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Determination of Zinc Isotopic Composition in Biological Tissues

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Determination of Zinc Isotopic Composition in Biological Tissues

by

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Abstract

Reliable measurement of zinc (Zn) isotopic composition in biological tissues is crucial for understanding zinc metabolism and its role in health. Zinc isotopic ratios, particularly δ^{66}Zn, provide insights into zinc uptake, distribution, and excretion within the body. The primary aim of this study is to develop and refine advanced analytical methods to accurately measure Zn isotopic composition in biological samples, specifically liver and kidney tissues.

This study addresses the challenges posed by the inherently narrow range of natural mass-dependent fractionation in zinc, typically confined to ±1‰. Using multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) and the double-spike method, the analytical method achieves precision up to 0.1‰. The method ensures low blank levels (below 5 ng) and a high yield recovery rate (95%) during processing. Certified Reference Materials (CRMs) such as Bovine Liver (NIST1577c); Human Hair (GBW07601, USGS42, USGS43); Whole Blood (SeroNorm); Bone Ash (NIST1400); and Bone Meal (NIST1486) were used to validate the accuracy and precision of the method.

To further demonstrate the method's efficacy, Zn isotopic compositions were analyzed in biological samples from mice with different gut microbiota conditions: germ-free, specific pathogen-free (SPF), and Segmented filamentous bacteria group (SFB).

These analyses help elucidate the impact of gut bacteria on zinc homeostasis. The method developed provides a robust tool for accurately detecting subtle isotopic variations of less than 0.3‰, which is essential for understanding the complexities of zinc metabolism in biological systems.

The successful development and validation of this analytical method mark a significant achievement, setting a new standard in the field of zinc isotopic analysis. This advancement not only enhances the precision and reliability of Zn isotopic measurements but also lays the groundwork for future studies investigating zinc dynamics in biological contexts, thereby contributing to a deeper understanding of zinc metabolism and its implications for health.

Preface

This thesis, titled "Determination of Zinc Isotopic Composition in Biological Tissues" was performed at the Stable Isotope Lab at the University of Calgary and led by Professors Michael Wieser[1] and Keith A. Sharkey[2]. Part of this work was presented as a poster at the 2024 Winter Conference on Plasma Spectrochemistry.

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List of Symbols, Abbreviations, and Nomenclature

Symbol or Abbreviation	Definition
α	Natural mass-dependent fraction of the samplewith respect to the standard
β	Analytical mass fractionation caused by sample preparationand analysis on MC-ICP-MS
δ	Delta
Zn	Zinc
DS	Double-spike
ESI	Elemental Scientific Inc.
ERM-DB001	Standard reference material for human hair
GF	Germ-free group (uncolonized)
GBW07601	Standard reference material for human hair
GBW09101	Standard reference material for human hair
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HNO_3	Nitric acid
ICP	Inductively Coupled Plasma
IRMM-3702	Certified reference material for Zn isotope amount ratios
PCal	Certified reference material for Zn isotope amount ratios
m_i	Mass of the isotope in the numerator of the R ratios
m_{ref}	Mass of the reference isotope
MC-ICP-MS	Multi-Collector Inductively Coupled Plasma Mass Spectrometer
MQ (H_2O)	Milli-Q water
NIST SRM 1400	Standard reference material for bone ash
NIST SRM 1486	Standard reference material for bone meal
NIST SRM 1577c	Standard reference material for bovine liver
$R_{M,i}$	ith isotope amount ratio of the sample-DS mixture
$R_{S,i}$	ith isotope amount ratio of a known standard
SD	Standard deviation
SeroNorm Whole Blood L-3	Standard reference material for whole blood
SFB	Segmented filamentous bacteria group
SPF	Specific Pathogen-Free group
SRM	Standard Reference Material
USGS42	Standard reference material for human hair
USGS43	Standard reference material for human hair
V	Volt

Chapter 1

Introduction

1.1 Motivation for Research

Developing a reliable analytical method for measuring zinc isotopic composition in biological samples is pivotal for understanding zinc's intricate roles in biological systems. Zinc isotopes (^{64}Zn, ^{66}Zn, ^{68}Zn) are crucial in various biological processes, including enzyme catalysis and cellular metabolism [1]. Accurately measuring the relative amounts of these isotopes in biological matrices presents significant analytical challenges. However, developing a reliable method is fundamental for uncovering zinc's biochemical pathways and their impact on health.

1.2 Zinc Isotopic Composition

1.2.1 Isotopic Composition

Isotopic composition refers to an element's abundance of different isotopes in a sample. For zinc, this involves measuring the ratios of its isotopes (^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, and ^{70}Zn) using techniques like mass spectrometry. Accurate measurements of isotopic abundance ratios can reveal information about the sources, pathways, and processes that affect zinc within biological systems.

1.2.2 Role of Zinc in Biological Processes

Zinc is essential for numerous biological functions, including enzyme catalysis, cellular metabolism, and immune function. Zinc functions as a cofactor for many enzymes, aiding in catalysis and maintaining the structural integrity of proteins. It is involved in DNA synthesis, cell division, and protein synthesis, making it vital for growth and development. Additionally, zinc plays a critical role in maintaining immune system function.

