 2009
North Carolina,	Funk et al., 2013
Iowa, U.S.	Carrel et al., 2017
Nebraska, U.S.	El-Hajjar, 2007
U.S. (Michigan)	Montrose 2020
U.S. (Nebraska)	Peck et al., 2009
Tanzania (Geita)	Nyanza et al., 2019
Tanzania (Geita)	Nyanza et al., 2021
Lille, France.	Vacchina, 2014.
Denmark.	Pedersen, 2017
Las Vegas U.S. specimens provided by the Wisconsin State Lab. of Hygiene 2004	Cizdziel, 2007
Spain (Corunia)	Moreda-Piñeiro et al., 2017
Taiwan	Hsieh et al., 2009
Taiwan	Hsieh et al., 2011
Michigan, U.S. (1996.1997)	Yee and Holtrop, 1997
New York, U.S. 1990-1991	Verebey et al.,1991
Conecticut, U.S	Cooke et al., 1974
U.S. (N.Y)	Piomelli
U.S. Wisconsin	Schlenker et al., 1994
U.S. Wisconsin (PT progam)	Stanton et al., 1999
U.S. (Michigan)	Yee et al., 1995
U.S. (NY)	Parsons et al., 1997
U.S.	Schonfeld et al., 1994
Catanzaro, Italy.	Di Martino et al., 2004
Italy (calabria)	Michniewicz et al., 2015
Spain (Zaragoza)	Rello et al., 2015
Spain	Resano 2007
U.K (Scotland)	Moore et al., 1977
Great Britain	Morgan et al., 1979
London	Cernik and Sayers, 1971
Ontario (1984-1987 collection), Toronto (DBS collection)	Wang and Demshar, 1992
Nigeria, Jos	Pfitzner et al., 2000
France	Joselow et al., 1972
China	Shen et al., 2003
U.S. (Utah)	Moyer, et al., 1999
Canada	Rodriguez-Saldana et al., 2020
U.S. (Boston)	Specht et al., 2021

Instrumentation	DBS sample studied	Filter cards	Sample size (# of individuals)
ICP-MS	3/16" punch (estimate 11.5	Not mentioned	249 (50 with elevated BLLs and 199 with very low BLLs)
ICP-MS	1/4" punches (6.35 mm in diameter)	Whatman no. 903	Not specified (table 11b report a performance summary of 18 individuals)
ICP-MS	One half of DBS (~30 uL)	Whatman #903	49 samples (newborns)
ICP-MS	1/8 " diameter punches	Not specified	2300 DBS samples as part of routine newborn screening over a 5-month
ICPMS	6 mm punches	Schleicher and Schuell #903	28 DBS samples (prepared with excess patients speciments and spiked with
HR-ICP-MS	2 (3 mm punches)	Whatman 903	DBS from children 129 (40 from 12-24 months -todlers, 39 from preschool
Initially ICPMS	single 6 mm circular punch	Not addressed	Samples were 422 remainder bloodspots from 138 patients (an average of 3
ICPMS	8-14 mm punches	Whatman 903	DBS from 40 pregnant women living in areas with artisanal and small-scale
ICPMS	8-14 mm punches	whatman 903	DBS from439 woman in their second trimester between 2015 and 2017
ICP-MS	Not addressed (membranes	PVDF filter membranes	2 (9 DBS from each volunteer)
ICP-MS	3.2 mm punches	Whatman no. 903	20 (from table 3), three lots for the validation
LAICP -MS	Not specified (200 um spot size beam)	Not specified (special designed filter paper)	5 (DBS samples). The materials were part of a prior FP proficiency testing program (year 2004). The Pb target values for these samples were established
LA-ICPMS	3 mm punches	Whatman 903	7 DBS from newborns
LA-ICPMS	110 um	PTFE filter membrane	Aqueous and matrix matched standards (4 RM and 3 replicates)
LA-ICPMS	110 um	PTFE filter membrane	CRM Seronorm
GF-AAS	1/4" punches	# 903 Schleicher & Schuell,	163 samples from 6 urban sites
FAAS	1/4" punches	# 903 Schleicher & Schuell,	6 samples (spotted 10 times) ("matrix matched standards"(human blood)
AAS	3/16 inch (5 mm)	# 903 Schleicher & Schuell,	170 clinical patients (children 1-5 years old)
AAS (Delves cup)	Whole circles and punches	Filter paper	15 healthy adults (volunteers)
GF-AAS	Not applicable	Capillary blood vs Venous blood	295 (60 from pilot population) children btw 6 months and 6 years (method
Mostly electrothermal at-	Different methods used, laboratories from A-F	Schleicher & Schuell no. 903 filter	Volunteer donors were recruited from a cohort industrial workers of mixed sex and race with occupational lead exposure. 6 more non-exposed donnors
GF-AAS	1/4 inch diameter paper punch	Schleicher & Schuell no. 903 filter	Initially 10 samples and a follow up validation with 100 (2 samples per
GF-AAS	Not applicable	Capillary vs Venous blood	Matched venous and capillary blood specimens were collected from 533
GF-AAS	3/16 inch punches	Schleicher & Schuell no. 903 filter	1546 samples from 172 children (only for filter paper tests, between 6 months
GF-AAS	6.3 mm punches	Schleicher and Schuell no. 2992	20 (hospital patients)
GF-AAS	6.3 mm	Schleicher and Schuell no. 2992	Residual DBS Neonatal screening tests (130 DBS samples per region, 9
HR-CS-ETAAS	3.2 mm punches	Whatman 903.	DBS samples (n = 526) collected at the same time from newborns (n = 229),
SS-GFAAS	3.2 mm punches	Schleicher & Schuell 903	18 patients
GF-AAS	3 mm punches	P.K.U. cards	77 DBS (41 from children with intellectual disabilities and 36 of controls)
GF-AAS	small punch samples from 1-	PKU cards	50 DBS from newborns (6-14 days old)
AAS (Delves	8-9 mm diameter	Whatman #4 (9 cm diameter)	85 (workers from 5 factories, male)
GF-AAS	3/16" punches (6.7 mm^3 of	Schleicher and Schuell no. 903	425 (newborns)
GF-AAS	Verebey et al., cited in	"filter paper" no brand mentioned	218 children between 6 and 35 months (cross-sectional study) (48 different
GF-AAS	Whole discs and punches	Filter membrane (DELVES)	Children samples clinic, unclear if field conditions used children or adults
GF-AAS	0.6-cm filter paper disk	Schleicher & Schuell 903	Children 159 (clinical conditions)
Discussing AAS	NA	Discussing Filter paper in general	NA (Editorial, included for the limitations discussion)
TXRF	1 entire circle and 3 mm	Whatman 903	40 (non-exposed individuals) and 41 (ocupationally exposed individuals)
EDXRF	whole circle/area	Whatman 903	22 blood samples from children (Boston Children's Hospital) and 10

Central measures value (median, mean, etc)	% non-detect	Detection limit
Not specified (< 5 ug/dL from table 1)	92% (<5 ug/L)	Not mentioned
0.41± 0.10 ug/dL (lot W011), 0.82 ± 0.12 ug/dL (lot W031) and 0.32 ± 0.07 (lot W041)	Not addressed	Estimate= 0.36 ug/dl, based on three times the 0.12 mg/dl SD
13.3 ug/L (1.33 ug/dL)	100% detected	Low ppt range
2.47 ug/dL	N=18 (0.78%)	Not specified (low ppt)
Not applicable	Not addressed	Lowest concentratin std = 2
Median = 0.55 µg/dL (<LoD-	16% < LoD	0.07 ug/dL
Not applicable	Not applicable	Not applicable
Median= 26.3 ug/L (17.0-	100% of samples above LoD	0.080 ug/L (0.008 ug/dL)
Median= 27.2 (25–30.3) ug/L	100% detected	0.080 ug/L (0.008 ug/dL)
27 ± 3 ug/L and 35 ± 18 ug/L (volunteer A and B respectively, capillary blood)	Not addressed	3 ug/L (from table 1)
<3.72 ug/dL (from table 3)	Not addressed	0.18 umol/L (3.72 ug/dL
1±0.79; 7±0.5; 15±1.26; 20±1.43, and 30±1.94 ug/ dL	Not addressed	0.9 ug/ dL
61.1± 5.4 , >LoD, 43.1± 3.8,	14% of samples (1 out of 7 samples From table 4)	11.9 ug/L (1.19 ug/dL)
Not applicable	Not applicable	0.1 ng/mL
Not applicable	Not applicable	0.13 to 2.8 ng mL−1
1.5 ug/dL (batch 1), 1.2 ug/dL	34.07% (estimated from table 1)	Not specified (< 2 ug/ dL)
98 ug/dL (batch 1), 92 ug/dL	Not addressed	Not specified (< 4 ug/dL)
389 ug/L (38.9 ug/dL)	Not addressed	Not specified
mean= 14.6 ug/dL	Not addressed	Not specified
Mean capillary blood= 0.77	Not addressed	Not addressed
Mean= 20.8 □g/dL	Not applicable	Elsewhere
2.5-24.1 ug/dL (mean = 8.9	Not addressed	Not addressed
median □ 78 □g/L, SD □ 111	Not addressed	< 20 ug/L (< 0.2 ug/dL)
Median= 14 ug/dL and mean	Not addressed	Not addressed
6.58 ug/mL (DBS + EDTA), 3.62 ug/mL (DBS-EDTA)	Not addressed	Not mentioned
Median 1.52-3.93 ug/dL (3.93	0-6% from table 2	0.06-0.027 ug/dL (0.22 ug
Not addressed	Not addressed	1 ug/L (0.1 ug/dL
Not specified (between 40 and	Not specified (most samples lower than 30 ug/L)	0.25 ug/dL
1-23 +/- 0-43 umol/1 (25.49	Not addressed	Not addressed
mean 1.81 + 0.77 Umol/L	Not addressed	Not addressed
Mean from the group of	Not addressed	Not addressed (lowest
0.19 ± 0.13 umol/ dm3 (3.93 ug/dL)	4.7% (estimated from fig. 3)	0.05 umol/ dm3 (1.035 ug/dL)
Mean= 15.2 ± 1.4 µg/dL	Not addressed 12 % chldren had BLL < 10 ug/dL)	Not addressed (verebey cited)
Not addressed	Not addressed	Not addressed
9.5 (3.7) ug/dL (mean)	Not addressed	Not addressed
NA	NA	NA
0.78 (±0.46) and 3.78 (±3.01)	0%	0.28 ug/dL
21 ± 12 µg/dL (Boston's	NA	1.7 µg/dL

Precision	Linearity	Upper measurement value
0.78 (at the 5μg/dL),0.60 (at the 20 μg/dL) and 0.68 (at the 50	0.05-1 μg/dL, R^2= 0.9999	Not specified (≥10 ug/dL)
Not addressed	Not addressed	1.88 ug/dL (from table 11b)
10.2 %	Not addressed	Not addressed
< 10%	Not addressed	4.59 ug/dL
10.9 % >= 4 ug/dL in blank samples (18 blanks)	R2= 0.986	Not applicable
This analysis method incorporates daily quality assurance and	Described elsehwere	5.27 ug/dL
Not applicable	Not applicable	Not applicable
Repeatibility coefficient 5.8 (5.2-6.5) and ICC values above 0.9	R2=0.969	24.9 ug/L (2.49 ug/dL)
<= 10 % RSD	R2= >0.9	145 ug/L (14.5 ug/dL)
< 12%	R^2= 0.995	Not addressed
Concentrations were lower than LoQ	44-132%	Not specified (<LoQ)
< 15 %	R^2= 0.996	30 ± 1.9 ug/ dL
> 12% RSD	R2= 0.985	122.76 ug/L (12.27 ug/dL)
5-9 % RSD (Seronorm reference material)	R2=0.996-0.998	Not applicable
>5.7% RSD	R2= 0.998	Not applicable
5.8 % (at 8.3 ug/dL within-run precision)	R^2= 0.975	45 ug/ dL (from table 1)
3.45 (at 42ug/dL)	R^2= 0.988 (compared with MIKB extracio	288 ug/ dL (from table 3)
False negative = 1.8 % False positive= 19.4%	R2= 078	400 ug/L (40 ug/dL)
Not addressed (duplicates never exceeded 1 ug/dL)	Not addressed	Not addressed
Sensitivity = 86-96% and specificity= 94-100%	r2= 0.96-0.98%	22.5 ug/dL (from table 2, method 4 home
Of the 34 results falling outside the acceptable range, 29 (85%) fell below the minimum threshold, whereas only 5 (15%) were	r2= 0.94-0.99 % (relationship to FP program values)	1–38 □g/dL
within-run precission at a mean of 6.85 ug/dL 15% CV (N=15).	r2= 0.993 (correlation with venous whole	24.1 and 23.1 ug/dL from methods 1 and
as SD, is □ 2.5 □g/L (0.25 ug/dL) in the range 100–200 □g/L	Not applicable	~ 790 ug/L (79 ug/dL from Figure 1)
False positive rates were 19.1 and 31.4 at thresholds 15 and 25	r2= 0.83	73 ug/dL
Not addressed	Not addressed	18.5 ug/dL (estimated from Fig. 2,
0.27–0.65 µg/dL in blank filter paper	R2= 0.995±0.006	3.93 ug/dL
RSD > 10 % The rate of true positives detected was always	r2= 0.97 between ETAAS and HR-CS-	> 50 ug/dL
6.2% RSD	R2= 0.995	> 160 ug/L (16 ug/dL)
8-4 +/- 6-81% (CV in standard cards) 7’ 65 +/- 4-82% (CV in	Not addressed (only a correlation btw	> 2 umol/L (41.44 ug/dL)
= 12%	Not addressed (Moore et al., cited as	2.61 +/- 0.54 umol/L (54.08 +/- 11.19
SD from 12 replicates at 3 levels of concentration (SD ranged	Not addressed only correlation between	87 ug/dL (reported as ug per 100 ml)
11.8% (at 24.01 ug/dL); 7.8 (at 53 ug/dL)	R^2= 0.980	> 0.50 umol/dm3 (>10.35 ug/dL)
Not addressed	Not addressed	> 60 ug/dL (one children)
50% capillary blood samples 4 ug/dL higher than venous blood	R2=0.9	Not addressed
sensitivity = 82%, 85% and 100% at the target cut-offs of 10, 15	Not reported (only correlation coefficient	26.5 ug/dL
NA	NA	NA
14.9% (ranged from 9.9 to 19.1%)	R2= 0.997	95th percentile of 2.18 and 11.43 ug/dL
CV= 0.27 % (300uL blood spot) and 0.08 % (150 uL blood spot)	r2=0.997	40 µg/d (Boston's hospoital samples)

IRB Approval	Accuracy
NO	28% (at the 5μg/dL), 6% (at the 20 μg/dL) and 9% (at
NA	120% (without background subtraction) and 80% (with background subtraction)
NO	Recovery of Pb was in the range of 48-230%
YES	Not addressed
NA	101 +/- 5%
YES	Described elsewhere
NO	Not applicable
YES	102.80%
YES	Slope close to 1.0 between DBS and venous whole
NO	85-115 %
YES	95.1- 121.5%
NO	Not addressed
NO	105.5-113.0 % (calculated from table 4)
NA	97.8-112.8 %
NA	102%
NO	125.6% (0-5 ug/dL), 86% (>30 ug/dL)
YES	92.8-112.5 % recovery or proficency samples (from
NO	Not addressed
NA	Not addressed
YES	false negatives= 1-8% and false positives= 3-5
YES	Because the highest PT specimen concentration was 1.83 □mol/L (38 □g/dL), the predicted results at the
NO	98.6-119.5% recovery
YES	better than 15 □g/L below 400 □g/L, and rarely
YES	Not addressed
NO	Not addressed
YES	Not addressed
YES	Not addressed
NA	104.03 % (recovery from table 4)
NO	Not addressed
YES	Not addressed
NO	Not addressed
NA	97 ± 10 %
YES	Not addressed
NO	Not addressed
YES	specificity was 92%, 99% and 100%
NA	NA
YES	111.1% (97.0–129.7%)
NO	100-105%

QC
QC samples were prepared by spiking sheep' s blood with aqueous solutions containing known amounts of lead. These spiked blood pools were
Reconstituted reference material (RRM) samples were prepared from a freeze-dried, human whole-blood toxicology control (level 1). Two sets of RRM internal blank and blood punch pairs were analyzed after every 10 sample pairs. Additional quality control samples with whole human blood
Blank samples, spike standards and internal standards
Blood-based external standards and filter paper blanks (>100)
Bloodspot standards were then prepared by bolus addition of50µl of the whole blood standards to filter paper
NIST SRM 1640a was used as an external quality control standard to check the calibration and NIST SRM 955c caprine blood was used to assess
Control samples (standards prepared from an EDTA whole blood sample of normal hematocrit)
26 field blanks (0.020 0.020 ug/L) 26 lab blanks (0.009 0.017 ug/L) , CRM
Seronorm reference material (SRM), L-2®, (from Sero AS, Billingstad, Norway each 10 samples batch
Seronorm reference material (5 replicates) and blank samples (10 replicates)
Seronorm and CRM for toxic metals in bovine blood
Blank FP sheets and five spotted blood Pb specimen cards were provided by the Wisconsin State Laboratory of Hygiene (WSLH). The materials were part of a prior FP proficiency testing program (year 2004).
YES (Seronorm II and III)
YES, BOVINE BLOOD, CRM SERONORM TRACE ELEMENTS
YES, BOVINE BLOOD, CRM SERONORM TRACE ELEMENTS
Pb standard (FisherLead Reference Solution certified to contain1000+ 10mg lead/L) and blank samples (n=69)
Matrix matched standards (goat blood) sent by the state of NY and external Pb standards
A series of standards was run with each set of samples. For the standards, "A blood with a known " normal" lead concentration was used "
Samples in duplicate, blank samples taken from each samples cards)
Comparison agains venous blood samples. Material tested for Pb contamination
A,E,F used blood-based reference materials and C and D used aqueous-based standardas
Aqueous standards increasing concentrations, two 1/4 inch diameter paper punch blanks
100-□L aliquots of reference low-Pb animal blood. Materials assessed for Pb contamination.
Control samples with known concentrations
Yes (seronorm as reference material and Pb standard solutions for calibration)
YES (Seronorm, each 10 samples)
Matrix-matched reference materials created with venous blood samples and Pb standards
Yes (CRM ERM -CE194, 5 replicates and blank samples)
YES, standard cards made with known concentrations
2 punch samples from blank cards, blood standards
Ambient conditions tested with replicates, comparison between methods (POL vs AAS). 12 replicates, samples with known concentrations and
Blood standards M1 and M2 from the Behring Institute (Marburg, Germany) and human whole-blood controls from Bio-Rad (Anaheim, CA, USA)
Not addressed
Not addressed
Stability tested, quality control program participation
Discussing contamination and capillary/venous blood differences
3 blanks were included in each batch of 22 DBS samples, 4 different matix-matched references materials at increasing concentrations
Pb aquous standards for the ideal calibration and samples with known concentration for the quality control

Volume (calculated, assumed, ignored?)
Controlled, 6.7 uL (assumed to be the volumen in a 3/16 inch DBS
The blood was added until the dotted, printed circle was filled, which corresponds to a total blood volume of about 75 µl (according to clinical and laboratory standards institute, 2007). This value was calibrated during this study too. The authors stated their concern about the blood volumen
Estimated by normalization of dried blood mass (30 uL)
Estimated as 6 uL
This paper is focused on the area, volume and concentration of annular specimens, thus volume was controlled (50 uL)
assumed to contain 3.1 µL of blood based on previous studie
initially estimated as < 16 uL of whole blood in 6 mm circular punch. Apparent blood volume calculated from image analysis in the area of DBS
One drop per circle (Gravimetric analysis used to estimate the amount of dry blood per circle, 50 μL of blood assumed for blanks)
the mass of dry blood in each spot was first measured. The range of dried blood mass was between 45-55 uL. 50 uL of blood was assumed for
Determined by weighing the membrane before andafter sampling
(5 drops of blood on each card, i.e. approximately 275–375 µL. Different hematocrit levels evaluated
Ignored, nondestructive method
20 uL for seronorm DBS and analyzed different volumes (15, 20, 25 and 30 mL) finding no significant differences. Assumed in Newborns DBS,
0.5 uL blood sample
0.5 uL blood sample
Volume controlled= 16 uL
Volume controlled= 20 uL
Estimated as 10 uL of blood per disc
Not mentioned (filter discs and punches used)
100-500 uL capillary blood and 2 mL specimens of venous whole blood
10 mL blood specimens (3 tubes combined in a 50-100 mL baker and mixed) then spotted in filter paper
6-8 drops of blood onto filter paper, second phase used 50 mL of venous blood spotted onto filter paper
usually 50 uL, sometimes the was not a sufficient volume of capillary blood
5-8 drops to fill a circle, delves cup method
Volume was calculated as follows: was determined by the following procedure: 1 mL of blood with 44% HCT was mixed with 50 µL of 125I
Estimated from liquid samples (same in Di Martino)
Samples analyzed directly not extraction needed
Calculated (different volumes of reference materials spotted on filter cards)
Between 4-6 punches placed as sandiwich in the muffle furnace, samples were analyzed by GFAAS
"Blood volume involved having been determined from Standard blood samples from blank, uncontaminated cards".
10 uL (calculated, discs weighted)
50 mm3 aliquot of blood spotted onto filter cards. A 3/16 inch dried blood spot represents assumed 6.7 mm3 of whole blood
Not addressed (not contamination reported, differences btw hematocrit levels where assessed)
Not addressed
Not addressed
NA
First whole circles used (60 uL), then 3mm punches. Sub-samples also weighted.
Volume controlled and different volumens tested

Supplementary table S2-2. Quality assessment of each study included

First author, year	Easy to identify	Research question clearly stated	Aim clearly stated	IRB approval stated	Study design explained	Sample acquisition explained	Sample storage described	Sample preparation described	DBS type explained	QC/QA reported	Analysis explained	Statistical methods reported	Limitations discussed	Conflict of interest reported	Score
Archer et al., 2012	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Carrel et al., 2017	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Cernik and Sayers, 1971	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	12
Chaudhuri et at., 2009	Yes	Yes	Yes	NA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Cizdziel et al., 2007	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	12
Cooke et al., 1974	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Di martino et al., 2004	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	12
El-Hajjar et al., 2007	Yes	Yes	Yes	NA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Funk et al., 2013	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Hsieh et al., 2009	Yes	Yes	Yes	NA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Hsieh et al., 2011	Yes	Yes	Yes	NA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Joselow et al., 1972	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	10
Michniewicz et al., 2015	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	12
Montrose et al., 2020	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	14
Moore et al., 1977	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Moreda-Piñeiro et al., 2017	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Morgan et al., 1979	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	13
Nyanza et al., 2019	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	14
Nyanza et al., 2021	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	14

Parsons et al., 1997	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NA	Yes	Yes	Yes	Yes	Yes	13
Peck et al., 2009	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	12
Pedersen, 2017	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	14
Pfitzner et al., 2000	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	13
Piomelli et al., 1980	Yes	Yes	Yes	NA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Rello et al., 2015	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	13
Resano et al., 2007	No	Yes	Yes	NA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	11
Rodriguez-Saldana et al., 2020	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	14
Schlenker et al., 1994	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NA	Yes	Yes	Yes	Yes	Yes	13
Schonfeld et al., 1994	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	14
Shen et al., 2003	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	14
Specht et al., 2021	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Stanton, 1999	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	12
Vacchina, 2014	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	No	11
Verebey et al., 1991	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	14
Wang and Demshar, 1992	Yes	Yes	Yes	NA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Yee et al., 1995	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	11
Yee and Holtrop, 1997	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	11

A score “1” was given for each reported item.