1.3 The Study of Zinc Isotopes

1.3.1 Isotopic Analysis in Biological Systems

Isotopic analysis of zinc allows for a detailed study of zinc's pathways and transformations within biological systems. Zinc isotopes undergo redistribution during biochemical processes such as absorption, enzyme reactions, and cellular uptake. This fractionation leads to variations in isotopic composition [2]. Understanding these variations is vital for comprehending the intricate metabolic activities within organisms. For example, organs involved in zinc metabolism, like the liver and kidneys, may exhibit an enriched isotopic signature. In contrast, bones and teeth might show a depleted isotopic signature, indicative of the isotopic composition at the time of their formation.

1.3.2 Analytical Methodology for Zinc Isotopes

This study aims to accurately measure zinc isotopes by analyzing reference materials and mouse kidneys. Reference materials are crucial for validating analytical methods and ensuring accuracy [3]. By measuring zinc isotopes in these materials, methods can be refined, and a solid foundation for biological analyses can be established. The kidneys in mice are selected for their critical roles in zinc metabolism. The kidneys are essential for zinc excretion, making them ideal for studying zinc isotopic fractionation in biological systems [1].

1.4 Challenges in Isotopic Measurement

1.4.1 Spectral Interferences and Contamination Control

Accurate isotopic measurement in biological samples is fraught with challenges. Spectral interferences in mass spectrometry techniques like MC-ICP-MS can result in inaccurate isotopic ratios if not adequately corrected [2]. These interferences often arise from other isotopes and molecular species in the sample, necessitating the development of sophisticated correction techniques. Blanks and contamination from reagents or the laboratory environment can significantly affect the results, especially in samples with low zinc concentrations. Ensuring the purity of reagents and maintaining a controlled laboratory environment is thus imperative for reliable isotopic analysis.

1.4.2 Chemical Complexity of Biological Matrices

The chemical complexity of biological matrices also poses a significant challenge. The varying compositions and concentrations in biological samples can lead to matrix effects, affecting ionization efficiency and leading to additional interferences in isotopic measurements [3]. The extraction and purification of zinc from these matrices require meticulous and specialized techniques to ensure accurate isotopic analysis.

1.5 Advancements in Analytical Techniques

The calibration of instruments and validation of new reference materials are ongoing efforts to enhance the reliability of zinc isotopic data across various research facilities [3]. As analytical techniques advance and our understanding of zinc's geochemical behavior deepens, the accuracy and precision of zinc isotopic measurements in biological samples continue to improve. This is crucial for advancing our understanding of zinc's role in biological systems and exploring its potential implications in health and disease.

1.6 Outline of Thesis

Chapter 2 explores the role of zinc isotopes in understanding biological and environmental processes. It starts with an introduction to naturally occurring zinc isotopes and their significance in scientific research, highlighting their importance in health and environmental studies. The chapter then discusses the concept of isotope fractionation, covering mass-dependent mechanisms like equilibrium and kinetic processes that influence zinc's behavior in different contexts. It also examines how zinc isotopes are metabolized in the human body, their potential as disease biomarkers, and their varied applications across disciplines like biomedicine and environmental science. The literature review section provides insights into recent research findings on zinc isotopes, emphasizing their diagnostic and therapeutic potential. Finally, advanced analytical techniques, including the double spike method for precise isotopic measurement, are outlined to underscore the methodological approaches used in zinc isotopic studies.

Chapter 3 details developing and validating a refined analytical method for zinc isotopic analysis, emphasizing the challenges of achieving high precision due to the narrow range of natural zinc isotopic fractionation. It introduces advanced techniques like the double spike method and Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICP-MS), tailored to enhance accuracy and reduce contamination. The method's efficacy is validated using certified reference materials and applied to complex biological samples to ensure reliability across different testing scenarios. This approach significantly improves the precision of zinc isotopic measurements in biological systems.

Chapter 4 presents the results from the zinc isotopic analysis of biological samples, focusing on chicken liver from a local store and mouse kidneys from three experimental groups, validated against certified biological materials. The data confirm the method's reliability, highlighting the influence of spike ratios on the accuracy of zinc isotopic values. Detailed comparisons with certified reference materials demonstrate the method's precision, aligning closely with established literature values. The study also explores zinc isotopic variations in mouse organs. Overall, the validated method proves robust across various biological matrices, establishing a reliable framework for future isotopic analysis in biological and environmental studies.

Chapter 5 expands on the findings presented in Chapter 4, discussing the broader implications of the zinc

isotopic variations observed in chicken liver, certified reference materials, and mouse kidney samples. The chapter delves into the impact of methodological approaches, particularly addressing the challenges and solutions associated with blank, Ba^{2+} isobaric interference and the accuracy of isotope abundance. These elements are critically evaluated to suggest improvements and identify potential areas for future research, enhancing the reliability and application of zinc isotopic analysis in biological research.