CONNECTING PARAGRAPH

Chapter 2 presented a systematic evaluation of the available literature and an identification of the areas for further research on the use of DBS in the assessment of Pb exposure.

One of the gaps on the application of DBS sampling for Pb biomonitoring and other metals is the potential differences between venous and capillary blood, and the lack of information on the agreement between the two blood sources.

Chapter 3 is centered on determining if Pb concentrations in capillary blood is similar to that in venous blood from the same individual. This chapter is focused on three comparisons: association of samples, significant differences, and agreement analysis.

Pb biomonitoring was carried on along with other metals which toxicity is relevant in human health, such as As, Cd, and Mn and essential elements including Cu, Fe and Se. Thus, evaluating these elements is valuable for the application of multi-element analysis. We evaluated the agreement between venous and capillary whole blood for those selected elements along with Pb.

This chapter was co-authored by the candidate's supervisor Dr. Niladri Basu. This manuscript was submitted to the Journal of Trace Elements in Medicine and Biology on August 23, 2021.

3. **CHAPTER 3**. Comparison and agreement of toxic and essential elements between venous and capillary whole blood

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3.1 Abstract

For practical and ethical reasons there is an interest in the use of capillary blood as an alternative to classical human biomonitoring methods that use venipuncture. However, with the exception of Pb, few elements have been studied in detail to understand potential differences in measures between capillary and venous blood.

To increase our understanding of the agreement in concentrations of select toxic metal(loid)s and essential minerals in venous and capillary whole blood.

Concentrations of As, Pb, Cd, Mn, Cu, Fe, and Se were measured with Inductively Coupled Plasma-Mass Spectrometry in venous and capillary whole blood samples obtained from 49 healthy adult members of the McGill University community in 2016.

Measured concentrations of all elements were similar to values reported in background populations. There were strong correlations (i.e., $p<0.001$) in measures taken for all these elements between capillary and venous blood. Using Bland-Altman, a deeper investigation of the agreement between the measures found relatively good agreement for blood As, Pb, Cu, and Fe. For blood Cd, Mn, and Se, the agreement between the measures taken in capillary and venous blood were less consistent, though more research is needed to determine if the method can be improved and/or if there are real differences.

For the seven elements under investigation, there was relatively good correlation and agreement in measures taken between capillary and venous blood from the same individual. Further research is needed to confirm these findings (particularly from more diverse

populations groups), expand the number of elements analyzed, and explore the utility of capillary sampling in biomonitoring programs that take samples using traditional venipuncture methods.

3.2 Introduction

Human biomonitoring studies of trace elements, including toxic metal(loid)s and essential minerals, largely rely on measures taken in venous whole blood (Bergdahl and Skerfving, 2008; Sanders *et al*., 2009). Even though venous whole blood is a preferred matrix for gauging exposure to many metal(loid)s, there remain outstanding limitations to the widespread adoption of venipuncture, particularly among vulnerable populations including young and old individuals as well as communities situated in remote and/or resource-limited regions. Foremost is the invasive nature of venipuncture, which may pose ethical, behavioral, or physiological challenges to some groups. Additional challenges of venipuncture include the need for clinical settings and well-trained personnel, specialized materials and reagents, and particular conditions for sample transportation and storage (Barbosa *et al*., 2005; Nyanza *et al*., 2019). Taken together, these challenges have motivated the search for alternative methods to venipuncture for biomonitoring human exposures to trace elements.

Capillary blood sampling (i.e., via pricking the finger in adults and the heel in small children and babies) is widely viewed as a viable alternative to venipuncture (Tang *et al*., 2017). The blood derived from a capillary source is principally arterial blood along with interstitial fluids and some venous blood (Rowland and Emmons, 2010; Enderle *et al*., 2016). While there are physiological differences between these two blood types (e.g., differences in oxygen and

carbon dioxide concentrations; Tang *et al*., 2017), there are few publications that have examined for possible differences in metal(loid) concentrations between capillary and venous blood. Most of the studies in this area have focused on Pb exposure. Taken together, these studies have reported relatively high correlation in Pb measures between the two sources of blood from a given individual (r^2= > 0.96 to > 0.99) even though the studies included different sample preparation methods and analytical instrumentation (Schlenker *et al*., 1994; Parsons *et al*., 1997; Resano *et al*., 2007). Besides Pb, to our knowledge, no other element has been studied in depth to understand differences between capillary and venous blood.

The aim of this study was to increase our understanding of the agreement in concentrations of select metal(loid)s in venous and capillary whole blood. Specifically, we focused our study on toxic metal(loid)s (As, Pb, Cd, Mn) that are of notable health concern (i.e., on the U.S. Center for Disease Control's Agency for Toxic Substances and Disease Registry's 2019 Substance Priority List, they are ranked number 1, 2, 7, and 140, respectively; ATSDR 2019), as well as three minerals that are essential for human health (i.e., Cu, Fe, Se). The study population were healthy members from the McGill University community, from whom venous and capillary whole blood samples were obtained and analyzed for the target elements with Inductively Coupled Plasma-Mass Spectrometry.

3.3 Materials and methods

3.3.1 Blood collection and preparation

Ethics approval was obtained for this work from the McGill University Institutional Review Board (#A05-M26-16B). Venous and capillary whole blood was sampled from 49 volunteers (age range: 19 to 53 years) at the Mary Emily Clinic (demographic data are presented in **Supplementary table S3-1**) by a trained nurse (Macdonald campus, McGill University) in 2016 as previously described (Santa-Rios *et al*., 2020).

Study participants are considered to be healthy adults with no known occupational or environmental sources of prominent metals exposure. Following recommendations from the "USAID Biomarker field manual" (USAID, 2012), capillary blood from each individual was collected by a finger prick with a lancet (BD Microtainer®, High flow, 1.5mm blade, Franklin Lakes, NJ). Venous whole blood was collected into trace metal-free dipotassium ethylenediaminetetraacetic acid K_2EDTA tubes (BD Vacutainer®, Specialty Tubes, K_2EDTA 10.8 mg, Franklin Lakes) by venipuncture. Nitric acid (65% HNO_3 Omnitrace grade, EMD Chemicals, NJ), hydrochloric acid (12 N HCl, Merck, Germany), and hydrogen peroxide (35%, Merck, Germany) were used to digest the blood samples. A multi-element whole blood reference material from the Institut National de Santé Publique du Québec (INSPQ) Centre de Toxicologie du Québec (CTQ) was used to gauge method accuracy.

3.3.2 Metal(loid)s analysis

Blood samples were acid digested at room temperature following protocols outlined previously by us (Takyi *et al*., 2020; Rodríguez-Saldaña *et al*., 2021). Following digestion, samples were analyzed by Inductively Coupled Plasma Mass Spectrometer model 820-MS Series (Varian Inc., California, USA). Gallium was added to the samples as an internal standard at 10 µg/L

(prepared from a stock standard solution of 1000 mg/L, Merck, Germany). Every ten samples, three different blood reference materials with varying elemental concentrations were analyzed, along with blank samples (**Supplementary table S3-2**).

3.3.3 Data analysis

All statistical analysis were carried out using Microsoft Excel 2019 and R version 3.6.3, with figures created using the latter program. The accuracy (% recovery) and precision (relative standard deviation, % RSD) of the method was determined through the analysis of the blood reference materials and replicate analysis of samples, respectively.

To determine the comparability of metal(loid) measures between the two different sources of blood (venous and capillary whole blood) I evaluated: a) the differences in mean values between the two blood types using a t-test of paired samples ($\alpha = 0.05$, normality of the data was previously determined); b) the relationship of measurements between the two blood types using regression analysis; and c) the agreement between the two blood types using Bland-Altman plot analysis. From the Bland-Altman plots, data analysis focused on constant and proportional bias, the line of equality, and the lower and upper limits of agreement (LoA).

3.4 Results and discussion

3.4.1 Precision and accuracy

The analytical accuracy and precision of the method was within the predefined criteria, according to the International Conference Harmonisation guidelines (I.C.H, 2005). These results are presented in the supplementary information (**Supplementary table S3-3**).

3.4.2 Differences between venous and capillary whole blood

We used paired venous blood and capillary samples from the study population (n=49) to evaluate differences between the two sources of blood in terms of metal(loid) and essential element concentrations (**Table 3-1**). In the case the toxic metal(loid)s, there were no significant differences in the mean concentrations of As and Pb between measures taken in venous and capillary whole blood. However, for Cd and Mn, differences were calculated with values in the capillary blood being 26 % and 34 % higher, respectively, than values measured in venous blood. These differences were also of statistical significance. Regarding the essential elements of interest (Cu, Fe, Se), we did not find statistically significant differences in the concentrations between the two sources of blood.

Table 3-1. Mean (± SD) concentrations (μg/L, otherwise indicated) and 5-95th percentiles (μg/L, otherwise indicated) of metal(loid)s in paired venous and capillary whole blood samples from 49 volunteers as analyzed by ICP-MS.

Blood source	**Toxic metal(loid)s**				**Essential elements**		
	As	**Cd**	**Mn**	**Pb[a]**	**Cu**	**Fe**	**Se**
Venous (mean values)	2.4 ± 0.9	0.2 ± 0.1[b]	8.8 ± 2.0[b]	0.9 ± 0.3	767.5 ± 166.3	413044.0 ± 35026.4	160.0 ± 17.1
Percentiles							
5th	1.0	0.0	5.2	0.4	599.2	351754.1	138.7
25th	1.7	0.1	7.1	0.6	655.8	391444.0	146.1
50th	2.2	0.2	8.7	0.8	752.7	412884.4	158.6
75th	2.5	0.3	9.8	1.1	837.5	435967.7	175.1
95th	4.9	1.2	12.0	1.4	1155.2	481650.9	190.4
Capillary (mean values)	2.3 ± 0.9	0.3 ± 0.1	12.4 ± 2.5	0.9 ± 0.4	751.2 ± 164.0	407127.1 ± 32508.2	155.2 ± 16.1
Percentiles							
5th	1.0	0.0	8.2	0.4	588.1	347216.7	134.8
25th	1.5	0.1	10.5	0.6	671.2	382556.0	143.8
50th	2.0	0.3	11.8	0.7	734.9	409559.3	155.9
75th	2.4	0.4	13.3	1.2	803.4	435977.9	168.5
95th	4.5	1.6	15.5	1.9	1124.9	458780.2	179.8

[a] Concentrations in µg/dL

[b] Concentration values between venous and capillary blood are significantly different ($p < 0.05$)

3.4.3 Association (regression analysis) between the two sources of blood

For all elements studied, the correlations between measured concentrations in venous and capillary blood were of statistical significance (i.e., $p<0.001$ in all seven cases) (**Fig. 3-1**). The strongest positive correlations were found for As ($r^2= 0.87$; $y = 0.97x + 0.01$), Pb ($r^2= 0.96$; $y=0.94x + 0.08$), and Cu ($r^2= 0.79$; $y= 0.88 x + 79.98$), and less so for Cd ($r^2= 0.67$; $y = 0.94x + 0.10$), Mn ($r^2= 0.44$; $y = 0.53x + 8.10$), Se ($r^2= 0.44$; $y= 0.63x + 55.60$), and Fe ($r^2= 0.43$; $y= 0.60x + 155628$). Upon visual inspection of the scatterplots, we observe that the variability in measures for Cu, Cd, and Mn were higher at the lower concentration ranges while for Fe and Se the variability was higher in the medium concentration range.

To our knowledge there are few studies that have compared the concentrations of toxic metal(loid)s and essential minerals between capillary and venous blood. For Pb, a few studies have compared measured concentrations in capillary and venous blood and reported correlation coefficients ranging from 0.96 to 0.99 (Cernik and Sayers, 1971; Schlenker *et al*., 1994; Resano *et al*., 2007) similar to what we report here ($r^2=0.96$; see more details in the **Supplementary table S3-4**). For Se and Fe (as ferritin), similar to here, others found no significant difference in measures taken in capillary and venous blood (Van Dael *et al*., 1994; Lu *et al*., 1987). For the four other elements studied here, we are unaware of past studies that have compared measured concentrations between capillary and venous blood.

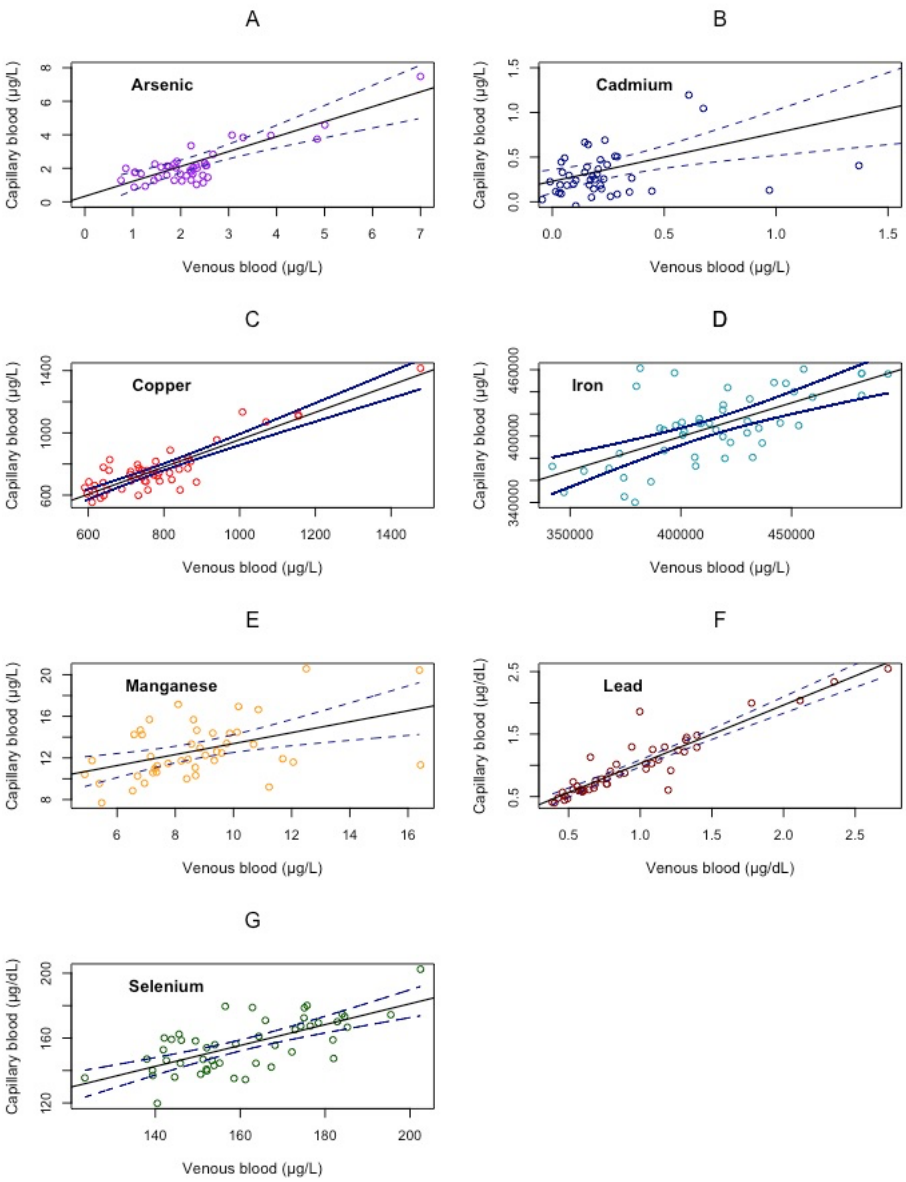

Figure 3-1. Scatterplots of toxic metal(loid) and essential element concentrations between venous and capillary blood sources from McGill University members analyzed by ICP-MS. Data is provided for: A) As (data= purple, p-value= <0.001; r^2=0.87); B) Cd (data= dark blue, p-value= <0.001; r^2=0.67); C) Cu (data= red, p-value= <0.001; r^2=0.80); D) Fe (data= greenish-blue, p-value= <0.001; r^2=0.43); E) Mn (data= orange, p-value= <0.001; r^2=0.44); F) Pb (data= dark red, p-value= <0.001; r^2=0.96); and G) Se (data= green, p-value= <0.001; r^2=0.44). Points refer to measures taken in an individual, regression lines are indicated as a solid line, and the corresponding 95% confidence interval band is indicated with dashed lines.

3.4.4 Agreement between venous and capillary whole blood

A strong association does not necessarily imply a good agreement between two measures (Bland and Altman, 1986; Giavarina, 2015; Doğan, 2018) and thus examining the variability across the measurement range is necessary to identify systematic errors and outliers. Here, agreement analyses were performed using Bland-Altman plots (toxic metal(loid)s in **Fig. 3-2** and essential elements in **Fig. 3-3**). In doing so, for each element we calculated the mean difference in measures between venous and capillary whole blood (i.e., constant bias), the lower and upper limits of agreement (LoA), the range between the LoA limits, and examined the 1:1 line of equality. To determine if there were changes over the measurement range, we calculated proportional bias by plotting a regression line in the Bland-Altman diagram.

Arsenic

The constant bias value for blood As was 0.13 µg/L (95% CI: -0.37 to 0.63 µg/L), and the line of equality fell within the 95% CI and so we conclude that there is no systematic bias in the measures. There was no proportional bias in measurement comparison with a calculated slope of -0.03 (95% CI: -0.43 to 0.37). In general, these results suggest good agreement in As measures between venous and capillary blood, even at the lower concentration range. The lower and upper LoA were -3.15 µg/L (95% CI: -4.02 to -2.29 µg/L) and 3.40 µg/L (95% CI: 2.54 to 4.27 µg/L), with a range of 6.56 µg/L. We acknowledge that this LoA range is relatively large, and that two measurements at the higher concentration range (between 5-6 µg/L) fell outside the LoA. To assess the relevancy of these blood As findings for the general population, we considered data from several biomonitoring studies performed in healthy, non-occupationally exposed adults (Heitland and Köster, 2006; Ding *et al*., 2014; Freire *et al*., 2015) carried out in diverse countries (Germany, China, and Brazil, respectively). These studies reported venous blood As levels ranging from 0.9 to 4.2 µg/L.

These are similar to the values we report in the current study though are also values that span the LoA range. We end by noting that most research and biomonitoring studies focus on As measures in urine.

Cadmium

The constant bias value for blood Cd was -0.11 µg/L (95% CI: -0.22 to 0.00 µg/L), and the line of equality was barely inside the confidence interval. There was no proportional bias in the measurements with a calculated slope of -0.20 (95% CI: -0.58 to 0.17). These results suggest that Cd measurements in venous blood are acceptable and consistently, yet slightly, lower than measures taken in capillary blood. The lower and upper LoA were -0.83 µg/L (95% CI: -1.01 to -0.64 µg/L) and 0.61 µg/L (95% CI: 0.42 to 0.79 µg/L), respectively, with a range of 1.43 µg/L between them. Three measurements fell outside the LoA, and these were at the higher concentration range (0.5-1.0 µg/L). To assess the relevancy of these blood Cd findings, we compared the bias value (-0.11 µg/L) and the LoA range (1.43 µg/L) with blood Cd data from the Canadian population. For example, from CHMS cycle 5, the mean blood Cd concentration was 0.28 µg/L and the 95th percentile value was 2.9 µg/L. These blood Cd concentrations are similar to what we report here on our study population. While the bias value is lower than blood Cd concentration from the average Canadian, the LoA range is higher.

Manganese

The constant bias for Mn was -3.95 µg/L (95% CI: -4.79 to -3.12 µg/L). As the line of equality fell outside the confidence interval, the results suggest a systemic bias in the measurement with an overestimation of Mn concentrations in capillary whole blood compared to venous whole blood. There was no proportional bias in the measurements with a

calculated slope of -0.17 (95% CI: -0.54 to 0.20). The lower and upper LoA were -9.33 µg/L (95% CI: -10.76 to -7.89 µg/L) and 1.42 µg/L (95% CI: -0.01 to 2.85 µg/L), respectively, with a range of the LoA of 10.74 µg/L. To understand the relevancy of these findings, for the general population we note that Mn in whole blood ranges from 7.0 to 12 µg/L (Flora, 2014), and in the Canadian population (according to the CHMS, cycles 1-3) the mean concentration was reported to be 9.2 µg/L with a 95th percentile value of 15 µg/L. There is overlap in the expected blood Mn concentrations from the general population with the constant bias (~4 µg/L) and LoA (~11 µg/L) values reported here, thus raising concerns about the suitability of measures taken from capillary blood as an alternative to those taken from venous blood.

Lead

We calculated a constant bias value of -0.04 µg/dL (95% CI: -0.09 to 0.02 µg/dL) for blood Pb, and the line of equality fell within this confidence interval. There was no proportional bias between the measurements, with a calculated slope of -0.01 (95% CI: -0.13 to 0.12). The lower and upper LoA were determined to be -0.42 µg/dL (95% CI: -0.51 to -0.32 µg/dL) and 0.35 µg/dL (95% CI: 0.25 to 0.44 µg/dL), respectively. The range between the LoA was 0.76 µg/dL with two samples falling outside this range. Taken together, these data suggest relatively good agreement between Pb measures taken in capillary and venous blood from the same individual. The LoA range may warrant further consideration given the value nears mean blood Pb concentrations found in the general Canadian population (i.e., the CHMS from cycle 5 reported mean Pb concentrations in venous blood of 0.93 µg/dL in the Canadian population, and a 95th percentile value of 2.5 µg/dL). However, when the LoA is compared to widely used reference values for blood Pb levels (5µg/dL or 10 µg/dL for most countries), the range seems appropriate. Further, the constant bias value is very small compared to blood Pb levels in the general population. Assessing Pb exposure through capillary blood sampling

is commonplace in many jurisdictions worldwide and given that an estimated 632 million children in 34 countries have high blood Pb levels (Ericson *et al*., 2021) these findings provide further evidence that capillary blood can serve as an alternative to venipuncture.

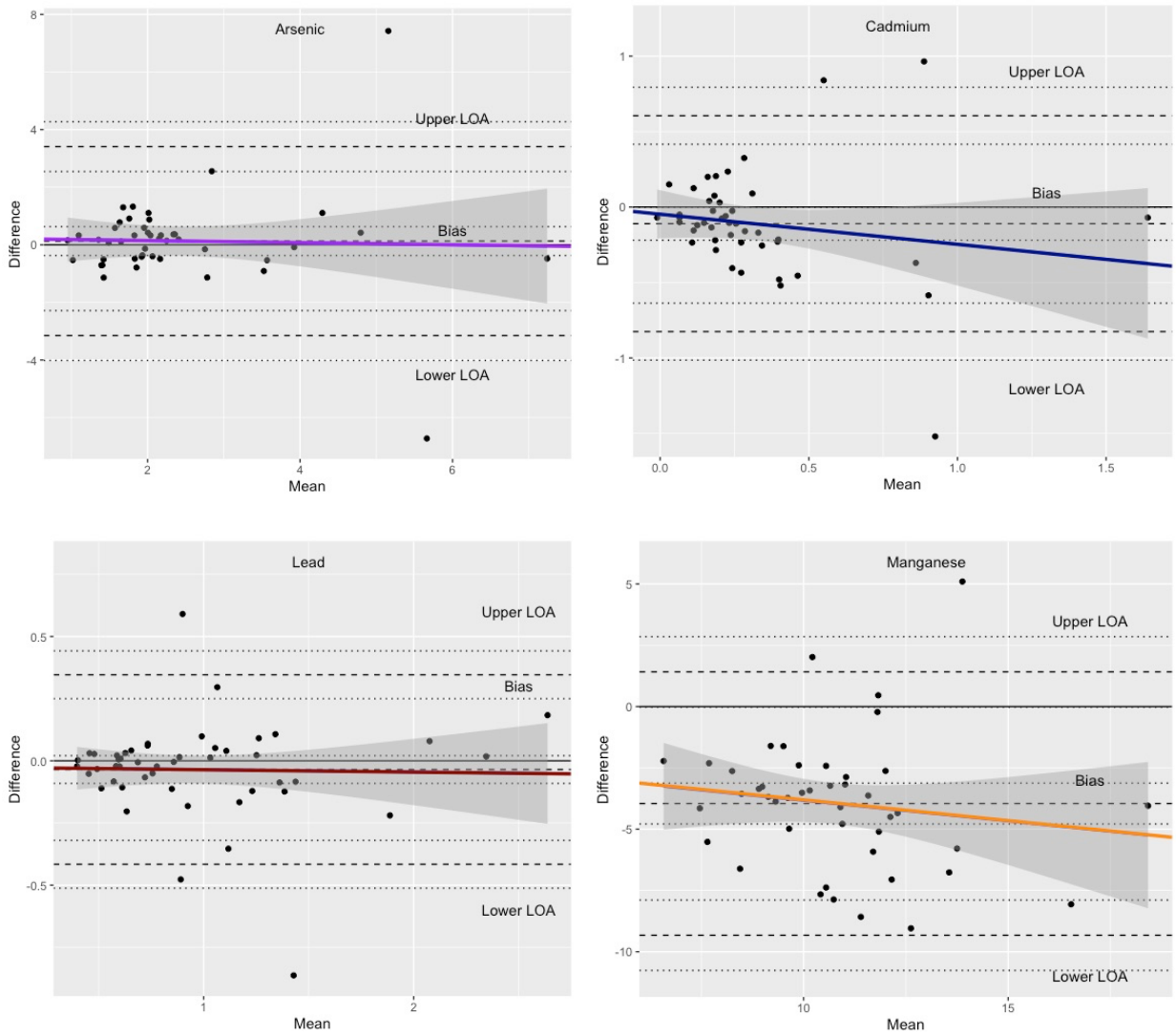

Figure 3-2. Bland-Altman plots of differences (y axis) in metal(loid)s concentrations (μg/L for As, Cd, and Mn; μg/dL for Pb) in whole venous blood and capillary blood versus the mean of the two measurements (x axis). Each plot has 3 dashed (- - - -) black lines, and from the top of the plot to the bottom of the plot these indicate the upper LOA, constant bias, and lower LOA values, respectively. For each of these 3 dashed lines, the corresponding upper and lower 95% confidence intervals are provided as dotted (· · · ·) black lines. Finally, in each plot a solid-colored line indicates the proportional bias line.

Cooper

A constant bias of 16.52 µg/L (95% CI: -5.82 to 38.87 µg/L) was calculated for blood Cu, and the line of equality fell within the confidence interval. There was no proportional bias between the measurements with a calculated slope of 0.01 (95% CI: -0.13 to 0.16). In general, these results suggest a good agreement in Cu measures between venous and capillary blood. The lower and upper LoA were -135.99 µg/L (95% CI: -174.45 to -97.53 µg/L) and 169.05 µg/L (95% CI: 130.59 to 207.51 µg/L), respectively, with a LoA range of 305.04 µg/L. To understand the relevancy of these findings, we note that blood Cu levels in the general population range between 800 and 1,300 ug/L based on a review of 16 datasets (Iyengar & Woittiez, 1988), with values measured in the current study population falling on lower end of this scale. The constant bias value as well as the LoA we report here are much smaller than the 95th percent reference values for Canadians (i.e., 1,300 ug/L for females and 1,000 ug/L for males) as calculated by Saravanabhavan *et al*., 2017.

Iron

The constant bias value was 6,077.13 µg/L (95% CI: -1,981.80 to 14,136.08 µg/L) for blood Fe, and the line of equality fell within the 95% confidence interval. There was no proportional bias in the measurements, with a calculated slope of 0.09 (95% CI: -0.17 to 0.36). In general, these results suggest good agreement in Fe measures between venous and capillary blood. The lower and upper LoA were -48,914.78 µg/L (95% CI: -62,781.65 to -35,047.91 µg/L) and 61,069.06 µg/L (95% CI: 47,202.19 to 74,935.93 µg/L), respectively, with a LoA range of 109,983.84 µg/L (~110 mg/L). To ascertain the relevancy of these findings, we note that blood Fe levels in the general population range between 309 and 521 mg/L based on a review of 13 datasets (Iyengar & Woittiez, 1988). The blood Fe values we measured in the current study population fall near the middle of this range. In addition, the

constant bias value and the LoA range are relatively small compared to the expected measures in a given study population.

Selenium

The constant bias value was 4.87 µg/L (95% CI: 1.07 to 8.67 µg/L) for blood Se. As the line of equality fell outside the confidence interval, the results suggest systemic bias in the measurement with an overestimation of Se concentrations in venous whole blood compared to capillary whole blood. We do note that there was no proportional bias in the measurements with a calculated slope of 0.07 (95% CI: -0.18 to 0.32). The lower and upper LoA were -21.08 µg/L (95% CI: -27.62 to -14.53 µg/L) and 30.83 µg/L (95% CI: 24.28 to 37.27), respectively, with a LoA range of 51.91 µg/L. To understand the relevancy of these findings, we note that blood Se levels in the general population range between 58 and 235 ug/L based on a review of 20 datasets (Iyengar & Woittiez, 1988). The blood Se values measured in the current study population fall near the middle of this range. While the constant bias value is relatively small compared to blood Se levels in the general population, the LoA range is not as small. Taken together, the agreement in Se measures between venous and capillary whole blood is not as strong as the other elements studied here.

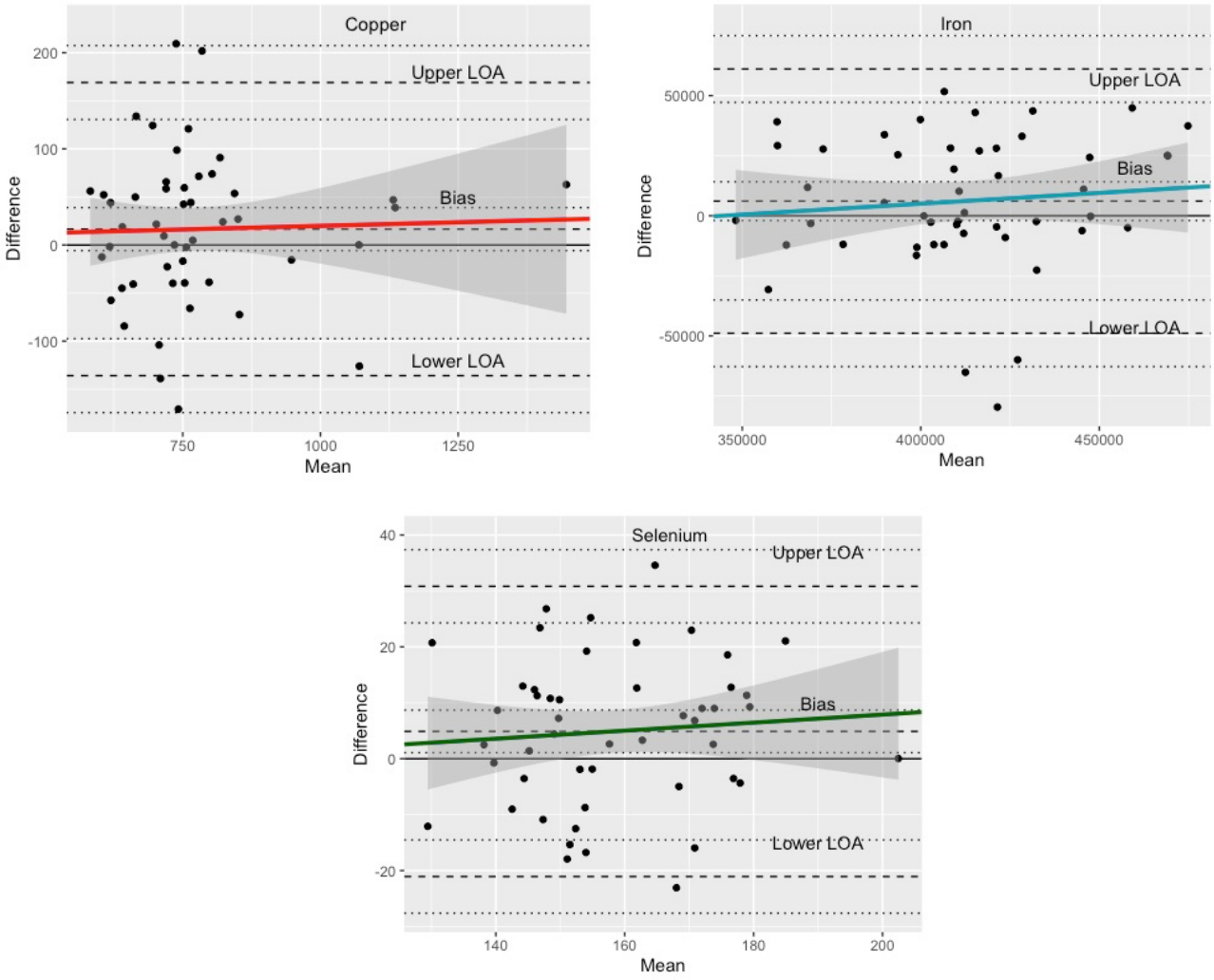

Figure 3-3. Bland-Altman plots of differences (y axis) in essential element concentrations (μg/L) in whole venous blood and capillary blood versus the mean of the two measurements (x axis). Each plot has 3 dashed (- - - -) black lines, and from the top of the plot to the bottom of the plot these indicate the upper LOA, constant bias, and lower LOA values, respectively. For each of these 3 dashed lines, the corresponding upper and lower 95% confidence intervals are provided as dotted (· · · ·) black lines. Finally, in each plot a solid-colored line indicates the proportional bias line.

3.5 Conclusion

For biomonitoring of human exposures to toxic and essential elements, there is an interest in the use of capillary whole blood as an alternative to classical methods that use venipuncture.

However, with the exception of Pb, we are not aware of another element that has been studied in detail to understand potential differences in measures between capillary and venous blood. In the current study, we focused our research on seven key toxic metal(loid)s and essential minerals of relevance to human health, and in doing so we found that there were strong correlations in measures taken for all these elements between capillary and venous blood. It is important to point out that when using different matrices (or different methods) to measure the same analytes it is expected that their measurements are correlated to some extent, but it the agreement analysis what can really demonstrate their equality. In this sense, a deeper investigation of the agreement between the measures (including an examination of the relevancy of the results for background or highly exposed populations) found there was relatively good agreement for blood As, Pb, Cu, and Fe. For blood Cd, Mn, and Se, their correlation was less strong and the agreement between the measures taken in capillary and venous blood were less consistent, thus, steps can be taken to improve the method and/or investigate further if there are indeed differences (e.g. Investigate other hematological parameters to further understand these differences). Moving forward, for all the elements studied here, additional research is needed to confirm these findings particularly from more diverse populations groups (e.g., ones with high environmental exposures or nutritional deficiencies). Future studies can also expand the number of elements analyzed, as well as take blood chemistry measures which may be helpful when interpreting the results (e.g., some elements bind preferentially to erythrocytes). Finally, to understand the applied possibilities, biomonitoring programs that take samples using traditional venipuncture methods could also take capillary blood from the same individual.

3.6 Author contributions

This manuscript was written by all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally.

3.7 Conflict of interest declaration

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

3.8 Acknowledgment

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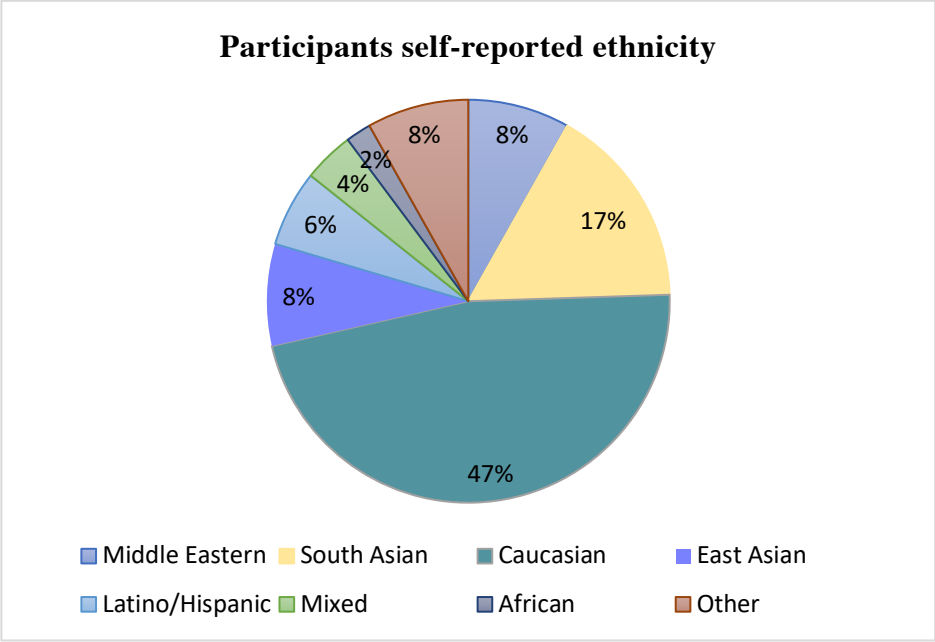
Supporting information

Supplementary table S3-1. Demographic information of the study participants

Variable	Male	Female
Sex	41% (n=20)	59% (n= 29)
Age (years)	27.8 ± 5.5	28.0 ± 9.1
Venous hemoglobin (g/L)	148.9 ± 9.2	131.9 ± 6.4
Capillary hemoglobin (g/L)	149.7 ± 8.03	128.1 ± 6.9



Supplementary table S3-2. Whole blood reference materials used from the Institut National de Santé Publique du Québec (INSPQ), and their certified multi element concentrations at low, medium, and high concentrations (μg/L)

Element	QME-B-Q-1314 (Low QC)	QM-B-Q-1505 (Medium QC)	QM-B-Q-1506 (High QC)
As	2.68	5.88	11.42
Cd	3.05	10.33	13.23
Cu	2236.81	3037.49	3094.69
Mn	8.73	19.61	22.52
Pb	47.86	158.92	501.42
Se	172.15	290.61	411.43
Zn	58.28	7910.98	10853.08

Supplementary table S3-3. Quality control parameters for the analysis of capillary and venous whole blood samples by ICP-MS

Element	Parameter			
Toxic metal(loid)s	Precision (% RSD)	Accuracy (% Recovery)	Linearity* (r2)	Limit of detection (μg/L and μg/dL for Pb)
As	0.7-6.1	97.8-126.4	0.999	0.008
Cd	0.4-1.7	100.6-108.7	0.999	0.002
Mn	0.2-6.0	90.5-97.6	0.998	0.52
Pb	0.8-2.1	94.4-95.2	0.999	0.34
Essential elements	Precision (% RSD)	Accuracy (% Recovery)	Linearity* (r2)	Limit of detection (μg/L)
Cu	0.9-2.0	91.4-95.4	0.997	5.08
Se	0.7-1.2	92.7-98.4	0.992	3.48
Zn	0.5-2.9	88.4-96.7	0.980	4.50

*Based on a 4-point calibration curve with matrix-matched reference materials

Supplementary table S3-3. Details on the association between capillary and venous blood for Pb measurements among past studies.

Reference	Geographic location	Sample size (n=)	Correlation coefficients (r^2)	Remarks
Cernik and Sayers, 1971	United Kingdom	85	0.91	AAS compared to other techniques. Capillary blood from ear prick
Schlenker *et al*., 1994	United States	295	0.96	GF-AAS
Resano *et al*., 2007	Spain	18	0.98	SS-AAS
This study	Canada	49	0.96	ICP-MS

CONNECTING PARAGRAPH

Chapter 2 presented the existent knowledge and the main limitations of DBS sampling applied to Pb exposure assessment, from which key gaps were identified for experimental studies. Chapter 3 reported findings on the differences and agreement of Pb levels between venous and capillary blood, which represented a potential limitation for DBS application. Another major drawback reported on the literature of DBS application for Pb analysis (from Chapter 2) was the low volume and sensitivity-related issues. As new technology develops the analytical capacity improves and thus, some advantages as less sample volume requirements and improved limits of detection.

Chapter 4 focuses on the development, validation, and application of a new method to measure Pb in DBS samples, applying a relatively new technique called Total Reflection X-Ray Fluorescence. This analytical technique allows for microanalysis, it offers efficient limits of detection, reduced costs, among other advantages compared to traditional techniques. Detailed information and supplementary figures related to the method development and validation results, quality control results, statistical results, and agreement between the developed method and traditional methods is included in the Supporting Information.

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4. **CHAPTER 4.** Lead (Pb) exposure assessment in dried blood spots using Total Reflection X-Ray Fluorescence (TXRF)

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4.1 Abstract

Lead (Pb) exposure is often determined through the analysis of whole blood though venipuncture poses ethical, economic, and logistical barriers. Dried Blood Spots (DBS) may help overcome such barriers though past studies measuring Pb in DBS have been challenged with quality control, small sample volumes, and other issues. Total Reflection X-Ray Fluorescence (TXRF) may help address some of these challenges but has yet to be used to measure Pb in DBS. As such, the aim of the current study was to develop, validate, and apply a method to analyze Pb in DBS samples using TXRF for use in human biomonitoring studies. First, I developed a novel method (tested a range of parameters), and then used blood reference materials to validate the method against performance criteria listed in ICH Q2A and Q2B and the European Bioanalysis Forum. Finally, we applied the method to two populations who exemplify divergent conditions (41 university members with relatively low Pb exposures sampled in a clinical environment; 40 electronic waste workers with relatively high Pb exposures sampled in a contaminated field setting). The limits of detection and quantification of the method were 0.28 and 0.69 µg/dL, respectively. The overall precision and accuracy of the method were 15% and 111%, respectively. The mean (±SD) DBS Pb levels by TXRF in the university members and e-waste workers were 0.78 (± 0.46) and 3.78 (± 3.01) µg/dL, respectively, and these were not different from Pb measures in venous whole blood using ICP-MS. Bland-Altman plot analyses indicated good agreement between DBS Pb measures by TXRF versus whole blood Pb measures by ICP-MS in both groups. By combining data from the two population groups, there was no significant constant bias (intercept of 0.02 µg/dL) or proportional bias (slope was -0.02) between the two measures,

and the lower and upper LoA were -0.86 and 0.91 µg/dL, respectively, with a LoA range of 1.77 µg/dL. These results demonstrate that TXRF-based analysis of Pb content in DBS is a good alternative to the gold standard (i.e., ICP-MS analysis of whole blood), and helps overcome some of the challenges associated with current methods.

4.2 Introduction

Lead (Pb) exposure remains of public health concern worldwide as recognized by notable institutions including WHO (WHO, 2010), the U.S. Centers for Disease Control (ATSDR, 2017), and Health Canada (Health Canada, 2019). Exposure to Pb is most often determined through measuring Pb levels in blood (CDC, 2017), and values can be compared to regulatory guidelines to assess health risk. However, there remain several challenges associated with blood sampling including the need for specialized clinical settings, medical equipment, and skilled technicians as well as the use of invasive techniques (Sanders *et al*., 2009). Sampling blood is particularly challenging for vulnerable populations (e.g. children and elderly) and in remote or resource-limited regions, though these represent groups particularly at-risk for Pb exposure.