Chapter 2

Isotopic Fractionation and the Application to Study Zinc Metabolism

2.1 Introduction to Zinc Isotopes

Zinc, an essential transition metal, is pivotal in various biological and environmental processes. An essential metal is necessary for the normal functioning of biological systems. Zinc is crucial for enzyme function and immune system support and acts as an antioxidant in biological systems [4]. It also contributes to nutrient cycling and indicates pollution in environmental contexts [5]. Furthermore, zinc's impact on human health is significant, influencing nutritional status and being involved in metabolic disorders [6]. Its isotopic composition, which includes several stable isotopes, offers a unique perspective for scientific investigation.

The naturally occurring isotopes of zinc are ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, and ^{70}Zn. ^{64}Zn is the most abundant, constituting approximately 48.63% of natural zinc, followed by ^{66}Zn at about 27.9%, ^{68}Zn at 18.75%, ^{67}Zn at around 4.1%, and ^{70}Zn at about 0.62% [1].

Despite being chemically identical, each zinc isotope has a unique atomic mass contributing to slight differences in physical and chemical behaviors. These isotopic variations are crucial for tracing biochemical pathways within organisms and understanding environmental geochemical cycles. For instance, isotopic differences can offer insights into reaction mechanisms and environmental processes due to the slight variations in how lighter and heavier isotopes react or move [2].

The isotopic analysis of zinc goes beyond merely measuring abundance; it aims to understand the subtle differences caused by variations in atomic mass and their implications. These differences affect how

isotopes participate in chemical reactions and biological processes. For example, lighter isotopes may react more quickly or escape from reaction sites more readily than heavier isotopes, influencing both enzymatic activities and metabolic pathways. In human health studies, variations in zinc isotopic composition in tissues can reveal information about an individual's nutritional status or metabolic disorders, providing critical insights into the mechanisms of disease [3]. Similarly, in environmental science, changes in zinc isotopic composition in soils or water can indicate sources of pollution or shifts in geochemical processes. This makes zinc isotopes invaluable in both detecting environmental issues and understanding health-related concerns, as they help elucidate complex biochemical and geochemical processes [3].

The isotopic analysis of zinc goes beyond merely measuring abundance; it aims to understand the subtle differences caused by variations in atomic mass and their implications. These variations influence how isotopes participate in chemical reactions and biological processes. Lighter isotopes, for example, may react more quickly or escape from reaction sites more readily than heavier isotopes, affecting biochemical pathways and environmental cycles. Understanding these causal relationships enhances our ability to trace nutrient cycling, monitor environmental pollution, and explore the underlying mechanisms of diseases linked to zinc metabolism.

Therefore, the study of zinc isotopes is instrumental in deepening our understanding of numerous biological and environmental phenomena, offering both quantitative data and qualitative insights into the complex mechanisms governing zinc's behavior [1; 2; 3].

2.2 Isotope Fractionation

Isotope fractionation refers to the variations in the relative abundances of isotopes of an element due to physical or chemical processes. This section focuses on zinc isotope fractionation to help understand the transformations zinc undergoes in various environments, categorized into mass-dependent and mass-independent mechanisms [7].

2.2.1 Mass-Dependent Fractionation

Mass-dependent fractionation encompasses both equilibrium and kinetic processes arising from differences in the physical properties of isotopes, primarily their mass. While the chemical properties of isotopes of the same element are nearly identical, their differing masses result in slight variations in their physical behavior. Equilibrium fractionation occurs when isotopes participate in reactions that reach thermodynamic equilibrium, minimizing the system's Gibbs free energy. In these processes, lighter isotopes, such as ^{64}Zn, often participate more readily due to their slightly higher kinetic energy, which makes them more reactive in certain conditions compared to heavier isotopes like ^{68}Zn and ^{70}Zn. This distinction is due to differences in how atoms form chemical bonds, where lighter isotopes are favored

because of their ability to move and react more quickly [8; 9].

Kinetic fractionation, on the other hand, involves differences in reaction rates based on mass. For instance, the lighter zinc isotope ^{64}Zn exhibits greater ability to move and reactivity compared to its heavier counterparts (^{66}Zn, ^{67}Zn, ^{68}Zn, and ^{70}Zn), allowing it to diffuse more quickly and escape from reaction sites more readily. This differential behavior of zinc isotopes due to their mass differences is fundamental to understanding their distribution and reactivity in various environmental and physiological contexts [10; 11].

In biological contexts, kinetic fractionation is predominant, affecting the isotopic composition of tissues through processes like enzymatic reactions and ion or molecule transport, which may exhibit mass-dependent preferences. Knowledge of these processes is integral to interpreting isotopic data across scientific disciplines, offering insights into the functioning of natural systems, historical climates, and complex metabolic pathways [12; 2].

Furthermore, diseases can significantly alter the isotopic composition of elements such as zinc in major organs through either depletion or enrichment of specific isotopes, potentially serving as biomarkers for disease diagnosis or monitoring progression. These alterations are mass-dependent processes. For instance, the altered isotopic composition of zinc in organs could indicate metabolic disruptions or organ-specific diseases [13; 14].