Dried Blood Spots (DBS) have been successfully used as an alternative to classical venipuncture (whole blood sampling) for about 40 years with many analytical methods validated and subsequently applied in a range of real-world settings (Spooner *et al*., 2009; Nys *et al*., 2017; Li and Lee, 2014). Sampling blood using DBS has been regarded as a feasible alternative to venipuncture in that it is less invasive, associated with lower costs, and more amenable for use in remote or resource-limited areas (Li and Lee, 2014). While there is interest in the use of DBS for gauging Pb exposure there remain outstanding concerns. For example, in our review of 13 prior studies (summarized in Chapter 2), seven of the studies relied upon relatively small sample sizes (< 20 individuals), seven of the studies reported a limit of detection which averaged near 1 µg/dL (close to mean blood Pb levels in the Canadian and US populations, i.e., 0.95 and 1.12 µg/dL respectively; CHMS, 2017; CDC, 2019), and key quality control aspects such as accuracy and precision were missing from many of the works.

The current "gold standard" for the determining blood Pb levels is the analysis of venous whole blood by Inductively Couple Plasma Mass Spectrometry (ICP-MS). Despite the high sensitivity of ICP-MS for detecting Pb in blood samples, this instrument has notable limitations for working with DBS such as the need for a relatively high sample volume (~200-500 µL versus a single 3 mm DBS punch which may be ~3 µL), the presence of matrix effects, potential for polyatomic and non-polyatomic interferences, and relatively high costs associated with procurement and operation. As an alternative to ICP-MS, Total Reflection X-Ray Fluorescence (TXRF) is proving feasible for the analysis of DBS as it is a multi-elemental analytical technique that allows for the analysis of small volumes (≤ 10 µL) (Stosnach, 2007; Dhara and Misra, 2011; Towett *et al*., 2013; Pawly *et al*., 2019). Compared to ICP-MS, TXRF-based analyses may have relatively low background noise and few interferences, detection limits in the ppb range (thus comparable to ICP-MS for several elements), lower operational and maintenance costs, and samples for TXRF analysis can be prepared in hours (versus hours-to-days) (Bilo *et al*., 2014).

To date some studies have determined Pb levels in whole blood using TXRF (**Supplementary table S4-1**), though to our knowledge no study has measured Pb in DBS using TXRF. As such, the aim of the current study was to develop, validate, and apply a method to analyze Pb in DBS samples using TXRF for use in human biomonitoring studies. The realize this aim, our study consisted of three phases: 1) method development phase to establish initial assay parameters; 2) method validation phase with blood reference materials to refine assay parameters; and 3) application phase with real samples from human subjects. During phase 1 (method development) I tested different sample preparation methods and internal standards, and in phase 2 (method validation) I evaluated the method against performance criteria listed within ICH Q2A and Q2B (Guideline ICH, 2005) and the European Bioanalysis Forum (EBF) (Timmerman *et al*., 2011). Finally, in phase 3 the method was applied to evaluate Pb content in DBS from a group of university members (Santa-Rios *et al*., 2020) and workers from an electronic waste (e-waste) recycling site in Ghana (Srigboh *et al*., 2016). From both populations I was able to compare Pb measures in capillary DBS samples via TXRF versus the 'gold standard' which is Pb measures in venous whole blood via ICP-MS. These two populations exemplify divergent conditions (i.e., university members with relatively low Pb exposures sampled in a clinical environment

versus workers with relatively high Pb exposures sampled in a contaminated field setting), which help demonstrate the wider applicability of the method.

4.3 Experimental section

4.3.1 Blood reference materials and chemicals

Whole blood reference materials were obtained from the Institut National de Santé Publique du Québec (INSPQ) Centre de Toxicologie du Québec (CTQ). I used five reference materials with assigned Pb concentrations ranging from 1.02 to 50.14 µg/dL (**Supplementary table S4-2**). The reference material at the lowest concentration is a Pb-specific standard (i.e., a standard prepared and certified only for Pb), while the other reference materials were prepared for multiple elements. Internal standard (IS) solutions of Ni, Sr, and Ga were prepared from 1,000 mg/L stocks (Merck, Germany). Nitric acid (65% HNO_3 Omnitrace grade, EMD Chemicals, NJ) and hydrochloric acid (12 N HCl, Merck, Germany) diluted to 6 N were used to prepare the samples. Methyl Isobutyl Ketone (MIBK, analytical grade, Merck, Germany) was used for iron extraction.

4.3.2 Instrumentation

The levels of Pb were measured in samples using Total X-Ray Fluorescence (model S2 Picofox, Bruker AXS Microanalysis, Berlin, Germany) and an ICP-MS (820-MS Series, Varian Inc., California, USA). I have previously outlined the operation of both instruments (Pawly *et al*., 2019; Srigboh *et al*., 2016). To assure that samples were free of contamination, I used metal-free microcentrifuge vials and pipette tips. The acrylic discs for TXRF analysis were cleaned using RBS^{TM} 50 cleaning solution (5%), 10% HNO_3, and deionized water, and blank discs were regularly tested for contamination.

4.3.3 Phase #1: Method development

Method development is an iterative process, and here I summarize key steps taken while reserving greater details to the Supporting Information (S3-S7). I based the current method

on our past study that measured levels of copper, selenium, and zinc in DBS using TXRF (Pawly *et al*., 2019) with some slight modifications. Aforementioned whole blood reference materials were pipetted (60 µL) onto Whatman® 903 protein saver cards (hereafter, filter paper) to realize lab-based DBS samples. Our studies focused on the analysis of one circle of a DBS sample (60 µL blood) or 3 mm diameter punches (3.1 µL blood, based on Li and Lee, 2014) added to a 1.5 mL microcentrifuge tube. I evaluated digestion solutions (high purity 65% HNO_3 and a combination of HNO_3 and 30% H_2O_2) and settled upon the mixture of HNO_3 and H_2O_2 (**Supplementary figure S4-1**). A range of digestion times (30 – 90 min range) and temperatures (90 – 165 º C range) were tested, and I settled upon a 45 min digestion at 130 º C. The digested samples were evaporated to dryness and taken up with 6 N HCl, following which they were heated for 15 min at 120 ºC. At this stage, the internal standard was added to ensure that it bound to the sample matrix. I evaluated the performance of three internal standards (Ga, Ni, and Sr) previously studied for TXRF-based analysis of Pb (Silva *et al*., 2013; Stosnach, 2009; Martinez *et al*., 2004, Bounakhla, 2003), and decided upon the use of Ni at a final concentration of 150 ppb (**Supplementary figure S4-2**). As previous studies (including our own pilot efforts) have shown that the relatively high iron levels in blood interfere with TXRF-based detection of some elements (Prangue, 1989; Khunder, 2006), we included Methyl Isobutyl Ketone (MIBK) to the digested samples to extract out the iron (more details on the extraction process are in Supporting information S6). After iron extraction, an 8-10 µL aliquot of the aqueous phase was pipetted onto acrylic discs which were then heated at 55 ºC on a hot plate until the sample dried. The Pb concentrations in the dried sample were then analyzed by TXRF applying a measuring time of 1,000 seconds. Operational conditions of the TXRF were based on our past study of copper, selenium, and zinc in DBS (Pawly *et al*., 2019) and updated with additional suggestions by Riaño *et al* (2016).

4.3.4 Phase #2: Method validation

Method performance was evaluated following the recommendations of the European Bioanalysis Forum (EBF) on the method validation and analysis of DBS samples, as well as

validation criteria outlined by ICH Q2A and Q2B guidelines. The linearity and the working concentration range of the method was evaluated by building a calibration curve using DBS created with the five aforementioned whole blood reference materials that had Pb concentrations spanning from 1 to 50 µg/dL. The calibration curve was generated by linear regression and linearity was assessed by Fisher´s test. The LoD_{method} (mean concentration of blanks + 3x SD) and LoQ_{method} (mean concentration of blanks + 10x SD) were calculated from Pb measurements in blank filter paper treated with sample preparation reagents. Accuracy was determined by measuring Pb in DBS created from the aforementioned blood reference materials. Precision was determined by calculating the percent relative standard deviation % RSD, based on replicate analysis (n=9 separate cards) of the reference materials. It is important to mention that TXRF is a highly selective method for the analysis of trace elements due to its analytical principle. Specifically, the method can identify elements by its characteristic lines or peaks (usually compared to a fingerprint) (Riaño *et al* (2016), therefore, selectivity was not a required component of this validation.

4.3.5 Phase #3: Method application

The method was applied to characterize Pb concentrations in whole blood and DBS samples in two different groups (members of the McGill University community in Montreal, Canada, and e-waste workers situated in Ghana).

McGill University members

Samples were collected from volunteers (n=40) by a trained health professional at the Mary Emily Clinical Nutritional Research Unit at Macdonald Campus as previously detailed (Santa-Rios *et al*., 2020). Briefly, DBS samples were stored at ambient conditions in plastic bags, while the whole blood samples were placed at – 80º C until analysis. The IRB (human ethics) approval for this work was obtained from McGill University (A05-M26-16B).

Ghanaian e-waste workers

Samples were collected from e-waste workers (n=41) from Agbogbloshie (Accra, Ghana) as described elsewhere (Srigboh *et al*., 2016). Collected samples were transported to McGill University where they were stored at -80 °C (whole blood) and room temperature (DBS). The IRB (human ethics) approval for this work was obtained from the Noguchi Memorial

Institute for Medical Research at the University of Ghana (IRB00001276) and McGill University (A06-M30-16B).

4.3.6 Data analysis

Statistical analysis was conducted using Microsoft Excel 2017. All reported Pb concentrations were measured using TXRF unless indicated. For the method development stage, comparisons between the different sample preparation conditions were carried out using unifactorial ANOVA. In addition, I used ANOVAs to assess if the Pb concentrations varied across batch runs. Finally, Bland-Altman plots were constructed to evaluate the agreement between a range of comparisons (e.g., Pb in DBS versus whole blood; measurements in TXRF versus ICP-MS). All figures were generated using R version 3.6.3.

4.4 Results and discussion

4.4.1 Method validation

Linearity and working range

The calibration curve relating Pb measurements in DBS created with the five blood reference materials and TXRF count values was linear with a coefficient of determination (r^2) greater than 0.997 (**Supplementary figure S4-2**). The lower end of this curve (once dilution factors were accounted for) covers relevant blood Pb levels in Canada and the U.S. where recent national surveys estimate mean blood Pb levels to be 0.95 and 1.12 µg/dL, respectively (CHMS, 2017; CDC, 2019). The middle range of this curve spans regulatory guidelines (e.g., the blood Pb intervention level is 5 and 10 ug/dL according to the U.S. CDC and Health Canada, respectively), and the upper range spans a "level of action" where oral chelation therapy may be considered (CLSI, 2013; CDC, 2019).

Lower limits of detection and quantification

The LoD and LoQ of the method were calculated to be 0.28 and 0.69 µg/dL, respectively. These detection limits are relevant for human biomonitoring. For example, from the latest cycle of the CHMS (cycle 5: 2017-2018), blood Pb levels in the 10th percentile of Canadians (aged 3-79 years old) was 0.43 µg/dL (95% CI: 0.36-0.42 µg/dL). These detection limits also

compare well technically against established methods such as ICP-MS and Atomic Absorption Spectroscopy (AAS) where Pb detection limits are in the low ppb range (LoD ~0.22-2.4 µg/dL; **Supplementary table S4-2**). Moreover, the TXRF-based method I demonstrate here may serve as an alternative to portable Pb analyzers (e.g., LeadCare® has a reported LoD of 3.3 µg/dL), though more comparative research would be necessary to test this assertion.

Accuracy and precision

In the method validation phase of our study, I characterized analytical accuracy and precision using DBS created with the whole blood reference materials. The overall mean accuracy was 111.1 % (97.0 - 129.7 %) (**Table 4-1**). While this overall value met pre-established acceptance criteria (80 - 120 %) from the ICH guidelines, I observed a tendency of overestimation at the lower concentration levels which start to approach the method LoQ (0.69 µg/dL). With additional research on this new method that I introduce here, I expect that improvements in its analytical accuracy may be realized with changes to, for example, optimization of TXRF measurement parameters, testing of different reference materials (especially certified ones from NIST such as SRM 955d), and setting up external calibration curves. For precision, the mean value was 14.9 % (ranged from 9.9 to 19.1 %) (**Table 4-1**). This value met the predefined precision acceptance criteria (RSD <15 %) based on ICH Q2A and Q2B.

Table 4-1. Analytical accuracy (recovery %) and precision (RSD %) of the method established by analyzing lead (Pb) levels in Whatman® 903 protein saver (filter paper) cards onto which 60 µL of whole blood reference materials were pipetted. Each reference material was independently tested on nine separate cards (n=9).

Blood reference material ID	Assigned concentration of Pb (µg/dL)	Measured concentration of Pb in DBS (mean ± SD, µg/dL)	Accuracy (% recovery, min-max in brackets)	Precision (mean RSD %)
PC-B-L1601	1.01	**1.31 ± 0.13**	**129.7%** (118.8 - 149.5)	**9.9%**
QM-B-Q1313	4.57	**5.66 ± 1.08**	**121.2%** (99.9 - 156.7)	**19.1%**
QM-B-Q1505	22.99	**22.24 ± 4.19**	**97.0%** (84.2 - 129.9)	**18.8%**
QM-B-Q1506	50.14	**51.74 ± 6.38**	**103.2%** (84.3 - 119.6)	**11.7%**

Concentration of Pb in blank samples

The potential contamination of filter cards intended for DBS research with Pb has been reported as an issue in past studies (Yee, *et al*., 1997; Chaudhuri, 2009). To increase understanding of the Pb values in the filter cards I used, I analyzed internal blanks along with the samples during the study phase #3. Thus, in ~14% of our samples (i.e., 3 blanks were included in each batch of 22 DBS samples), I determined Pb levels in a full blank circle spot (i.e., filter paper not containing blood) from a card containing a real participant's DBS sample. From this, I found an average of 0.02 ng (± 0.01) Pb per blank circle spot. These blanks came from filter papers processed in two divergent conditions (i.e., university members sampled in a clinical environment versus workers sampled in a contaminated field setting), and there was no difference in the values. When considering the estimated volume of a whole circle spot to be 60 µL blood, the estimated average concentration of Pb would be 0.03 ± 0.016 µg/dL (ranging from <LoD to 0.01 µg/dL). The correlation of Pb measures in the blank spots and their paired DBS samples was weak (R^2= 0.002), and the subtraction of blank Pb levels from the paired DBS sample had no impact of our results. I also note that the blank values are much lower than blood Pb levels of the general population (e.g., CHMS cycle 4 geomean is 0.95 µg/dL) as well as the current study (see further below; e.g., 13-fold and 26-fold lower than the minimum and median values, respectively, among the McGill University members). I thus conclude that the presence of Pb in the filter papers used here are very low and not influencing the results. We performed routine sampling practices in both study locations and did not take significant added steps to try and minimize contamination, which further gives us confidence that background contamination is likely not of major concern.

4.4.2 Method application

For the university members, the mean (±SD) blood Pb level in DBS was 0.78 (± 0.46) µg/dL and ranged from below the detection limit (in 7.5% of the cases) to 2.31 µg/dL (**Supplementary table S4-3**). These Pb levels measured in DBS using TXRF did not differ from Pb levels measured in the same person's venous whole blood sample using ICP-MS (e.g., mean was 0.86 ± 0.46 µg/dL and ranged from 0.39 to 2.35; **Supplementary table S4-4**). These values are comparable to the average Canadian (e.g., median blood Pb level was 0.95 µg/dL from CHMS, 2017). In the e-waste workers, the mean (±SD) blood Pb level in the

DBS was 3.78 (± 3.01) µg/dL and ranged from 1.79 to 19.67 µg/dL (**Supplementary table S4-3**). These blood Pb levels compare well against values previously reported on e-waste workers from the same site (**Supplementary table S4-5**).

A key goal of this study was to compare Pb measures in capillary DBS samples using TXRF versus the 'gold standard' which is Pb measures in the same person's venous whole blood using ICP-MS. In general, the comparisons were strong based on correlative analysis for the McGill university members (r^2=0.814; y= 0.916 x + 0.013, **Fig. 4-1 A**) and the e-waste workers (r^2=0.911; y= 0.974 x + 0.116, **Fig. 4-2 A**).

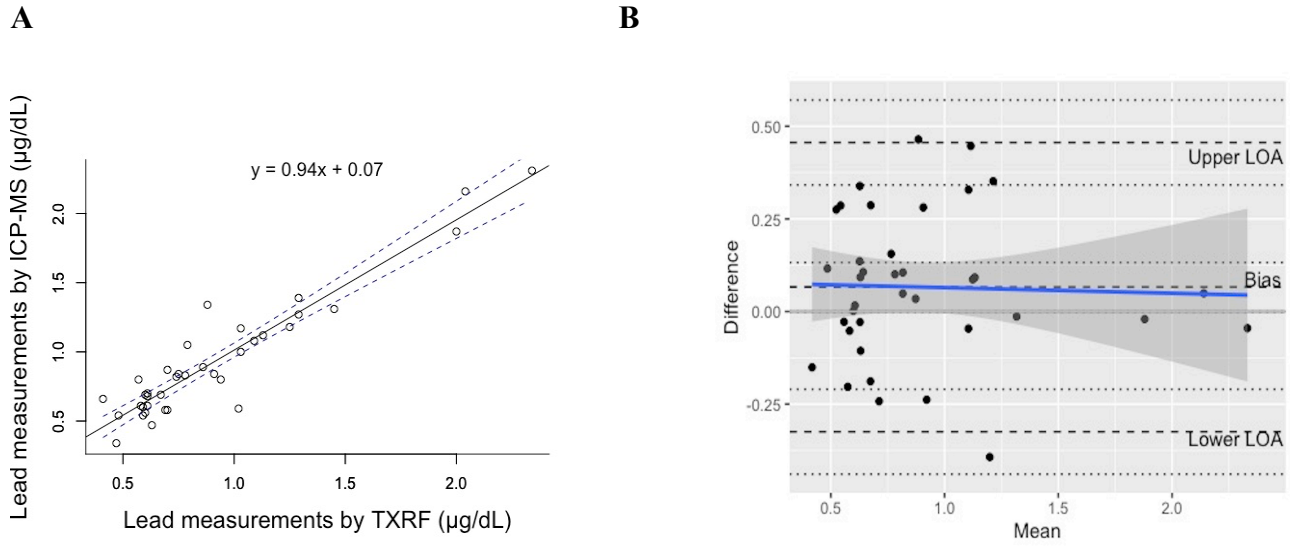

Figure 4-1. (A) Regression line of Pb measurements (µg/dL) in McGill University members comparing same-person capillary DBS samples analyzed by TXRF against venous whole blood samples analyzed by ICP-MS. The 95% confidence band is indicated with dashed lines; (B) Bland-Altman plot of difference (y axis) in Pb measurements (µg/dL) in capillary DBS analyzed by TXRF and venous whole blood analyzed by ICP-MS versus the mean of the two measurements (x axis). The dashed lines indicate the upper LoA, bias, and lower LoA (from the top of the figure to the bottom), and for each parameter the 95% CI are indicated with dotted lines. The blue line represents the proportional bias trend line.

To move beyond simple correlations, and determine if there were any systematic differences, Bland-Altman analyses were conducted to assess agreement between the two approaches (**Fig. 4-1 B** and **Fig. 4-2 B**). For the McGill university members, there was not a significant constant bias (intercept of 0.06 µg/dL; 95% CI: 0.00 – 0.13) or proportional bias (slope was -

0.01; 95% CI: -0.17 – 0.14) between the two measures. For this group, the lower and upper Limit of Agreement (LoA) were -0.32 (95% CI: -0.43 – -0.21) and 0.45 µg/dL (95% CI: 0.34 – 0.57), respectively, and the range of the LoA was 0.77 µg/dL with two samples falling outside the LoA. For the e-waste workers, there was also no significant constant bias (intercept of -0.01 µg/dL; 95% CI: -0.19 – 0.17) or proportional bias (slope was -0.02; 95% CI: -0.10 – 0.18) between the two measures. For this group, the lower and upper LoA were -1.17 (95% CI: -1.50 – -0.85) and 1.15 µg/dL (95% CI: 0.83 – 1.47), respectively, and LoA range was 2.32 µg/dL with one sample falling outside the LoA. I also combined data from both population groups together (**Supplemental figure S4-6**). From this combined analysis, there was no significant constant bias (intercept of 0.02 µg/dL; 95% CI: -0.07 – 0.12) or proportional bias (slope was -0.02; 95% CI: -0.06 – 0.02) between the two measures. The lower and upper LoA were -0.86 (95% CI: -1.03 – -0.68) and 0.91 µg/dL (95% CI: 0.73 – 1.08), respectively, and LoA range was 1.77 µg/dL with three samples falling outside the LoA.

The results from the Bland-Altman plot analyses demonstrate good agreement between the methods across a range of Pb exposures. Of the 81 paired samples tested, 3 of them fell outside the LoA range and had values less than 3 µg/dL. The relevance of the LoA (0.59 µg/dL for the university members and 2.32 µg/dL for the e-waste workers) seems appropriate for biomonitoring activities given that the values fall below regulatory intervention levels (5 and 10 µg/dL blood Pb according to the U.S. CDC and Health Canada, respectively).

A **B**

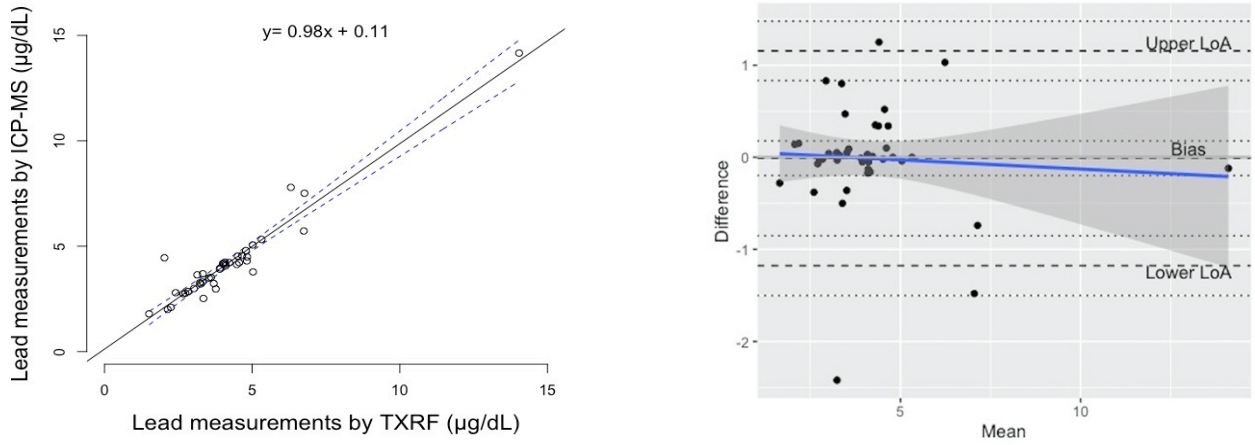

Figure 4-2. (A) Regression line of Pb measurements (µg/dL) in e-waste workers comparing same-person capillary DBS samples analyzed by TXRF against venous whole blood samples analyzed by ICP-MS. The 95% confidence band is indicated with dashed lines; (B) Bland-Altman plot of difference (y axis) in Pb measurements (µg/dL) in capillary DBS analyzed by TXRF and venous whole blood analyzed by ICP-MS versus the mean of the two measurements (x axis). The dashed lines indicate the upper LoA, bias, and lower LoA (from the top of the figure to the bottom), and for each parameter the 95% CI are indicated with dotted lines. The blue line **re**presents the proportional bias trend line.

Based on past studies in this field, I had initial concerns about the detection limit among the background exposed group (McGill members) though only 7.5% of the TXRF-based measures of the DBS were below the LoD. None of the samples taken from the e-waste worker fell below the LoD. For the e-waste workers who were sampled from a contaminated field site, I had concerns that sampling in such an environment may lead to external contamination of the filter paper with Pb though as detailed earlier this was not found to be a problem in the current study. This is further supported by the results of the Bland-Altman analyses which show no systematic bias especially constant bias which may have been expected if there was external contamination of the paper. I completed the analysis of samples from both populations across 6 batch runs (3 batch runs each) in ~2 weeks (**Supplementary figure S4-7**). I did not find statistical differences ($p > 0.001$) between real samples batch runs and I did not observe any changes in the precision and accuracy of the method through our inspection of the blood reference materials data.

4.5 Conclusion

This study enhances our understanding of DBS as a potentially novel approach to characterize Pb exposures in both the general population (in a clinical environment) as well as a highly exposed population (in a resource limited, logistically challenging, contaminated field site). I first developed a method and demonstrated that TXRF analysis of processed DBS filter paper samples yield results that are accurate and precise (when compared to blood reference materials with assigned levels). By purposefully applying the method to study two populations that typify divergent conditions (i.e., background population with relatively low Pb exposures, and e-waste workers with relatively high Pb exposures), I conclude that there is a relatively good agreement between the gold standard of blood Pb measures (i.e., ICP-MS analysis of venous whole blood) with the new method I outline here (i.e., TXRF analysis of capillary DBS).

The TXRF-based method I detail here helps overcome barriers associated with current strategies to measure Pb in DBS (e.g., past studies have paid limited attention to key quality control aspects such as detection limit, accuracy, precision; see **supplementary table S4-1**).

Further, the TXRF-based method detailed here (compared to ICP-MS) enables the analyses of blood to be completed in a shorter time frame with use of fewer chemical reagents and volumes thus supporting principles of green chemistry (Koel, 2016). To exemplify this, for the e-waste samples discussed in this paper I roughly calculated that measures of Pb in DBS using TXRF were 5-fold quicker (8 hrs versus 56 hrs) and used approximately 50% fewer chemical volumes than the Pb measures in whole blood that were acquired with ICP-MS (**Supplementary table S4-6**). Moving forward, there is a need to scale-up the use of this method in a range of population-based studies to better understand the opportunities and limitations in terms of adopting the method into routine biomonitoring efforts.

4.6 Author contributions

This manuscript was written by all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally.

4.7 Conflict of interest declaration

The authors declare no conflict of interest

4.8 References.

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Supporting information

Supplementary table S4-1. Compilation of studies on the analysis of lead in blood by TXRF

Reference	Stosnach *et al.*	Silva *et al.*	Martinez *et al.*	Prange *et al.*	Khuder *et al.*	Savage *et al.*	Viknsa *et al.*	Ayala *et al.*	Bounakhla *et al.*	Kubala Kukus *et al.*	Summary
Study location and year	Germany, 2009	Sri Lanka, 2013	Mexico, 2004	Germany, 1990	Syria, 2007	UK, 1998	Sweden, 2002	Guatemala, 1991	Morocco, 2003	Poland, 2016	Between **1991-2016**
Sample size	6 SRMs of different concentrations	50 individuals	Samples from 50 individuals taken since 2001	6 SMRs of different concentrations	46 samples	9 SRMs horse blood plasma	110 samples	Whole blood samples from 12 individuals	50 children	35 individuals	**Mean sample size of 39** individuals from 5 population-based studies
Sample amount	2 mL, 0.5 mL and 0.2 mL	2 mL	100 µL	1 mL	0.1 mL	Not provided	Not provided	Not provided	35 µL	0.8 mL	**Volume** used for **digestion** ranged **35-500 µL**
Sample preparation	Direct preparation (dilution), microwave digestion, savillex digestion and cold ashing	Digestion with HNO3 (65%)	Fe extraction with MIBK (treated with HCl)	Digestion with HNO3 detection limits improved with extraction with MIBK and methanolic NaDBDTC solution	Digestion with HNO3 (PTFE bomb) and Fe extraction with MIBK	Treated with modifiers and ashing microwave	Pipetted directly into the carrier	Ashed with low-temperature O2 plasma	Microwave digestion with HNO3 and H2O2	Only serum samples were used in addition to the internal standard	**50%** performed **digestion**, **30%** performed a MIBK **extraction**, and **20%** analyzed the sample **directly**
Internal standard	Ga	Ga	Ni	Ga	Ga	Mo	Not provided	Sr	Sr	Ga (and changed to Co to improve LoD)	**5/10 used Ga** as internal standard

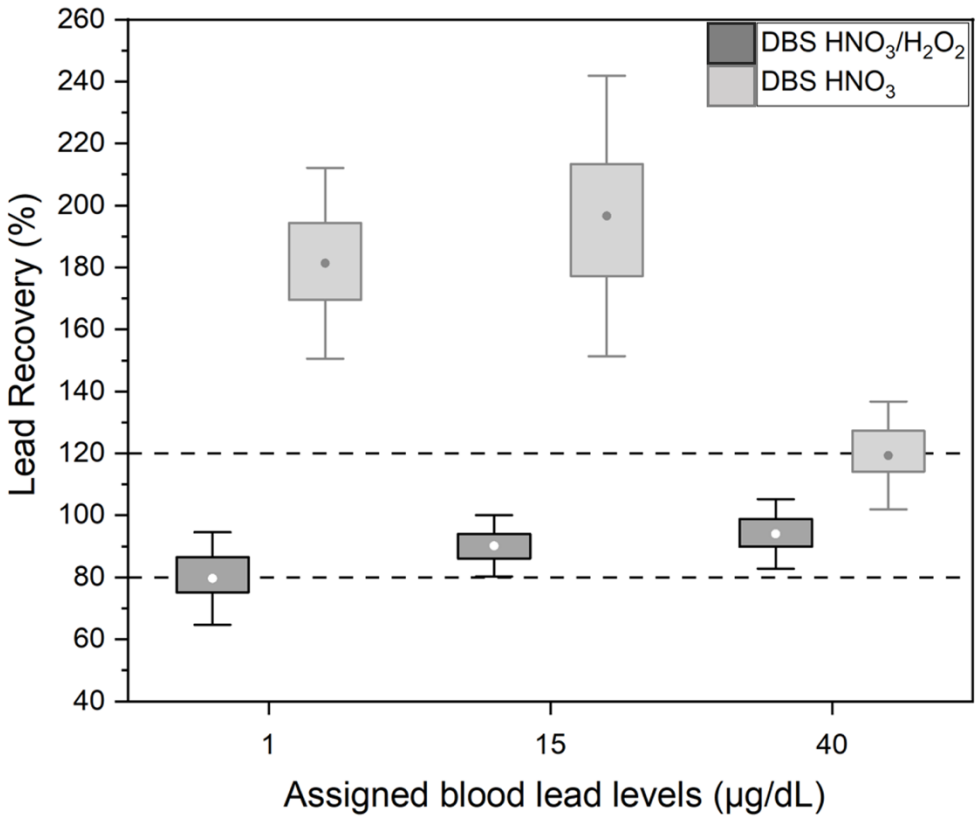
Other elements analyzed	P, S, Cl, K, Ca, Fe, Cu, Zn, Se, Rb, and Sr	Ni and Cd	No	P, S, Cl, K, Ca, Fe, Cu, Zn, Se, Rb, and Sr	Fe, Ni, Cu, and Zn	P, S, Cl, K, Ca, Fe, Cu, Zn, Se, Rb, and Sr	P, S, Cl, K, Ca, Fe, Cu, Zn, Se, Rb, and Sr	P, S, Cl, K, Ca, Fe, Cu, Zn, Se, Rb, and Sr	Fe, Cu, and Zn	Other elements during the validations (P, K, C, Fe, Cu, Zn, Au)	9/10 analyzed other elements besides Pb and **8/10 included Fe, Cu and Zn**
Precision	Not provided	3%	± 7%	16%	>9.8%	< 9.8%	Not provided	Not provided	Not provided	Not provided	**5/10 reported precision** with values **< 16%**
Accuracy	Not provided	Not provided	107%	90- 100%	Pb values not certified	90-100%	Not provided	Not provided	Not provided	Not certified	**3/10 reported accuracy** with values between **90-107%**
LoD and LoQ	Direct = 10 ppb Microwave= 5 ppb Savillex= 14 ppb Cold ashing= 10 ppb	1 ppb	0.03 ug /g (0.67 ug/dL)	30 ppb after Fe extraction and 2 ppb after alkaline earth separation	Not provided	Not provided	Pb values were under the LoD. Pb was analyzed by GFAAS	30 ppb	50 ppb	7 ppb after increasing measurement time and changing the internal standard	**7/10 reported LoD** with values between **1-50 ppb** **Mean LoD= 21.8 ppb**
Correlation with other Technique?	No	No	No	No	No	No	No	No	No	No	**Non of the studies compared** with **other** analytical **technique**

Supplementary table S4-2. Whole blood reference materials used from the Institut National de Santé Publique du Québec (INSPQ), and their certified Pb concentrations.

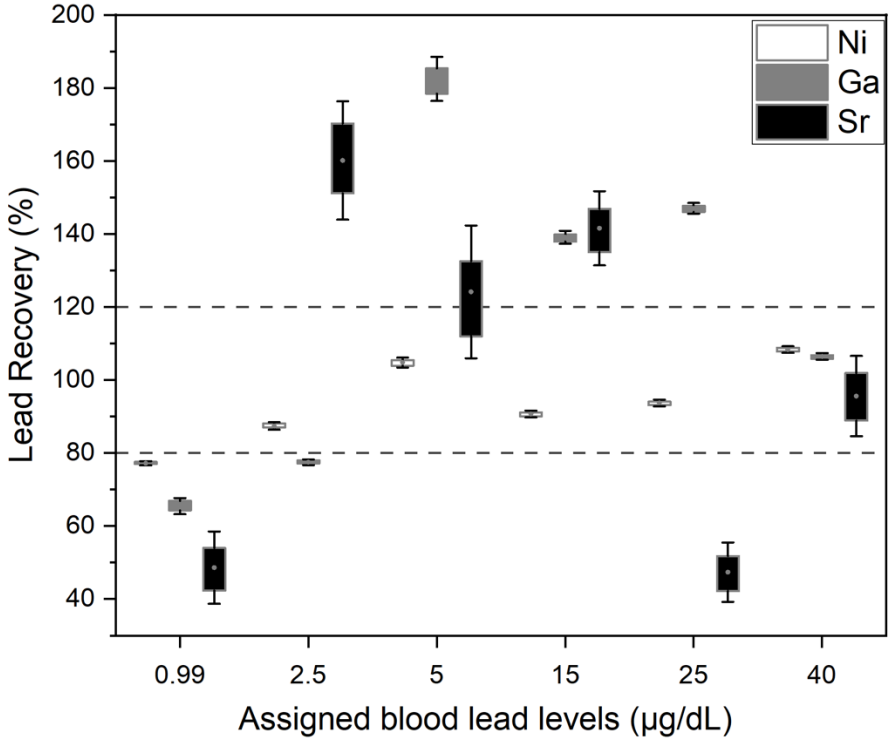
Standard reference material number	Certified concentration of Pb (µg/dL)
PC-B-L1601	1.02
QM-B-Q1313	4.79
QM-B-Q1314	15.89
QM-B-Q1505	22.92
QM-B-Q1506	50.14

Supporting information S4. Supplementary details on the development of the method – digestion and accuracy

We compared the performance of two digestion methods (HNO_3 alone and a mixture of HNO_3 and H_2O_2) to assess recovery of Pb from DBS created from the whole blood reference materials. The addition of H_2O_2 yielded digests that were visibly clearer (i.e. the blood was completely removed from the filter paper) because it aids in the complete oxidation of organic matter, improving the accuracy (i.e., mean recovery of Pb across 3 reference materials ranged from 81-96% versus 122-197%).



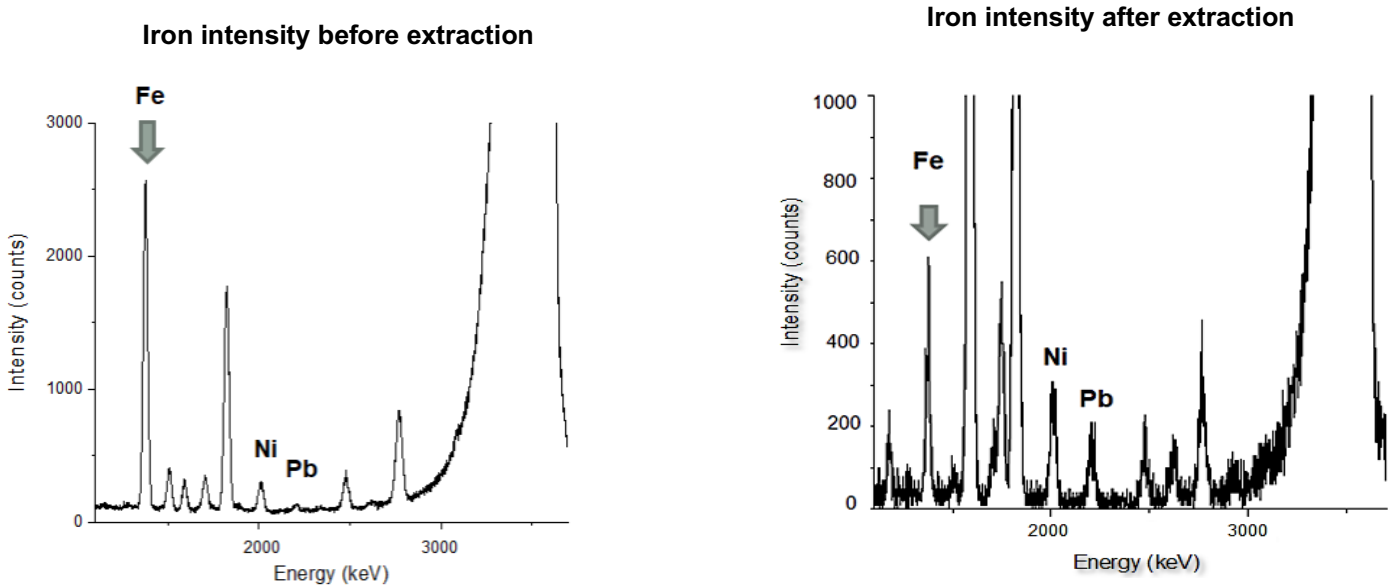
Supplementary figure S4-1. Analytical accuracy of lead (Pb) measurements taken in dried blood spots with absolute values shown as boxplots comparing two digestion procedures. Accuracy was determined by analyses of DBS that had been spotted with three different whole blood standard reference materials (SRM) from the Institut National de Santé Publique du Québec (INSPQ).



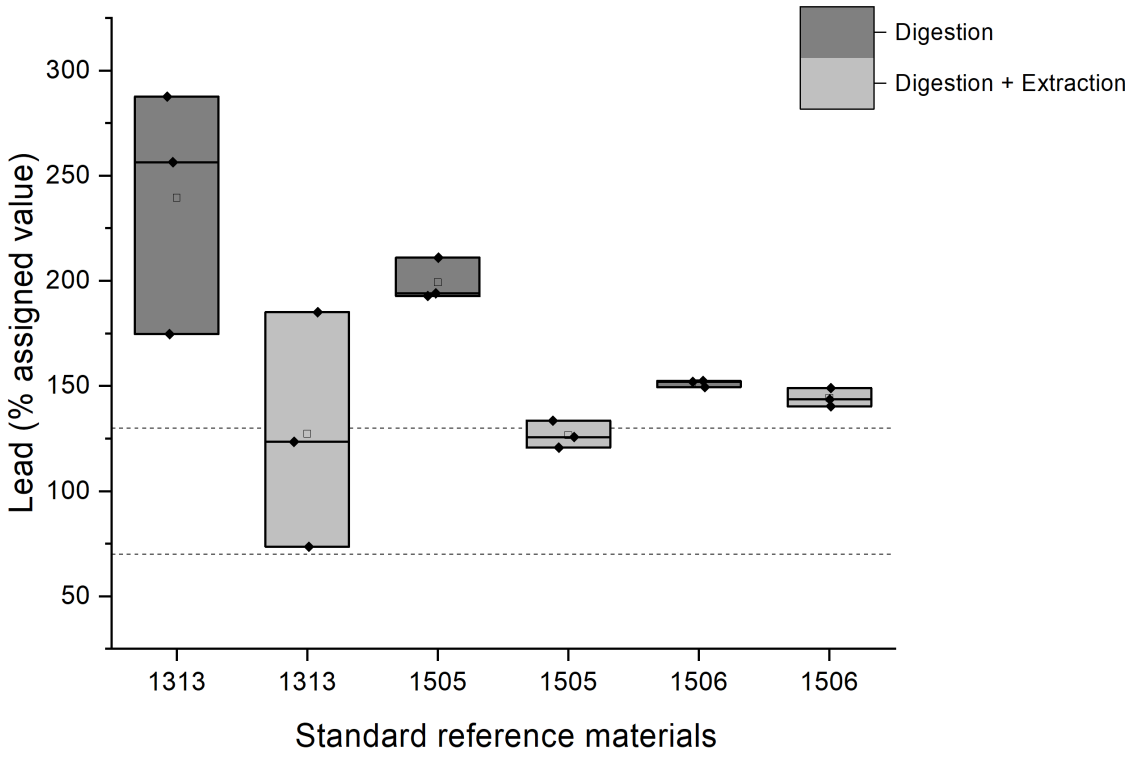
Supplementary figure S4-2. Analytical accuracy of lead (Pb) measurements taken in dried blood spots with absolute values shown as boxplots comparing three internal standards. Accuracy was determined by analyses of DBS that had been spotted with three different whole blood standard reference materials (SRM) from the Institut National de Santé Publique du Québec (INSPQ).

Supporting information S4. Supplementary details on the development of the method – iron extraction

Extraction of iron was performed by handshaking the samples (other novel micro-extraction techniques are now being explored in the lab); the supernatant was removed from the samples, and the extraction was repeated 2 times more using fresh solvent. The extraction of iron is a key step in the analysis of lead at low concentrations (<5µg/dL) as it decreases the background levels of radiation and thus help improve the limits of detection for lead (Supplementary figure S4-3) which is a critical requirement for the analysis of DBS samples. I also observed an improvement in the accuracy of the lead measurements by applying an iron extraction after digesting the samples (Supplementary figure S4-4), the samples analyzed without an iron extraction presented an overall accuracy of 196.68 % (± 48.39 %) across blood reference materials spanning 3 concentrations, while the overall accuracy after performing the extraction procedure was 126.21% (±23.54 %).

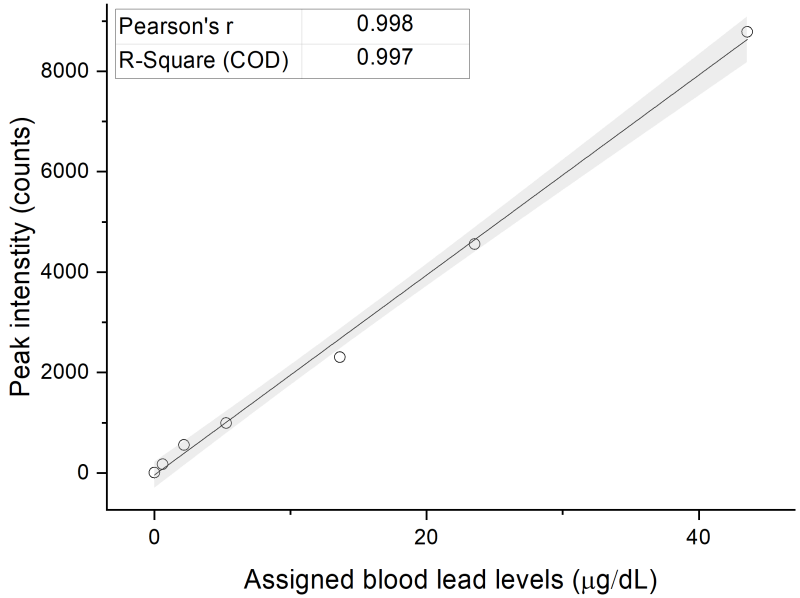


Supplementary figure S4-3. Iron intensity (counts) in DBS samples, before and after the extraction with methyl isobutyl ketone (MIBK)



Supplementary figure S4-4. Analytical accuracy of lead (Pb) measurements taken in dried blood spots with absolute values shown as boxplots comparing the effect of iron extraction (i.e., digestion without extraction and digestion + extraction). Accuracy was determined by analyses of DBS that had been spotted with three different whole blood standard reference materials (SRM) from the Institut National de Santé Publique du Québec (INSPQ).