2.2.2 Zinc Metabolism and Isotope Fractionation in the Human Body

Zinc plays a crucial role in various biochemical processes within the human body. Its metabolism and the dynamics of its isotopes—particularly ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, and ^{70}Zn—provide vital insights into physiological processes and health outcomes [15]. As depicted in Figure 2.1, zinc is primarily absorbed in the small intestine (GI Absorption) and then transported to various tissues, with the liver playing a key role in its regulation [16]. Isotopic fractionation of zinc occurs as it traverses through different organs, leading to subtle variations in its isotopic composition [17]. Factors such as dietary sources, the efficiency of absorption, and organ-specific utilization influence this fractionation[18; 19].

In studies monitoring zinc isotopes, fractionation manifests as either kinetic or equilibrium processes. Kinetic fractionation is noted during the transport and utilization of zinc isotopes in the body, while equilibrium fractionation is observed when zinc binds to proteins and other molecules [20]. Organs such as the liver and kidneys exhibit distinct zinc isotopic signatures, indicative of their respective roles in metabolism and excretion [1]. For example, the liver, a central organ for zinc storage and detoxification, might reveal an isotopic signature that differs from that of the kidneys, which are integral to excretion and maintaining homeostasis [21; 20].

Understanding zinc isotope dynamics in the human body is critical for evaluating nutritional status, diagnosing diseases, and examining the environmental impact on human health. Analyzing the fractionation

patterns and delta values of zinc isotopes in various organs offers a deeper understanding of the intricate interactions and functions of zinc at the molecular level[16].

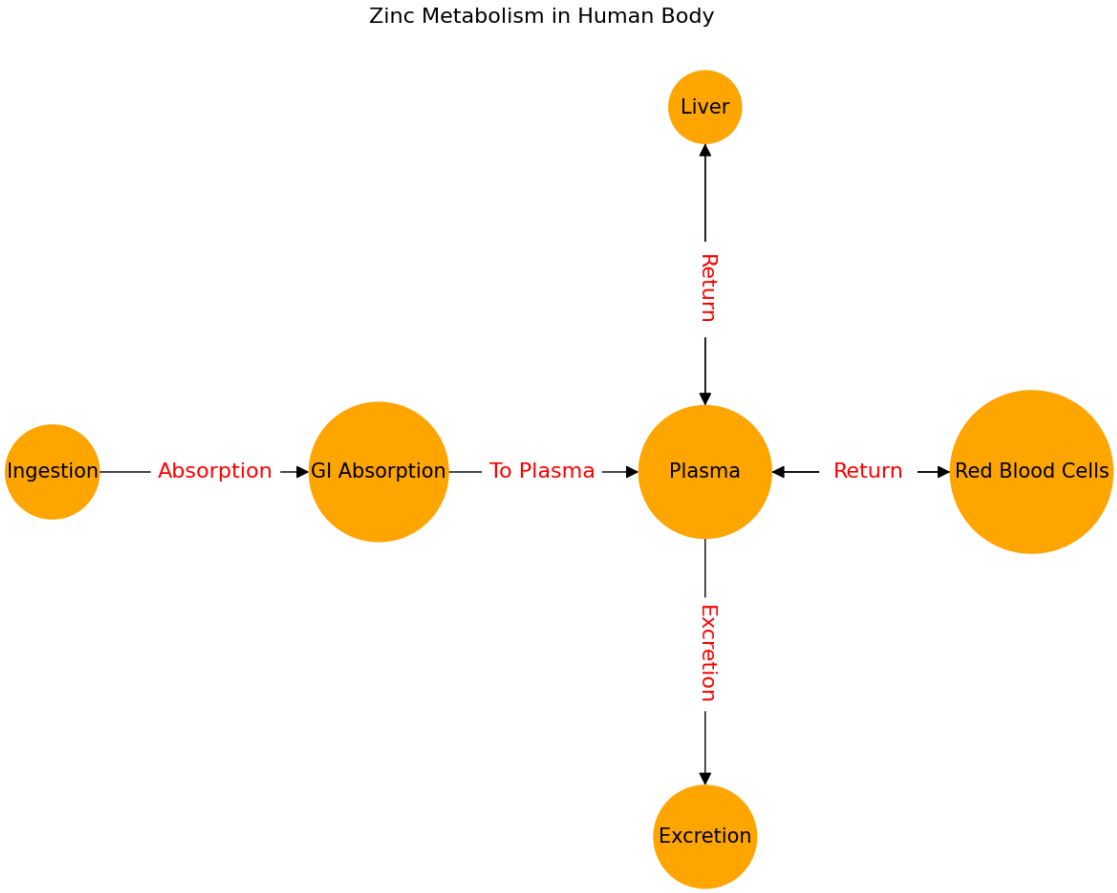


Figure 2.1: Overview of Zinc Metabolism in Humans.

2.3 Literature Review on Zinc Isotopic Composition

The diverse applications of zinc isotopes encompass several scientific fields, including environmental science, biomedicine, and geochemistry. Researchers explore stable zinc isotopes, notably ^{64}Zn and ^{66}Zn, for their diagnostic and therapeutic potential, their ability to trace environmental pollution, and their utility in understanding geochemical processes.