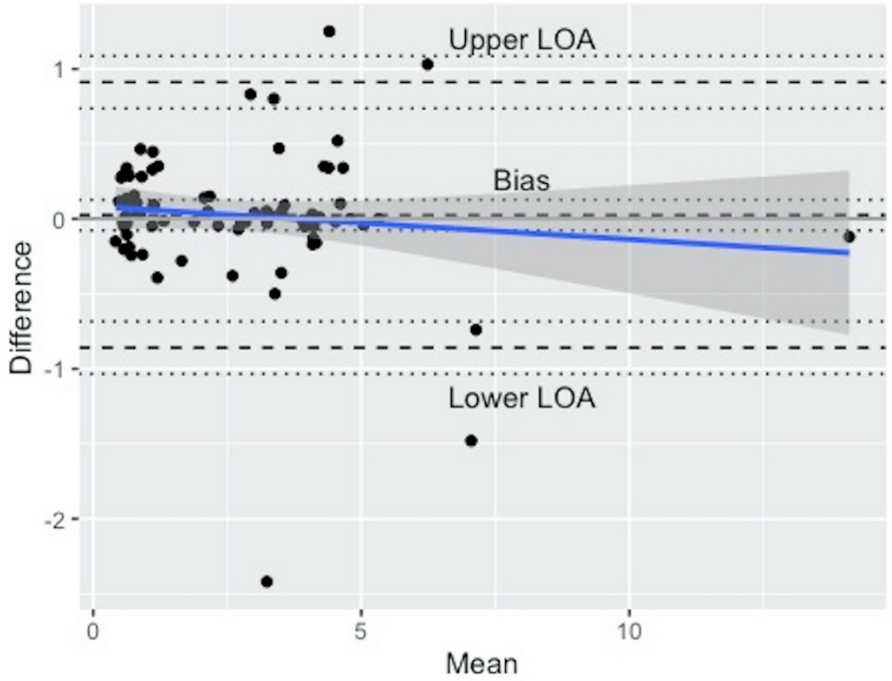
Supplementary information S4. Supplementary details on the development of the method - linearity



Supplementary figure S4-5. Analytical linearity of Pb measurements in dried bloodspots (DBS) using the TXRF method developed in this study. Linearity was calculated by linear regression analysis of peak intensity counts and Pb levels in DBS that had been spotted with seven **di**fferent whole blood standard reference materials (SRM) from the Institut National de Santé Publique du Québec (INSPQ). The slope and intercept of the calibration curve were 199.19 and -40.76, respectively. The coefficient of determination (R^2) was > 0.997.

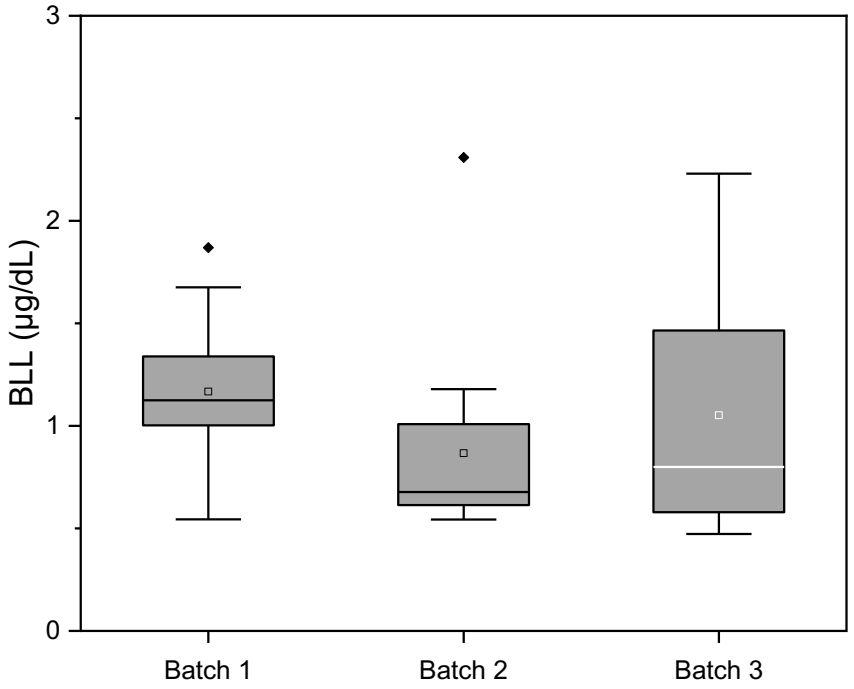
Supplementary table S4-3. Mean (±SD), geometric means (GM), range (min-max), and selected percentiles (10^{th}-95^{th}) of blood Pb levels (μg/dL) in a low exposed group (McGill university members) and a highly exposed groups (e-waste workers from Ghana).

	McGill University members			**E-waste workers**	
Statistic	Whole blood (ICP-MS)	DBS (ICP-MS)	DBS (TXRF)	Whole blood (ICP-MS)	DBS (TXRF)
Mean ± SD	0.86 (± 0.45)	0.93 (± 0.50)	0.78 (± 0.46)	4.15 (± 1.94)	3.79 (± 3.01)
GM	0.78	0.84	0.85	3.84	4.16
Range	0.39-2.35	0.39-2.73	<LoD - 2.31	1.51-14.04	1.79-19.67
5th	0.40	0.41	0.46	2.04	2.05
10th	0.45	0.47	0.54	2.27	2.70
25th	0.59	0.59	0.61	3.14	3.10
50th	0.75	0.77	0.82	3.84	4.07
75th	1.04	1.18	1.14	4.73	4.51
90th	1.61	1.31	1.71	6.44	7.79
95th	2.17	2.23	2.18	8.22	11.43
% < LoD (0.28 µg/dL)	0%	0%	7.5%	0%	0%
% < LoQ (0.69 µg/dL)	32%	32%	25%	0%	0%

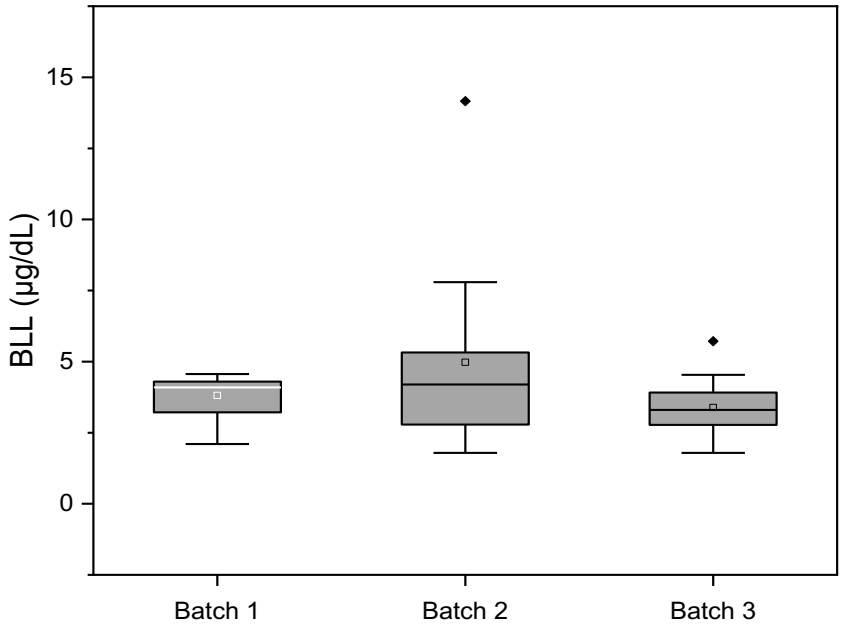


Supplementary figure S4-6. Bland-Altman plot of difference (y axis) in Pb measurements (µg/dL) in capillary DBS analyzed by TXRF and venous whole blood analyzed by ICP-MS versus the mean of the two measurements (x axis) in both population groups combined. The dashed lines indicate the upper LOA, bias, and lower LOA (from the top of the figure to the bottom), and for each parameter the 95% CI are indicated with dotted lines. The blue line **re**presents the proportional bias trend line.

A



B



Supplementary figure S4-7. (A) Boxplots of blood Pb levels (BLL) measured in capillary DBS samples by TXRF from McGill university members for each batch run; (B) Boxplots of BLL measured in capillary DBS samples by TXRF from Ghanaian e-waste workers for each batch run. There were 14 samples per each batch run.

Supporting information S4. Blood lead levels in studies of E-waste workers from Agbogbloshie, Ghana

Supplementary table S4-4. Comparison of blood Pb levels (BLL) among e-waste workers from Agbogbloshie, Ghana as reported by different studies and the present study (last line).

Study (Author and year)	Participants (n)	Analytical technique	Mean BLL (µg/dL)[a]	% of samples below the LoD
Caravanos *et al*., 2013	87	AAS	16.1 ("exposed workers")	NA[b]
Srigboh *et al*., 2016	69 (58 male and 11 female)	ICP-MS	7.93 (± 5.8)	13.0 % (LoD= 2.42 µg/dL)
Amankwaa, 2017	128 (81 e-waste workers)	AAS	3.49 (± 3.54; e-waste workers)	55.6% (LoD=0.001 µg/dL)
Wittsiepe *et al*., 2017	72	AAS	10.19 (31-351)	0%
Current study	41	TXRF	3.43 (± 3.0)	0%

[a] Data as mean ± SD unless indicated, [b] Not available

Supporting information S4. Resource comparison between ICP-MS analysis of whole blood and TXRF analysis of DBS

Supplementary table S4-6. Comparison of time and chemical reagents required to characterize blood Pb levels (BLL) in whole blood using ICP-MS versus DBS using TXRF. The example here specifically focuses on resources needed to analyze the 41 samples from the e-waste workers. In a prior study I detailed how the whole blood samples were analyzed via ICP-MS (Srigboh et al., 2016), and in the current study I detailed how the DBS samples were analyzed via TXRF.

	ICPMS analysis of whole blood	**TXRF analysis of DBS**
Time required[a]	1 day (24 hr period) for sample preparation and nitric acid digestion, 1 day (24 hr period) for H2O2 digestion, and 1 day (8 hr workday) for ICP-MS analysis = 3 days (56 hours)	1 day (8 hr workday) to prepare samples and analyze them in the TXRF = 1 day (8 hours)
Chemicals required[b]	100 mL concentrated nitric acid (to clean supplies), 40 mL concentrated nitric acid (digest samples), 20 mL H_2O_2 (digest samples) = 160 mL chemicals	25 mL RBS concentrate (to clean discs), 50 mL concentrated nitric acid (to clean discs), 0.5 mL Serva silicon solution (coat disc), 8 mL 6N HCl, 1 mL H_2O_2, 8 mL MIBK = 85 mL chemicals

[a] does not consider, for either method, the time needed to prepare samples and reagents (estimate [b] does not include reference materials, argon estimates for ICPMS.

CONNECTING PARAGRAPH

Chapters 3 and 4 presented findings on two of the main limitations of DBS sampling for Pb exposure assessment. Another major gap in DBS sampling is the so-called hematocrit effect, which can lead to potential errors during the analysis. There have been different approaches to correct for this effect, including color-based algorithms and using special blood collection devices. However, an ideal approach should be easy to apply, show low variation between individuals, not be affected by time, and use the same DBS samples that are used to measure the analyte.

Chapter 5 is focused on the correction of the hematocrit effect using potassium measurements as a proxy for the hematocrit content in DBS samples. The study is carried out in three phases: the validation of a method to measure potassium in DBS using AAS, the development of a correction factor for the hematocrit differences in DBS using potassium measurements, and the application of this approach with a test (larger) set of samples.

This chapter was co-authored by the candidate's supervisor Dr. Niladri Basu and Dr. Andrea Santa-Rios. The manuscript will be submitted for publication to the journal Environmental Health in September 2021.

5. **CHAPTER 5.** Correction of hematocrit for the analysis of metals and essential elements in dried blood spots using potassium measurements

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5.1 Abstract

Dried blood spots (DBS) is an advantageous sampling tool that has been applied in the field of exposure science. The benefits of this DBS are numerous, including low invasiveness, feasibility, and low costs. Yet, this approach has been challenged by limitations such as the so-called "hematocrit effect". The hematocrit effect refers to how the differences in hematocrit in the DBS result in an uneven blood spreading through the filter paper. One of the approaches to cope with this effect that has demonstrated reliability, is the use of potassium (K^+) as a marker of hematocrit. The goal of this study is to explore the potential of K^+ measurements as a correction factor for the hematocrit effect in the analysis of several elements, including As, Cd, Cu, Fe, Mn, Pb, Se, and Zn in DBS, to improve the agreement between whole blood and DBS. I validated a method to assess K^+ in DBS using atomic absorption. I built a calibration curve of whole blood samples with a range of hematocrit levels (0.25-0.60), and a fixed concentration of different elements and I created DBS using an aliquot of these samples. I obtained a correction factor from the K^+ measurements, and I evaluated the agreement between whole blood and DBS before and after the correction of the concentrations of elements in DBS. In terms of method performance, the limits of detection and quantification of the method were 1.76 and 5.80 ppm, respectively, and precision and accuracy values ranged from 0.02 to 0.33% and from 92.2 to 124.7% for these parameters respectively. For the % of differences, I observed a larger percentage of negative differences at a lower level of hematocrit (35-38%) for the elements of As, Mn, and Pb (mean % of differences: - 5.31, - 48.36, and -5.08, respectively), while the essential elements virtually not affected. For the agreement after correction, I observed an improvement in the agreement of DBS and whole blood measurements, with a reduction in the mean of differences (bias) from 0.41 (95% CI: 0.26 to 0.55) to 0.17 μg/dL (95% CI: 0.01 to 0.33) in the case of Pb and from -

3.96 (95% CI: -3.12 to -4.78) to -0.10 μg/L (95% CI: 0.59 to -0.79) for Mn. In the case of As, the constant bias value remained very similar from -0.08 (95% CI: 0.28 to -0.44) to -0.13 μg/L (95% CI: 0.54 to -0.31). This study presents two contributions, the development AAS-based method to measure K^+ in DBS samples and a correction method that could improve the measurement differences caused by different levels of hematocrit in DBS samples. The developed method showed a good performance and the correction method helped to reduce differences and improve the agreement between DBS and whole venous blood samples for some elements. However, it is important to point out the need to expand the hematocrit range in future studies, particularly at lower levels, as well as explore other elements and gather information about hematological parameters to further understand the differences between DBS and whole venous blood samples.

5.2 Introduction

Dried blood spots (DBS) have been successfully applied in biomonitoring studies over the years, with interest growing (Freeman, *et al*., 2018). DBS represent an alternative matrix in exposure assessment studies of chemicals of concern, including metals, as well as in the determination of elements essential to health. The benefits of this sampling technique when compared to traditional approaches that use venipuncture are numerous, and include being less invasive, having lower costs, and requiring fewer resources such as space and trained personnel (Zakaria *et al*., 2016; Li and Lee, 2014). These advantages allow DBS to help reach populations that were previously inaccessible and increase study participation (Li and Lee, 2014).

The assessment of human exposure to toxic and essential elements through DBS samples represents an advance towards making population studies more practical and accessible. Several studies have focused on the evaluation of DBS in metals exposure assessment, particularly for lead (Pb) (Chaudhuri *et al*., 2009; Funk *et al*., 2013; Nyanza *et al*., 2019; Rodríguez-Saldaña *et al*., 2021). In addition, studies are starting to characterize the potential of DBS to assess exposures to essential elements (Pawly *et al*., 2019; Vieira, 2021). While the results of these diverse studies lend support to the use of DBS in exposure assessment studies, there remain outstanding challenges which hinder its wider adoption.

One of the most outstanding challenges in the analysis of DBS is the "hematocrit effect". Specifically, hematocrit can influence the spread of the blood through the filter paper often in an

uneven manner (Li and Lee, 2014). Given that DBS are usually sub-sampled, the blood volume in a given sub-sample can vary greatly from within a single DBS thus affecting the measurement. Recently, several approaches have been proposed and developed to overcome this issue, with most of them focused on identifying an appropriate predictor for the hematocrit. One of the solutions that has demonstrated promise is the use of K^+ measurements to determine the hematocrit in the samples (Capiau *et al*., 2012). This approach relies on a) the simple determination of K^+, b) the stability of K^+ in blood, and c) the high abundance of K^+ in erythrocytes compared to serum or plasma (~30 times higher). Studies have documented that K^+ can serve as an effective hematocrit correction factor in the analysis of several analytes in DBS, including caffeine (De Kesel *et al*., 2014), paraxanthine (De Kesel *et al*., 2014), and creatinine (den Burger *et al*., 2015). Moreover, the authors of these studies pointed out the importance of properly validating and applying the spectrometric methods (e.g., Atomic absorption spectroscopy (AAS); Capiau *et al*., 2012) to measure K^+ so as to help improve the limits of agreement.

There is tangible and plausible evidence to suggest that K^+ can serve as an effective hematocrit correction factor with studying DBS, though to our knowledge this has not applied to the measurement of a panel of toxic and essential elements in DBS samples. Accordingly, the goals of this study were to: 1) develop a method to assess K^+ and several elements of interest (As, Cd, Cu, Fe, Mn, Pb, Se, and Zn) in DBS samples (phase 1 method development); 2) explore the potential of K^+ measurements as a correction factor for the hematocrit effect in the analysis of the aforementioned elements in DBS (phase 2 method validation) to help improve the agreement in measurements between DBS and whole blood samples; and 3) test this approach with an independent set of samples (phase 3 method application).

5.3 Materials and methods

5.3.1 General overview

This study was executed in three phases. In Phase 1 (Method Development), I set up a method to measure K^+ in DBS samples using Atomic Absorption Spectroscopy (AAS). In Phase 2 (Method Validation), the potential of K^+ as a proxy for hematocrit was evaluated in DBS with a range of values spanning normal to critical low limits of hematocrit (0.25-0.60). In Phase 3 (Method Application), I took the information gleaned from Phase 1 and Phase 2 and applied them in an independent study population to evaluate the percent differences and agreements in measures taken in whole blood and corresponding DBS before and after applying the K+ correction.

In Phase 1 and Phase 2, I used whole blood reference materials (**Supplementary table S5-1**). During Phase 2, I used whole blood samples from five volunteers (Institutional Review Board approval from McGill University: A09-M31-17B) and each of these samples was divided in three portions. One portion was used to determine the concentrations of the elements of interest (As, Cd, Cu, Fe, Mn, Pb, Se, and Zn) by ICPMS and K^+ by AAS, the second portion was used to analyze hematocrit content, and with the third portion I created DBS samples in the laboratory. In Phase 2 the ratio of the concentrations of toxic metals and essential elements in whole blood and DBS samples were plotted against the K^+ concentrations, and the equation generated was used to correct the DBS concentrations. The K^+ measurements were used to correct the bias due to the hematocrit

effect in the measurements of concentrations of the elements of interest in capillary DBS using reference set of samples (n=5).

Finally, in Phase 3, the efficacy of the correction was assessed with an additional test set of samples from another group of volunteers (n=45), more details about the group of volunteers and sample collection are provided elsewhere (Santa-Rios *et al*., 2020; Rodríguez-Saldaña *et al*., 2021) in which the elements of interest were analyzed by ICP-MS. The performance of the correction based on K^+ was assessed by determining the % of differences between the two types of samples and their agreement before and after correction.

5.3.2 Chemicals and reference materials

I used three multi-element whole blood reference materials from the Institut National de Santé Publique du Québec (INSPQ) Centre de Toxicologie du Québec (CTQ) as quality control samples to assess the precision and accuracy of the measurements and to confirm the linearity of the method through calibration curves (**Supplementary table S5-1**). Nitric acid (65% HNO_3 Omnitrace grade, EMD Chemicals, NJ), hydrochloric acid (12 N HCl, Merck, Germany), and hydrogen peroxide (35%, Merck, Germany) were used to digest samples. A stock standard solution of 1000 mg/L Ga (Merck, Germany) was used to prepared internal standard (IS) solutions for the ICP-MS analysis. A 5% Lanthanum solution was used during the analysis of K^+ by AAS.

5.3.3 Dried blood spots preparation

During Phase 1 and 2 (Validation), entire circles of DBS samples of whole blood were used. As part of the quality control of the analysis of metals and essential elements, I also used reference

materials at different levels of concentration (**Supplementary table S5-1**), spotting the whole blood onto DBS cards. During phase 2, I created DBS samples with different hematocrit levels. The hematocrit levels were created by adding or removing plasma from samples from voluntary donors. Finally, for phase 3, I used DBS samples obtained from volunteer donors (test set of samples).

5.3.4 Instrumentation

I used an Atomic Absorption Spectrometer model 2380 (Perkin-Elmer, Norwalk, USA) to perform the analysis of K^+ and the elements of interest during phase 2 and I used Inductively Coupled Plasma Mass Spectrometer model 820-MS Series (Varian Inc., California, USA) for the analysis of the elements of interest in the test set of samples (phase 3). The analysis of hematocrit was performed by an external laboratory using a hematology analyzer (Sysmex XN 9000).

5.3.5 K^+ analysis

The analysis of K^+ by ICP-MS is usually challenged by the high background of Argon plasma as documented in several studies (Murphy *et al*., 2002; Hammerli *et al*., 2013). Therefore, the measurements of K^+ were performed by Flame Atomic Absorption Spectroscopy (F-AAS) in whole blood and DBS samples. This approach is also preferred while working with concentrations in the ppm range. The samples were digested prior analysis using HNO_3 acid, HCl acid, and H_2O_2. The DBS samples were digested at room temperature, while the whole blood samples were heated in a hotplate at 120 °C. The samples were diluted 20 times and an aliquot of each sample was taken and treated with LaO_2 as a matrix modifier before the analysis.