In the realm of biomedicine, the study of zinc isotopes offers promising advances. Moynier et al. [22] explored the shifts in zinc isotopic composition within the human brain, specifically focusing on how these changes correlate with the progression of Alzheimer's disease. They found that zinc isotopes in the brain become isotopically lighter as Alzheimer's disease advances. This depletion in the heavier isotopes occurs because the disease alters the brain's zinc metabolism, possibly through the dysregulation of zinc transporters and enzymes that depend on zinc, which are critical for maintaining brain health and cognitive function.

This nuanced observation—that zinc becomes isotopically lighter—could be indicative of specific pathophysiological shifts in Alzheimer's disease. For instance, lighter zinc isotopes might accumulate due to the increased expression of certain metal-binding proteins in the brain, which preferentially bind lighter isotopes. These proteins are often upregulated in response to oxidative stress and inflammation, common features of Alzheimer's pathology.

The detection of these lighter zinc isotopes provides a direct insight into the biochemical changes occurring in the brain, offering a more detailed understanding of the disease's impact on metal homeostasis. This kind of molecular-level information is crucial for developing targeted therapies that aim to correct or stabilize these metabolic disturbances. Thus, by tracking how zinc isotopes vary with disease progression, researchers can not only detect Alzheimer's earlier but also gain valuable insights into its underlying mechanisms, paving the way for interventions that address specific biochemical pathways altered by the disease.

Novak et al. [23] embarked on an in-depth exploration of the therapeutic potential of ^{64}Zn-enriched compounds, focusing on their cytotoxic effects against various cancer cell lines. The study meticulously assessed how these enriched zinc isotopes, when incorporated into specific compounds, could selectively induce apoptosis in cancer cells more effectively than in normal cells. This selective cytotoxicity is crucial as it allows for targeted cancer treatment, minimizing damage to healthy tissues—an enduring challenge in oncology. The enhanced apoptosis observed suggests that the isotopic composition of zinc not only affects cellular metabolism but can be harnessed to improve the efficacy of anticancer agents. By demonstrating significant differences in the response between cancerous and non-cancerous cells to zinc isotopes, Novak et al. [23] highlight the potential for isotopic variants of elements like zinc to be developed into novel anticancer drugs that offer more precise targeting capabilities. This research underscores the innovative approach of using isotopic enrichment as a strategy to refine and enhance cancer treatment modalities, potentially leading to therapies that are both more effective in eradicating cancer cells and better at sparing healthy tissues, thereby improving therapeutic outcomes and reducing side effects. The implications of these findings encourage further clinical investigation to validate and extend the use of zinc isotopes in oncological applications.

Schilling et al. [24] conducted comprehensive in vitro experiments to delve into the phenomenon of zinc isotope fractionation within breast cancer tissues, specifically examining the dynamics of zinc uptake and efflux in a human breast cancer cell line. Their research illuminated the distinct isotopic discrimination that occurs between healthy and cancerous cells, where isotopic variations of zinc could reflect underlying changes in cellular zinc metabolism associated with cancer progression. By analyzing how different zinc isotopes are absorbed and expelled by cancer cells compared to normal cells, the study provided insights into the molecular mechanisms that drive cancer development. This differential handling of zinc isotopes by cancerous tissues suggests a potential for using these isotopic signatures as biomarkers in cancer

diagnostics. The ability to detect subtle shifts in zinc isotopic composition in tissues could lead to novel diagnostic tools that are both non-invasive and highly specific, offering a promising avenue for early detection and ongoing monitoring of cancer. Schilling et al. [24] underscore the need for further research to validate and refine the use of zinc isotopic analysis in clinical settings, potentially transforming how cancers are diagnosed and managed by leveraging the natural isotopic properties of elements involved in critical biological processes.

Larner et al. [25] explored the use of zinc isotopes as potential biomarkers for breast cancer by focusing on natural isotopic compositions and their variations in biological systems. They highlighted how isotopic fractionation—slight differences in isotopic composition during biological processes—can reveal subtle metabolic changes associated with diseases like cancer. The study found that lighter zinc isotopes tend to accumulate in cancerous tissues due to alterations in zinc transporter proteins and metallothionein, which are prevalent in tumor cells. This isotopic pattern offers a unique, quantitative measure that could potentially distinguish between healthy and cancerous tissues, making zinc isotopes a promising tool for early detection. By detecting isotopic changes at the onset of the disease, zinc isotopes could identify cancer earlier than current biomarkers, improving diagnostic specificity and patient outcomes. This research suggests that integrating zinc isotopic analysis into routine diagnostic procedures could enhance the sensitivity of existing biomarker tests, offering a non-invasive, safe, and effective diagnostic tool for early cancer detection. Larner et al. [25] advocate for more interdisciplinary research to further develop the diagnostic potential of zinc isotopes in medical science.

In environmental sciences, zinc isotopes are invaluable for tracing pollution and understanding biogeochemical cycles. Wang et al. [3] used high-precision zinc isotopic characterization of soil samples to study the movement and transformation of zinc in various environments. This method effectively identifies isotopic signatures that distinguish between heavier and lighter isotopes of zinc, indicating different sources and pathways of zinc within the ecosystem. For instance, lighter isotopes of zinc might be prevalent in areas impacted by industrial emissions, reflecting selective release during industrial processes. In contrast, heavier isotopes might accumulate where natural geochemical processes, such as mineral interactions in deeper soil layers, dominate. This ability to pinpoint isotopic variations enables researchers to trace zinc contamination sources more accurately and understand how zinc cycles through ecosystems. Such insights are crucial for developing targeted environmental management strategies, enhancing pollution remediation efforts, and supporting informed policymaking to preserve environmental health.