5.3.6 Hematocrit calibration curve

I used whole blood samples from each volunteer (n=5) to create a calibration curve with increasing levels of hematocrit by modifying the plasma content. An hematology analyzer was used to determine the hematocrit content. The hematocrit calibration points were 0.25, 0.35, 0.45, 0.55, and 0.60. This calibration curve was then used to determine the deviation of concentration of the elements of interest in DBS from whole venous blood values at different hematocrit levels from which a correction factor was realized. The calibration curves were carried out by performing an unweighted linear regression. Finally, the corrected and original concentrations of metals and essential elements in DBS samples were compared to the concentrations in whole blood samples.

5.3.7 Metals and essential elements

The analysis of concentrations of toxic metals and essential elements (As, Cd, Cu, Fe, Mn, Pb, Se, and Zn) in whole blood and DBS samples was carried out by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). I selected Ga as internal standard (IS), since it is an element that is rarely present in real whole blood samples, and I added 50 µL to each sample to a final concentration of 200 µg/L. More details on the digestion procedure are presented elsewhere (Basu *et al*., 2011). The samples were digested in the same way for the FAAS analysis; however, rather than using an IS as in ICP-MS analysis, I used external calibration curves.

5.3.8 Assay validation and quality controls

I included several quality controls during the analysis of each batch, such as filter paper blanks (n=3; sample-free), blood multi element reference materials at the upper, medium, and lower levels

of the concentration range (n=3; at the beginning and at the end of each run), and sample replicates (n=3). Evaluating the inter-card variability is not required when the filter cards are from the same type/manufacturer (European Bioanalysis Forum (EBF) recommendations; Timmerman, 2011).

5.3.9 Data analysis

All data were evaluated using the software MedCalc version 18.10.2 (MedCalc software, Mariakerke, Belgium) and Microsoft Excel 2017. Accuracy and precision of the AAS and ICP-MS methods applied was assessed by calculating the recovery % and the coefficients of variation (CV %) of replicate samples using whole blood reference materials. A linear calibration curve was created with 5 hematocrit levels. Unweighted linear regression analysis was performed to compare the K^+ and hematocrit levels and to obtain the correction equation from the whole blood/DBS ratio plotted against K^+ levels. The performance of the correction was determined by plotting the % of differences between whole blood and DBS samples across the relevant range of hematocrit levels before and after the correction. Bland-Altman plots were applied to assess the effect of the correction on the agreement between the concentrations of the elements of interest in DBS samples compared to the measurements of the same elements in paired whole blood samples. All figures were generated using R version 3.6.3.

5.4 Results and discussion

5.4.1 Contamination of DBS samples

The potential contamination of DBS samples was assessed from the data of filter paper blank samples analyzed along with the samples and it is presented in the **Supplementary table S5-2**. Considering that the volume in a whole circle in DBS samples contain about 60 μL of whole blood, I used the background levels obtained in ng to calculated concentrations in μg/L for the elements of interest in the blank filter paper samples. The estimated average concentrations were 0.00, <LoD, 0.80, 4.09, 0.12, <LoD, and 4.47 μg/L for As, Cd, Cu, Fe, Mn, Se, and Zn, respectively, and 0.03 μg/dL for Pb. Since I analyzed blank filter paper samples paired with DBS samples, it should be noted that the concentration of the elements of interest found in blank samples were never higher than the DBS samples. Finally, I found a very weak correlation between Pb measures in the blank spots and their paired DBS samples for all the elements of interest with coefficients of determination (R^2) of 0.04, 0.08, 0.03, 0.10, 0.14, 0.12, 0.02, 0.19 for As, Cd, Cu, Fe, Mn, Pb, Se, and Zn, respectively.

5.4.2 Phase 1: Method development (analysis of K^+ in DBS by AAS)

I developed a method to measure K^+ in DBS samples using AAS, and here I report on key validation parameters to demonstrate that the analytical approach was suitable for its intended purpose (**Table 5-1**). The calibration data showed a linear response (no weighting required) with a coefficient of determination of 0.999. The slope and the intercept of the linear equation were 1.024 and -0.066 µg/L respectively. The overall accuracy (recovery %) and precision (relative standard deviation, RSD %) across 5 levels of concentration of K^+ (0.2, 0.5, 1.0, 10.0, and 25.0 µg/L) were 103.4 ± 4.9% and 1.1 ± 0.6 %, respectively.

Table 5-1. Analytical validation parameters for the determination of K^+ (AAS), metals, and essential elements in DBS (n=20) created with SRM by ICP-MS

K^+ Analytical validation				
Parameter		**Value**		**Acceptance criteria**
LoD		1.76 ppm		Expected concentrations ~1000 ppm higher
LoQ		5.80 ppm		Expected concentrations ~1000 ppm higher
Linearity (R^2)		0.997		≥ 0.99
Precision (CV %)		0.02-0.33%		≤ 10 %
Accuracy (Rec %)		92.2-124.7%		80-120%
Metals Parameter	**Pb**	**As**	**Cd**	**Mn**
LoD (ppb)	0.19	0.02	0.004	0.24
LoQ (ppb)	0.55	0.04	0.08	0.41

Linearity (R^2)	0.990	0.974	0.996	0.886
Precision (CV %)	0.02-0.1	0.01-0.1	0.02-0.1	0.05-0.2
Accuracy (Rec %)	90.8-95.8	99.6-132.8	88.0-101.0	120.6-147.7

Essential elements Parameter	**Cu**	**Se**	**Zn**
LoD (ppb)	1.69	0.07	6.12
LoQ (ppb)	2.09	0.31	9.77
Linearity (R^2)	0.979	0.990	0.994
Precision (CV %)	0.02-0.02	0.05-0.08	0.01-0.02
Accuracy (Rec %)	94.0-101.7	107.1-120.4	89.9-100.7

5.4.3 Phase 2: Method validation

Analysis of metals and essential elements

The results of all the validation parameters were within the acceptance limits as shown in **Table 5-1**. The relevancy of the accuracy and precision of the elements of interest is based on the expected range of concentrations in the general population. Taking Pb as an example, the accuracy and precision were calculated across 5 levels of concentration (1.0, 2.0, 4.0, 10.0, and 20.0 µg/dL), covering the expected range of blood Pb levels in the population, based on the 10-95th% of individuals from Canada and U.S. (CHMS, 2017; NHANES, 2017).

Hematocrit association with K^+

I calculated an association between the K^+ concentrations and the hematocrit levels in both whole blood (**Supplementary figure S5-1**) and DBS samples. Specifically, the relationship between hematocrit in whole blood and K^+ in DBS samples showed a strong positive association (R^2= 0.96, **Supplementary figure S5-2**), intercept= 3.311 µg/L, 95% CI 2.01 – 4.52 and slope= 0.08, 95% CI -0.10 – 0.20). These results show that the hematocrit measured in whole blood and the hematocrit calculated based on the K^+ concentrations in DBS are comparable. These results align with validation data reported in a past study (Capiau *et al*., 2013) with a similar regression equation between hematocrit and calculated hematocrit based on K^+ measurements (intercept and slope of the calibration curve were 3.15 and –0.09, respectively).

5.4.4 Phase 3: Method application

Descriptive statistics of whole blood/DBS samples of the test set

The concentrations of the elements of interest (metals and essential elements) were determined in whole blood and DBS samples from 45 individuals. In this group, the geometric mean of hematocrit was 40.1% with a range of 30.2 to 47.9 %. The geometric mean concentrations as well as the 25th and 75th percentiles for all the elements of interest are presented in **Table 5-2**.

Table 5-2. Geometric mean (± SD), 25th and 75th percentiles of concentrations (µg/L, otherwise indicated) of the toxic metals and essential elements in whole blood and DBS samples analyzed by ICP-MS.

	Whole blood	**DBS**

Element	GM ± SD	25th PCTL	75th PCTL	GM ± SD	25th PCTL	75th PCTL
As	2.34 ± 1.5	1.71	2.51	2.19 ± 1.19	1.45	2.36
Cd	0.32 ± 0.27	0.10	0.26	0.38 ± 0.27	0.13	0.41
Cu	751.73 ± 121.72	646.36	815.64	730.24 ± 117.29	648.81	769.79
Fe[a]	415.92 ± 37.34	391.51	438.42	407.46 ± 34.76	380.82	438.42
Mn	8.78 ± 2.45	7.12	9.77	11.96 ± 2.01	10.52	13.34
Pb[b]	0.87 ± 0.38	0.59	11.5	0.89 ± 0.43	0.60	1.23
Se	160.01 ± 18.52	144.70	174.64	155.76 ± 16.53	143.78	169.84
Zn	5277.99 ± 559.38 µg/L	4883.50	5576.95	5149.79 ± 570.88	4810.58	5633.10

[a] Concentrations in mg/L; [b] Concentrations in µg/dL

Differences between whole blood and DBS samples before correction

I evaluated the differences between whole blood and DBS samples before K^+correction, with **Fig. 5-1** and **Fig. 5-2** showing the differences between the two types of matrices for metals and essential elements, respectively, against the hematocrit levels. I observed a larger percentage of negative differences at a lower level of hematocrit (35-38%) for the elements of As, Mn, and Pb (mean % of differences: - 5.31, - 48.36, and -5.08, respectively). The essential elements were less affected by variable hematocrit values, presenting a mean % of differences of 2.94, 1.10, 2.55, and 1.30 % for the elements of Cu, Fe, Se, and Zn, respectively. From these results I selected the elements As, Mn, and Pb, as the elements that could improve by applying a K^+ correction.

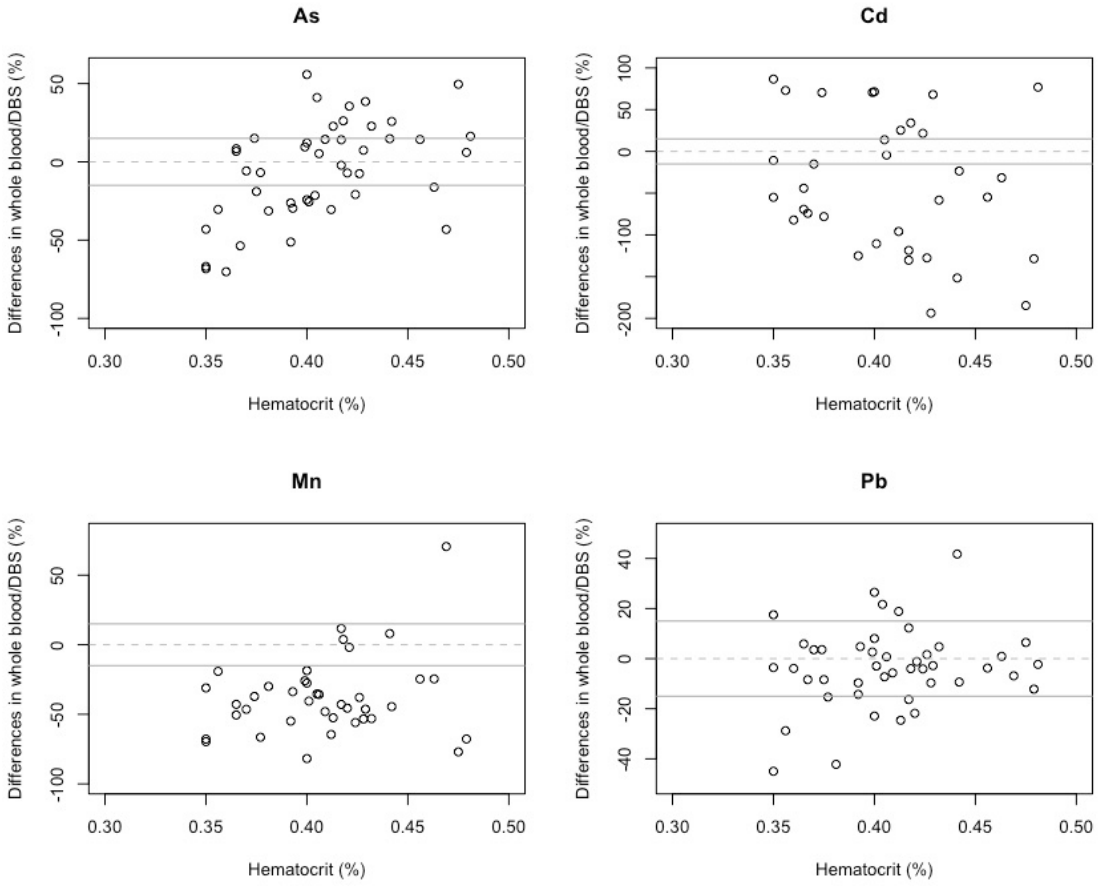

Figure 5-1. Percentage (%) of differences between DBS samples and whole blood for metal(oid)s across hematocrit levels (%) before correction. Gray solid lines indicate ± 15 % difference limits and dashed lines indicate 0 % difference.

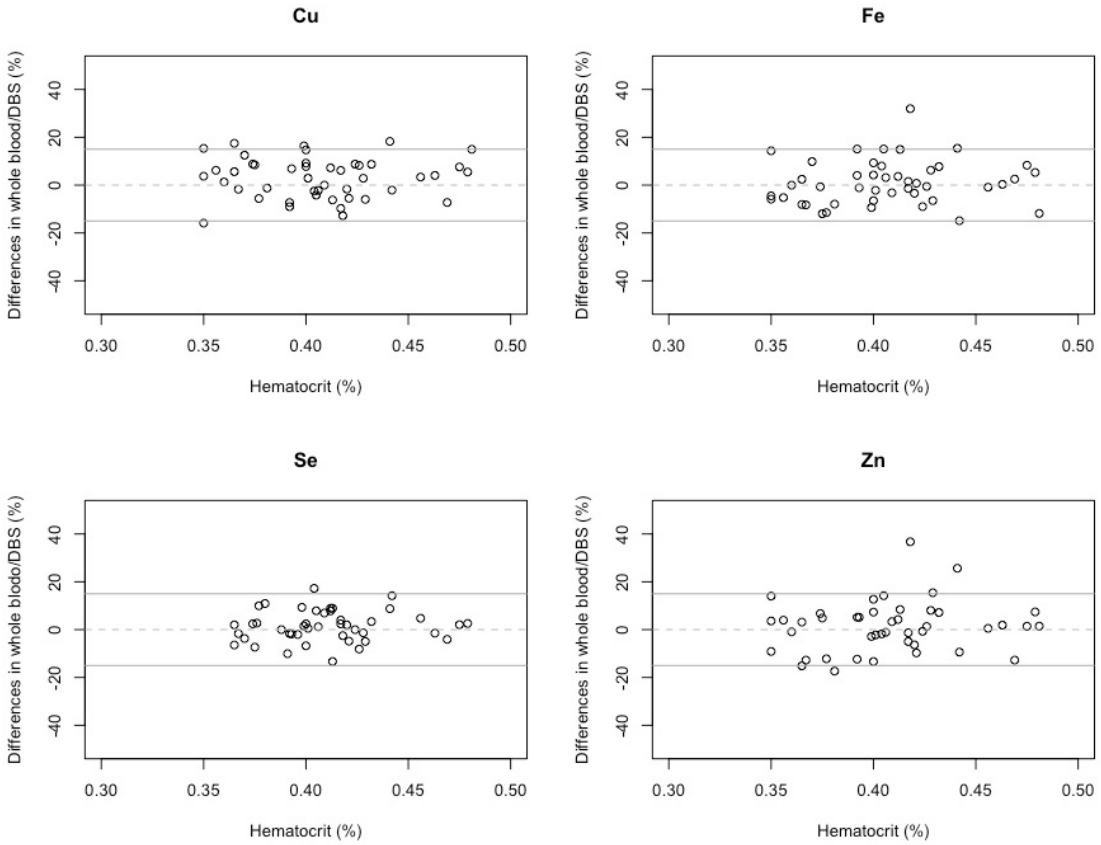

Figure 5-2. Percentage (%) of differences between DBS samples and whole blood for essential elements across hematocrit levels (%) before correction. Gray solid lines indicate ± 15 % difference limits and dashed lines indicate 0 % difference.

Differences between whole blood and DBS samples after correction

Based on a linear regression built from the ratio of DBS/Whole blood samples (from Phase 2) in which concentrations of the elements of interest were plotted against the K^+ measurements, I obtained equations to calculate new DBS corrected concentrations from a derived algorithm (*new element concentration in DBS= original element concentration in DBS* ((-slope * K^+ + intercept*)). In the case of Pb, for example, I obtained a slope of -0.23 (95% CI: -0.36 – -0.10) and an intercept of 1.11 (95% CI: 0.92 – 1.30), therefore, the corrected algorithm was: *new element concentration in DBS= original element concentration in DBS* ((-0.23 * K^+ + 1.11*)). After applying the K^+-based correction to the selected elements, I calculated the new % of differences between whole blood and DBS samples and I noticed an improvement in the mean % of differences of 3.57, 8.90, and 2.07 for the elements of As, Mn, and Pb, respectively (**Fig. 5-3**), versus the aforenoted % differences of - 5.31, - 48.36, and -5.08, respectively.

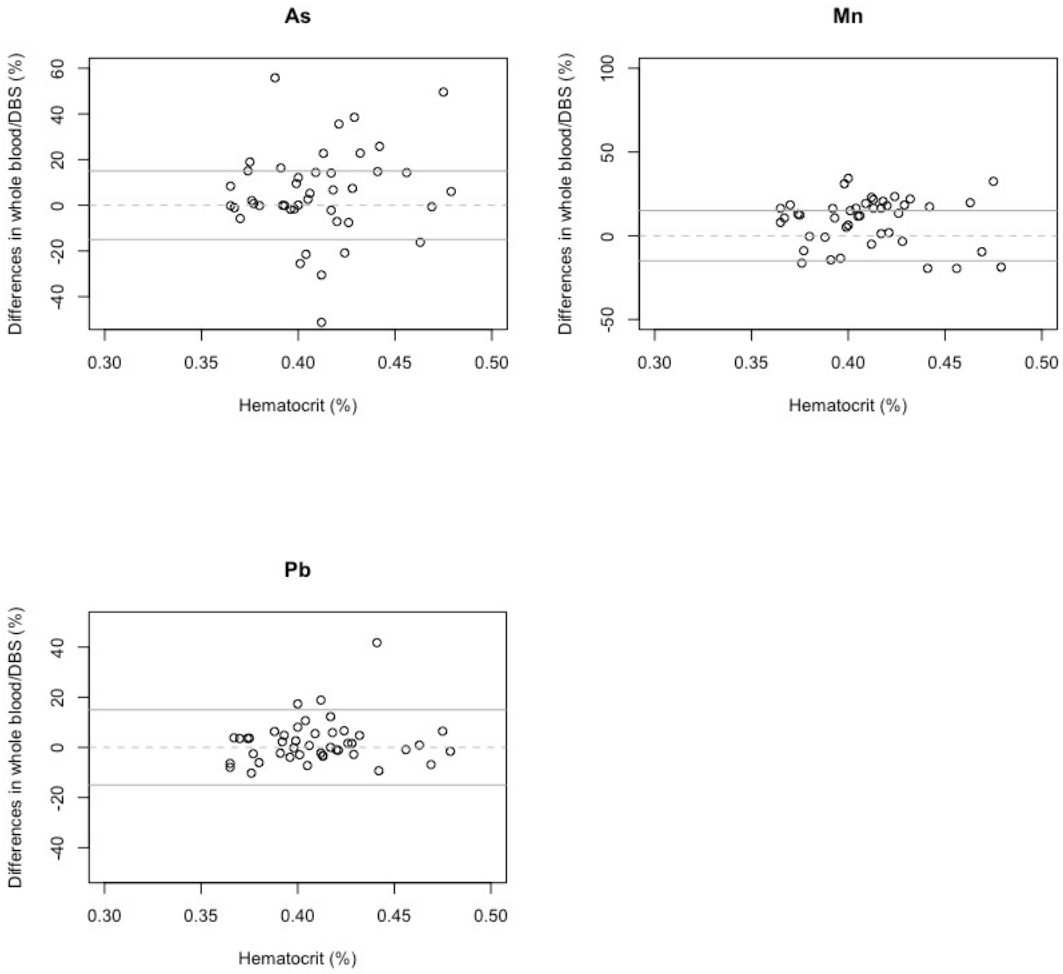

Figure 5-3.

Percentage (%) of differences between DBS samples and whole blood for selected elements across hematocrit levels (%) after correction. Gray solid lines indicate ± 15 % difference limits and dashed lines indicate 0 % difference.

Agreement between whole blood/DBS samples before and after correction

The agreement between the concentrations of the selected elements in DBS with corresponding whole blood samples was evaluated after the correction, and compared to the agreement of both type of samples before the correction of the hematocrit. I present key parameters of agreement, including the constant bias, upper and lower limits of agreement (LoA), range of the LoA, and the proportional bias (slope of the regression line) before and after the hematocrit correction (**Table 5-3**).

Table 5-3. Comparison of the agreement analysis parameters between whole blood and DBS samples for As, Mn, Pb, and Zn, before (initial) and after (new) applying a potassium-based correction of hematocrit.