Liang et al. [5] investigated zinc isotopes in suspended particulate matter from lakes in China to study the biogeochemical processes within these aquatic systems. Their research effectively utilized the distinct isotopic signatures of zinc to differentiate between the sources and transformations of zinc in the lake environments. This approach allows for the identification of lighter and heavier zinc isotopes, which are indicative of various environmental inputs and processes. For example, lighter zinc isotopes might indicate

anthropogenic influences such as industrial discharge, while heavier isotopes could be associated with natural geological processes in the lake beds. By mapping these isotopic patterns, Liang et al. were able to trace the pathways through which zinc enters and moves through aquatic systems, providing insights into the ecological dynamics at play. This knowledge is essential for understanding how environmental changes affect biogeochemical cycles in lakes, offering a basis for environmental monitoring and management strategies aimed at mitigating pollution and preserving aquatic ecosystems.

Araújo et al. [26] applied zinc isotope compositions in oysters to monitor and quantify anthropogenic zinc accumulation in marine environments, observing distinct isotopic signatures over four decades. Their research differentiated between heavier and lighter isotopes of zinc, which helped identify specific sources of zinc pollution. Lighter isotopes typically indicated more recent, anthropogenic sources such as industrial discharges, while heavier isotopes were associated with natural, geological sources. This nuanced understanding enhances traditional environmental monitoring by providing a more accurate method to trace and evaluate the impacts of pollution on marine ecosystems.

In geochemistry, the work of Xu et al. [27] focused on the behavior of zinc isotopes in mafic rocks during continental deep subduction, examining how isotopic stability varies under high-pressure conditions. Their study revealed that heavier isotopes of zinc tend to remain stable during these intense geological transformations, providing key insights into the recycling processes of crustal materials. Understanding these isotopic behaviors helps elucidate the broader zinc cycle within the Earth's crust and mantle, contributing to our knowledge of geological processes at great depths.

2.4 Double Spike

Isotopic fractionation during sample preparation and analysis occurs due to biases introduced during the sample introduction, ionization, and detection phases in mass spectrometry. These biases can stem from mass discrimination effects, matrix effects, and instrumental drift, all of which can alter the true isotopic ratios of a sample [28]. To ensure the accuracy, precision, and reliability of data, it is essential to perform internal calibration, which corrects these biases and compensates for factors.

Several methods are available for correcting the isotopic composition of zinc. Standard Sample Bracketing corrects for instrumental drift by analyzing known standards before and after the sample, allowing for compensation of any fluctuations in the instrument's performance over time [29]. Elemental Doping introduces elemental dopant of similar atomic mass to correct for mass bias, which helps to normalize the mass spectrometer's response and ensure more accurate isotope abundance ratio measurements [30]. In contrast, the Double Spike Technique employs two isotopic spikes to calculate the sample's true composition, thus addressing both fractionation and biases [31].

The double spike method involves adding a solution with a precisely measured combination of two Zn

isotopes, ^{64}Zn and ^{67}Zn, to the sample before the sample undergoes digestion and ion exchange during sample preparation. This process is illustrated in Figure 2.2, where the isotopic abundance in a sample is plotted with that in the Zn double spike and then shown combined in the mixture graph. The known quantity of the double spike in the solution serves as a reference, allowing for the correction of any isotope fractionation that may occur during chemical separation and isotopic measurement processes. Furthermore, the addition of the double spike enables the normalization of the measured isotopic ratios, a crucial step for correcting any instrumental or procedural biases. By optimizing the spike proportions within a range of 45% to 55%, uncertainties in the calculated natural fractionation factor (α) are minimized. The double spike method is particularly valuable for measuring the precise isotopic composition of small sample quantities, such as those less than 1 milligram, making it an excellent internal calibration method for limited samples [32].

The same mass-dependent and mass-independent effects that occur during biochemical processes also influence the analytical phase when analyzing a sample. The use of the double-spike method ensures that these effects are accounted for, providing consistent and reliable measurements, which is crucial for producing accurate data.

The double spike solution, with its established isotopic ratios, is used as a calibration standard. Adding this solution to the sample helps correct any isotopic fractionation that occurs during sample preparation and isotopic analysis. This correction is realized through a series of equations. Equation 2.1 adjusts the measured isotopic ratios of the spike $R_{s,i}$ and the mixture $R_{M,i}$ by applying the respective mass bias factors (α), which are instrumental in determining the zinc sample's true isotopic composition. Equation 2.2 then applies the factor β to adjust the isotopic ratios of the mixture $R_{M,i}$. This factor β represents the analytical mass fractionation that occurred during sample preparation and analysis. In these equations, $m_{a,i}$ represents the mass of the analyte isotope, and $m_{a,ref}$ the mass of the reference isotope used for calibration:

$$r_{s,i} = R_{s,i} \left(\frac{m_{a,i}}{m_{a,ref}} \right)^{\alpha} \tag{2.1}$$

Equation 2.2 is utilized to compute the true isotopic ratio of the mixture ($r_{M,i}$).

$$r_{M,i} = R_{M,i} \left(\frac{m_{a,i}}{m_{a,ref}} \right)^{\beta} \tag{2.2}$$

After the measured isotopic ratio has been calculated by Equation 2.2, the delta value δ^{66}Zn in parts per thousand (‰) is calculated by Equation 2.3:

$$\delta^{66}\mathrm{Zn}(‰) = \left(\frac{(^{66}\mathrm{Zn}/^{64}\mathrm{Zn})_{\mathrm{sample}}}{(^{66}\mathrm{Zn}/^{64}\mathrm{Zn})_{\mathrm{standard}}} - 1 \right) \times 1000 \tag{2.3}$$

The delta (δ) notation is used to express the isotopic composition of a sample relative to a standard[33]. It is expressed in parts per thousand (‰), also known as per mil, and indicates the relative difference between the isotopic ratios of a sample and a reference standard. This notation magnifies small differences in isotopic ratios, making it easier to detect and interpret isotopic variations. A positive δ^{66}Zn value indicates that the sample has a higher (^{66}Zn/^{64}Zn) ratio than the standard, meaning the isotopic composition is heavier. Conversely, a negative δ^{66}Zn value indicates a lower (^{66}Zn/^{64}Zn) ratio than the standard, meaning the isotopic composition is lighter.

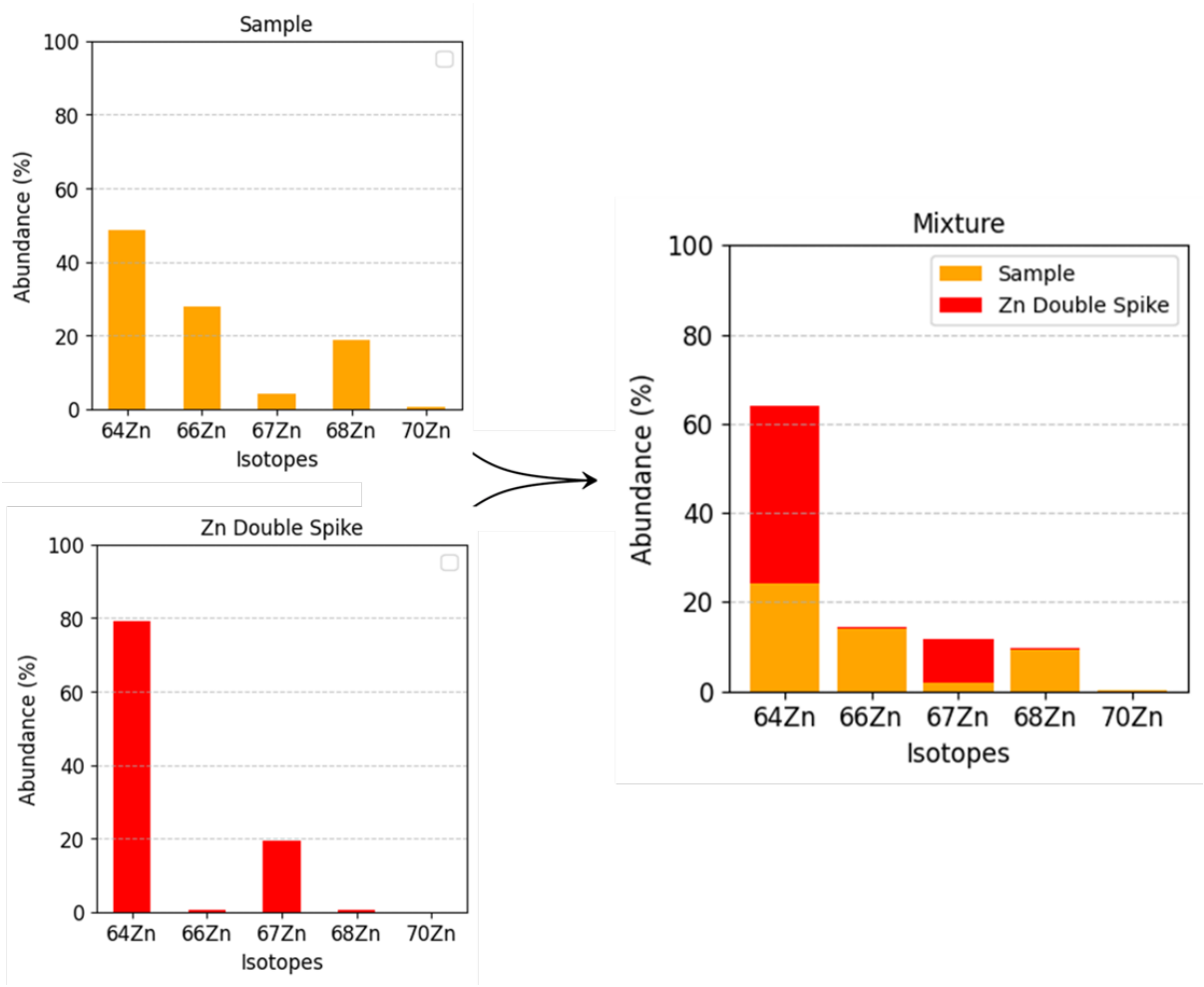


Figure 2.2: Distribution of Zinc Isotopes in the Sample, Zn Double Spike, and Their Mixture.