	Initial constant bias* (95% CI)	**Corrected constant bias** (95% CI)	**Initial LoAs** (Upper-lower) (95% CI)	**Corrected LoAs** (Upper-lower) (95% CI)	**Initial LoA range**	**LoA range after Correction**	**Initial proportional bias (slope)**	**Corrected proportional bias (slope)**
As	-0.08 (95% CI: 0.28 to -0.44)	-0.13 (95% CI: 0.54 to -0.31)	2.27 (95% CI: 2.90 to 1.64) and -2.44 (95% CI: -1.81 to -3.07)	1.09 (95% CI: 0.77 to 1.40) and -1.35 (95% CI: -1.03 to -1.67)	4.71	2.44	-0.38 (95% CI: -0.65 to -0.10)	0.05 (95% CI: -0.11 to 0.22)
Mn	-3.96 (95% CI: -3.12 to -4.78)	-0.10 (95% CI: 0.59 to -0.79)	1.42 (95% CI: 2.85 to -0.02) and -9.32 (95% CI: -7.90 to -10.76)	4.42 (95% CI: 5.62 to 3.23) and -4.63 (95% CI: -3.43 to -5.82)	10.74	9.05	-0.17 (95% CI: -0.54 to 0.20)	-0.32 (95% CI: -65 to 0.02)
Pb	0.41 μg/dL (95% CI: 0.26 to 0.55)	0.17 μg/dL (95% CI: 0.01 to 0.33)	-0.58 μg/dL (95% CI: -0.83 to -0.32) and 1.40 μg/dL (95% CI: 1.15 to 1.66)	-0.91 μg/dL (95% CI: -1.19 to -0.63) and 1.25 μg/dL (95% CI: 0.97 to 1.53)	-1.99 μg/dL	-2.16 μg/dL	0.55 (95% CI: 0.22 to 0.88)	0.007 (95% CI: -0.32 to 0.33)

*Values in μg/L otherwise indicated

Based on the agreement comparisons, I observe that there is an improvement in the agreement of DBS and whole blood measurements after applying the correction (**Table 5-3**), with a reduction in the mean of differences (constant bias) from 0.41 (95% CI: 0.26 to 0.55) to 0.17 μg/dL (95% CI: 0.01 to 0.33) for Pb, and from -3.96 (95% CI: -3.12 to -4.78) to -0.10 μg/L (95% CI: 0.59 to -0.79) for Mn. In the case of As, the constant bias value remained very similar from -0.08 (95% CI: 0.28 to -0.44) to -0.13 μg/L (95% CI: 0.54 to -0.31). In addition, the range of the limits of agreement (LoA) was improved for As and Mn, with values going from 4.71 to 2.44 μg/L and from 10.74 to 9.05 μg/L, respectively, while the range for Pb remained the same (-1.99 initially to -2.16 μg/dL). Furthermore, if we evaluate the proportional error based on the regression line drawn in the Bland-Altman plot of the elements of interest, we observed that the slope of the regression also decreased from -0.38 to -0.05 for As, and from 0.55 to 0.007 for Pb, demonstrating that the bias in the measurements could also be improved for these elements. For Mn, I did not observe an improvement in the slope (-0.17 to -0.32). Based on the visual observation of the plot and the percentage of differences between whole blood/DBS of Mn, I perceive an important variability between the measurements in these two matrices that prevails across the different values of hematocrit, even at the high levels. The Mn measurements are constantly higher in the DBS samples compared to whole blood, thus, by applying the correction factor I cannot improve this bias. In this case, these differences should be further explored to understand their nature and apply the necessary measures to ameliorate the agreement between DBS and whole blood samples.

5.5 Conclusion and study limitations

This study presents two important contributions. First, the development of a method to measure K^+ in DBS samples by AAS, with a limit of detection of 1.76 µg/L and an overall precision and accuracy values of 0.02-0.33% and 92.2-124.7%, respectively. Second, an approach to validate and apply a correction method based on K^+ measurements to reduce the differences between DBS and whole blood samples caused by the hematocrit effect. After applying the correction method for the elements of As, Mn, and Pb (elements with higher deviations due to hematocrit differences), their % of the mean of differences improved. Moreover, the agreement of these elements was also improved, reflected in reduced constant bias values and range values between their LoA. Besides these contributions, it is important to acknowledge the limitations of this study. Testing a wide range of hematocrit levels is key for the implementation of correcting algorithms among diverse vulnerable populations. While the range of hematocrit evaluated with the reference test of samples is representative of critical low and high values (25- 60%) of the population, the test set of samples had an average hematocrit of 40%, which is within the hematocrit interval expected in a healthy population. Therefore, further evaluation with samples of more individuals at the critical low range, ideally from patients with hematocrit values less than 25% is important to test the effectiveness of the correction method among other vulnerable populations. Moreover, this method was developed using AAS, which is a good analytical approach to assess both K^+ and other elements. However, one of the limitations of the analysis of several elements with AAS is that this is sequential, which makes the analysis time longer. It is recommended to further explored other technology to improve the processing and analysis of samples. Finally, gather information about other hematological parameters could expand our understanding of other factors influencing the differences between DBS and venous whole blood samples for the elemental analysis.

5.6 Author contributions

This manuscript was written by all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally.

5.7 Conflict of interest declaration

The authors declare no conflict of interest.

5.8 Acknowledgments

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Supplementary information

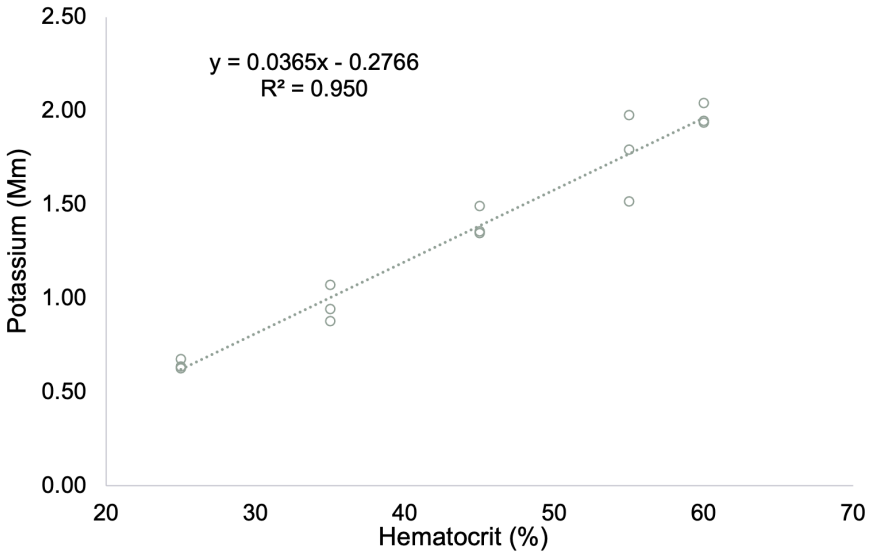
Supplementary table S5-1. Low, medium, and high concentrations (µg/L) of the elements of interest in standard reference materials (n=3) used during the validation phase and as analytical quality control.

Reference materials	QME-B-Q-1314	QM-B-Q-1505	QM-B-Q-1506
As	2.68	5.88	11.42
Cd	3.05	10.33	13.23
Cu	2236.81	3037.49	3094.69
Mn	8.73	19.61	22.52
Pb	47.86	158.92	501.42
Se	172.15	290.61	411.43
Zn	58.28	7910.98	10853.08

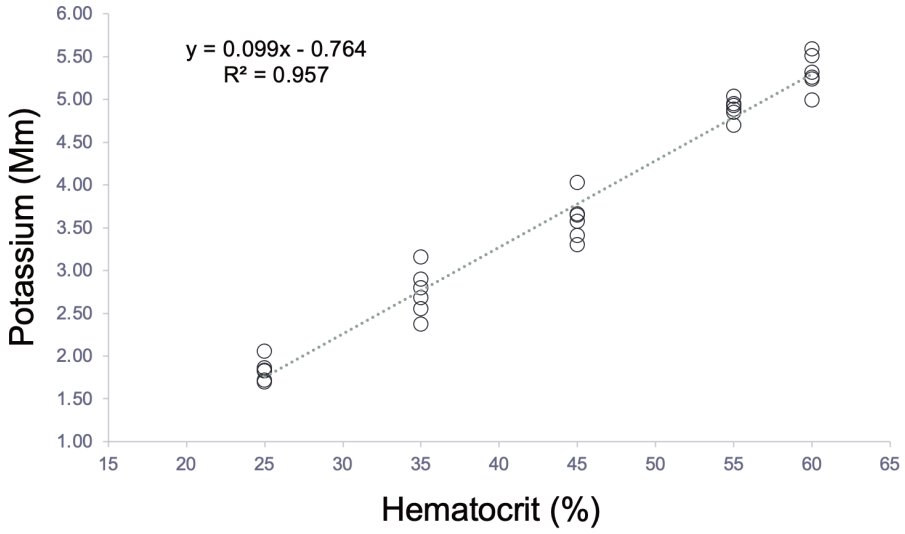
Supplementary table S5-2. Potential contamination track. Measurements of the elements of interest in filter paper blank samples (n=5), analyzed by ICP-MS.

Blank ID	As	Cd	Cu	Fe	Mn	Pb	Se	Zn
Bk 1	0.01	-0.03	1.49	4.33	0.15	0.49	-0.05	4.81
Bk 2	0.01	-0.02	1.49	4.32	0.16	0.52	-0.04	4.80
Bk 3	0.00	0.00	1.50	3.33	0.15	0.02	-0.07	3.77
Bk 4	0.005	-0.01	0.06	4.24	0.20	0.50	-0.03	4.55
Bk 5	-0.02	-0.01	1.61	4.31	0.03	0.00	-0.05	4.51

Supplementary figure S5-1. Relationship between potassium measurements (Mm) and hematocrit levels (%) in whole blood samples analyzed by AAS.



Supplementary figure S5-2. Relationship between potassium measurements (Mm) and hematocrit levels (%) in DBS samples analyzed by AAS.



6. GENERAL DISCUSSION

Monitoring Pb exposure is the best strategy in the prevention of Pb poisoning and it is necessary for the decision-making process and the creation of regulations to prevent Pb exposure among vulnerable populations (WHO, 2019). While whole blood testing is the most used method to assess Pb exposure, the need for alternative sampling methods has increased due to the numerous requirements that traditional venipuncture has. Among the most used alternative methods, dried blood spot sampling is one of the most popular ones since it is a microanalytical and low-invasive technique with fewer logistical requirements (Su *et al*., 2018). Nowadays, DBS is a technique that is broadly used in diverse applications, but some questions remained about its application and ultimately these gaps prevent it to be formally adopted in biomonitoring programs (Freeman *et al*., 2018).

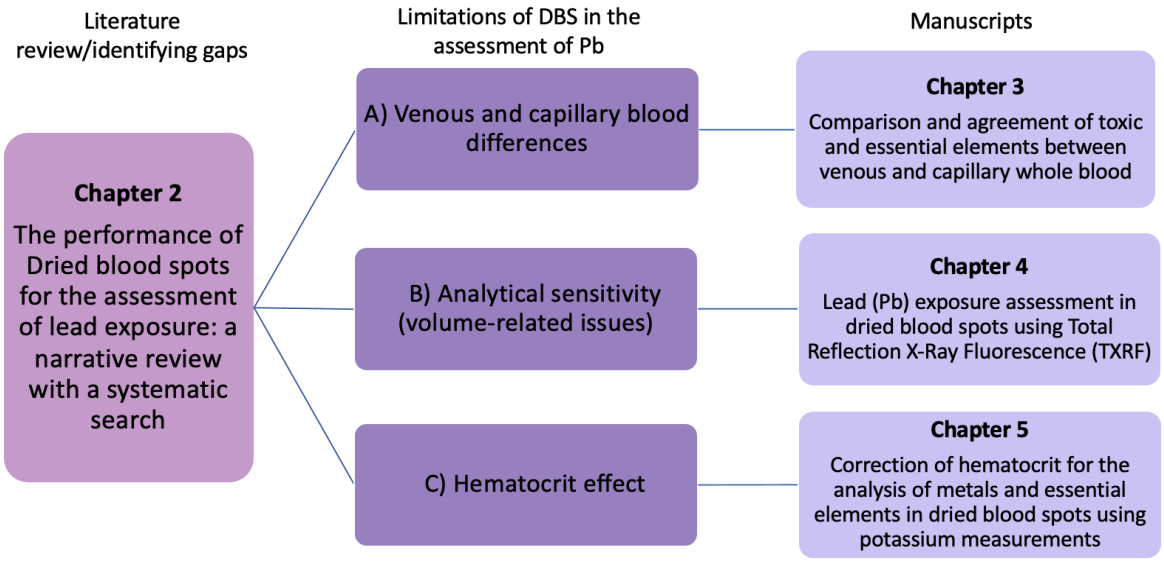
6.1 DBS limitations

The gaps in the application of DBS sampling have been reported across the literature since this technique was first developed in 1963 (Lim et al., 2018). Some studies have addressed some of the analytical issues with DBS that have been pointed out by other authors obtaining mixed results and conclusions on the impact of these aspects on the reliability of DBS. There are reviews that have discussed the advantages and barriers of DBS sampling in general in a narrative way (Nys *et al*., 2017; Lim *et al*., 2018; Freeman *et al*., 2018), however, there is a need to weigh the conclusions from the existing literature to reach a consensus on the real limitations in DBS application and how these barriers have been addressed and the different approaches that have been taken.

This thesis starts with an extensive systematic literature review designed to analyze all quality studies that used DBS sampling for the determination of Pb exposure. In doing so, the chapter

identified and discussed the main gaps in DBS application using qualitative and quantitative approaches. The findings from this chapter help synthesize for the broader research community the barriers that this sampling technique for the purpose of Pb biomonitoring, and also give focus on the thesis chapters. **Fig. 6-1** presents a diagram of the overview of this research and how the aims of this study were derived from our systematic review.

Figure 6-1. Schematic diagram of the thesis structure



6.2 Capillary and venous blood agreement in Pb analysis

The differences between capillary and venous blood have been addressed as an analytical concern in several studies that deal with Pb exposure determination (Parsons *et al*., 1997; Schlenker *et al*., 1994). These studies have compared the two sources of blood by finding a correlation between them and deciding if this value represents that both blood sources yield equal results. However, the correlation has been recurrently used to conclude that two methods/techniques are in agreement, while this argument is not always correct (Doğan, 2018). An agreement analysis is critical in the comparison of two methods to determine with confidence that if a novel method can replace a traditional one (Gerke *et al*., 2020). Moreover, the few studies that have reported an association between venous and capillary blood lack information on the quality control parameters and the information necessary when validating a method.

This research offers a side-by-side comparison between venous and capillary blood for the assessment of Pb exposure, not only reporting the association between the two sources of blood (see **Fig. 3-1**) but the differences (see **Table 3-1**) and the agreement between them (see **Fig. 3-2**). The results reported here provide an insight into the overall comparison between venous and capillary blood, demonstrating that these two types of blood are highly comparable for the analysis of Pb.

Furthermore, I decided to expand this study to other relevant elements that are commonly measured along with Pb in research studies and biomonitoring programs, including toxic elements such as As, Cd, and Mn and essential elements including Cu, Fe, and Se. The importance of measuring relevant toxic elements is key in biomonitoring programs, especially when the general population can be exposed to numerous toxic agents through different routes of exposure. Implementing alternative methods in biomonitoring programs can increase the participation and number of samples per participant, increasing the quality of the study and providing more information to guide future decisions. This study provides an understanding of the potential of capillary blood to be used in biomonitoring programs in the assessment of relevant toxic elements as a candidate as an alternative biomarker.

More research is needed to explore other elements of interest, as well as measuring hematological parameters to expand our understanding of how they can impact the agreement results (e.g., how differences of white or red blood cells counts in venous and capillary blood could affect the determination of some elements). Finally, to understand the boundaries of capillary blood application, it should be more widely applied in biomonitoring programs along with venous blood samples, as well as in research studies.

6.3 Analytical sensitivity and volume-related issues in DBS analysis

The low sample blood volume in DBS is an analytical issue that has been explored in several studies (Morgan *et al*., 1979; Chaudhuri *et al*., 2009; Peck *et al*., 2009). Despite the fact that the low blood volume in DBS is precisely what makes this technique less complex and more logistically feasible, it also poses challenges, especially concerning analytical sensitivity and detection limits.

I learned from our systematic review that ICP-MS has been increasingly used in the analysis of DBS samples in the last years, but this technique has several challenges as well (see **Table 1-3**). ICP-MS can only analyze liquid samples and it uses a relatively high volume of sample and chemicals (see **Supplementary table S4-6**), which can be a real problem considering that samples are often limited. Moreover, ICP-MS usually presents other analytical problems, including potential interferences, low tolerance to dissolve solids, and high operational and maintenance costs. These issues are particularly relevant with DBS sampling, which is often implemented in remote areas or in low-resource countries.

This research provided a novel method based on TXRF technology that helped alleviate the potential analytical issues of ICP-MS. To exemplify this, I determined some comparisons between the TXRF and ICP-MS performance (see **Supplementary table S4-6**), which includes a faster sample processing (5-fold quicker) and less volume from chemicals involved in the sample preparation (50%). This study offers advances in the field of microanalytical methods not only for DBS sampling but for TXRF-based methods as well. Finally, by comparing two exposed groups, I increased our understanding of DBS as an alternative method to determine Pb

exposures in both the general population (under laboratory conditions) and with a highly exposed population (in a resource-limited and contaminated field site).

Future research should focus on advancing the application of this method at a larger scale with diverse population groups and different settings (e.g., remote locations) to improve our comprehension of the strengths and weaknesses of its adoption in laboratory biomonitoring efforts.

6.4 Hematocrit effect in DBS samples

The effect or impact of the differences of hematocrit levels on the DBS samples is probably the most common reported limitation of DBS sampling after the low-volume issues (e.g., 25% of articles reviewed in Chapter 2 discussed the hematocrit effect). The hematocrit effect has been approached using different strategies and this inconsistency in the methods applied and most importantly, the lack of information on what strategies work for some analytes impede the widespread adoption of DBS sampling in the clinical field (its use with patients with critical low and high hematocrit levels).

The last chapter of this research focuses on the last reported limitation of DBS sampling in the literature; the hematocrit effect. Here, I reported the development and validation of an AAS-based method for the analysis of potassium in DBS samples in the first stage of this study, with adequate accuracy and precision values (based on regulatory guidelines; see **Table 5-1**). During the second phase, I introduced a validation and application of a potassium-based correction method to cope with this analytical issue in the assessment of relevant elements, particularly Pb, but other relevant toxic elements such as As, Cd, and Mn, as well as essential elements including Cu, Fe, Se, and Zn, were also included.

Our results demonstrated that the hematocrit differences particularly impacted the measurements of the elements of Pb, As, and Mn (see **Fig. 5-3**). I showed how the correction factor based on potassium measurements decreased the % of differences between whole blood and DBS samples

and improved the agreement between the two matrices and the systematic error observed in the agreement analysis (see **Fig. 5-4**).

Correcting the hematocrit bias by using a reliable marker is one of the most promising approaches in the implementation of a standardized correction method. Measuring potassium is more practical and feasible than other proposed markers for hematocrit (e.g., lipidomics profiling or UV-VIS reflectance; Miller *et al*., 2013; Liao *et al*., 2018). The potassium-based correction for the hematocrit effect has been used with diverse analytes (Capiau *et al*., 2012; De Kesel *et al*., 2014; Den Burger *et al*., 2015; Rufail *et al*., 2017) and it is used in this research with a selection of relevant elements, being the first time that this strategy is implemented in the field of exposure science.

Moving ahead, future studies can expand to explore more critical values of hematocrit (e.g., ideally < 0.25) to understand the effectivity of the potassium-based correction with samples from patients. Moreover, this correction could be expanded to other elements of interest, particularly those that can be measured at the same time with a multi-element analytical technique.

7. GENERAL CONCLUSSION

This doctoral thesis presents a collection of studies that focused on the understanding and the improvement of the limitations of DBS sampling in the determination of Pb exposure in human populations. This research aimed to provide a set of novel methods and strategies to cope with the most reported gaps in the execution of DBS sampling in both clinical/laboratory and field and conditions.

My PhD research findings help advance the understanding of the main gaps of DBS sampling, with the systematic identification of three main limitations including the capillary and venous whole blood differences, the low-volume issues, and the hematocrit effect. A deep analysis of these gaps, as well as their implications for Pb exposure assessment (and potentially other metals), was useful to set directions for the requirements that DBS sampling needs to fulfill in order to become a more widely used method in exposure science.

After understanding the gaps that need to be overcome, this research reports for the first time (to the best of my knowledge), a side-by-side comparison, including an agreement analysis, between capillary and venous whole blood for the analysis of Pb and several relevant elements in biomonitoring programs. The results from this study, suggest that although all the elements of interest presented positive correlations, the elements of As, Pb, Cu, and Fe were more consistent, having not only strong correlations and a lack of significant differences but a relatively good agreement. These findings are useful for future clinical research, however, I want to acknowledge some limitations of this study and thus areas where more research is needed. Some limitations include a) the need to compare the two types of blood at a wider range of concentrations (especially at higher levels) to determine if our results are still consistent, b) to better explain the differences found for some elements (e.g. Cd, Mn, and Se) it would be useful to determine several hematological parameters in both, venous and capillary blood, and c) this study is based on a non-occupationally exposed group, thus, collecting samples from vulnerable groups is required to better understand the challenges for capillary blood as an alternative sampling method.

Additionally, this thesis presents a novel method based on TXRF technology to help overcome some of the gaps in the analysis of Pb in whole blood, mainly low-volume related limitations. The method reported here compares well with existing gold standard methods for determining Pb, such as ICP-MS; e.g., both methods exhibit a limit of quantification in the ppb range. Though, in comparing the performance of TXRF vs ICP-MS, we found that the sample preparation time was shorter (~5 times) and the volume of reagents required was reduced (~50% less) for this novel TXRF method. There are limitations of the TXRF method, and so more research needed to evaluate the developed method with a) populations critically exposed (e.g. with blood lead levels higher than 10 μg/dL), b) more field studies, especially when contamination of samples is hard to minimize, and c) different modifications of the method to improve its limits of detection, such as trying external calibration curves and reference materials with certified concentrations.

Finally, this research addresses one of the most critical gaps of DBS sampling, the Hct effect. In the last chapter of this thesis, I present two contributions: first, a method to measure potassium in

DBS using AAS, and second, a potassium-based correction factor to correct for the Hct differences in DBS samples. The developed AAS-based method was suitable for measuring potassium in DBS (overall precision and accuracy values of 0.02-0.33% and 92.2-124.7%, respectively). Moreover, the potassium-based correction was useful to reduce the constant and proportional bias for Pb measurements between whole blood and DBS samples (from 0.41 to 0.17 μg/dL and from 0.55 to 0.007 (slope value), respectively). Moving forward, the following limitations/further research include: a) testing lower Hct levels, especially lower than 0.25, as this is key to understand the relevancy of the correction factor with samples from patients, b) analyzing more elements, as the hematocrit effect affects numerous analytes, and c) evaluating other alternative proxies of Hct to determine which is the best option to correct for the Hct effect for the analysis of metals.

Collectively, this research demonstrated that there could be a consensus on what the real limitations of DBS sampling are, and it showed several strategies that can be applied to overcome these barriers. These strategies can be further explored in the future in a range of population-based studies and may shed light on further opportunities and requirements to improve our testing efforts.

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