2.5 Mouse Samples and the Role of the Gut Microbiome

The gut microbiome, consisting of bacteria, fungi, viruses, and other microbes within the digestive tract, plays a crucial role in physiological processes such as digestion, nutrient synthesis, immune regulation, and pathogen defense. The intricate interactions between the human genome and the gut microbiome emphasize the role of human genes in nutrient digestion, immune regulation, and host metabolism [34]. Gut bacteria produce essential metabolites and modulate immune responses, contributing significantly to overall health [35]. The microbiota also plays a vital role in maintaining the intestinal barrier, which is crucial for gut integrity and function. Furthermore, the symbiotic relationship between gut microbes and their host supports digestive processes and immune health, highlighting the mutual benefits and protection against pathogens provided by this relationship [36]. This underscores the crucial role of the gut microbiome in various physiological processes and its potential for therapeutic modulation.

While this introduction lays the groundwork for understanding the significance of the gut microbiome in physiological and metabolic studies, the primary focus of this thesis is not on the interactions within the microbiome itself. Instead, it emphasizes the necessity of developing precise and reliable analytical methods tailored to meet the specific challenges of measuring zinc isotopes in organic materials, including those influenced by the microbiome. The following sections will therefore concentrate on detailing the advanced isotopic measurement techniques and methodologies that are critical for accurate assessments in such complex biological matrices.

Chapter 3

Method Development

This chapter outlines developing and applying an analytical method designed to explore zinc isotopic fractionation in biological systems. Accurately measuring zinc isotopes presents distinct challenges due to the inherently narrow range of natural mass-dependent fractionation in zinc, typically confined to ±1‰. Such precise measurements demand exceptionally high reliability and sensitivity from analytical techniques. This chapter aims to detail both the development and the validation of a method that enhances precision and minimizes errors in zinc isotopic analysis. It addresses significant gaps in current methodologies by offering improved strategies for managing low blank levels and ensuring reproducibility. The contributions made by this study extend the capabilities of zinc isotopic analysis and have the potential to significantly advance our understanding of zinc dynamics in biological contexts.

3.1 Development of the Analytical Method

To address the challenges, including the inherently narrow range of natural mass-dependent fractionation in zinc, the need to minimize contamination throughout the analytical process, and the complexity of biological matrices that contain interfering elements, the analytical method was refined to include double spike as a calibration technique and Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICP-MS). The double-spike method for internal calibration has significantly improved reproducibility, achieving precisions up to 0.1‰. This method also keeps blank levels below 5 ng and achieves a yield recovery of 95% during ion exchange processing. The use of Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICP-MS) is critical in this methodology, enabling precise isotope ratio measurements and effectively compensating for potential mass biases introduced by the instrumentation and sample preparation processes. The enhanced double-spike method, as a robust calibration strategy, is essential in standardizing measurements and ensuring the reliability of results.

3.2 Zinc Isotopic Analysis in Certified Reference Materials (CRMs) and Mouse Models

The efficacy of this analytical method was initially validated using Certified Reference Materials (CRMs) such as Bovine Liver (NIST1577c), Human Hair (GBW07601), Whole Blood Serum (SeroNorm), Bone Ash (NIST1400), and Bone Meal (NIST1486), Human Hair (USGS42), Human Hair (USGS43). These CRMs, being certified materials with known isotopic compositions, are crucial for verifying the accuracy and precision of the analytical techniques, thereby ensuring the validity of the method. The complexity of biological matrices, which include diverse mixtures of proteins, fats, and other organic and inorganic substances, poses significant challenges in zinc isotopic analysis. These complexities can alter the binding affinities for zinc, impacting the isolation and quantification of zinc isotopes. Moreover, biological matrices often contain interfering substances that require sophisticated separation techniques to effectively isolate zinc.

Table 3.1 lists the certified reference materials used for validation, providing information about their source, institution, and matrix.

Table 3.1: Certified Reference Materials

Biological reference material	**Source Institution**	**Matrix**
NIST SRM 1577c	NIST	Bovine Liver
SeroNorm Whole Blood L-3	SERO AS, Billingstad, Norway	Whole blood serum
GBW07601	National Research Centre for Certified Reference Materials (CNRM), Beijing, China	Human Hair
GBW09101	JRC - Joint Research Centre, Geel, Belgium	Human Hair
ERM-DB001	U.S. Geological Survey Reston Stable Isotope Laboratory	Human Hair
USGS42	U.S. Geological Survey Reston Stable Isotope Laboratory	Human Hair
USGS43	U.S. Geological Survey Reston Stable Isotope Laboratory	Human Hair

Following the development of the method using chicken liver samples obtained from a local grocery store (see Section 4.1), its accuracy and precision were thoroughly validated using Certified Reference Materials (CRMs) (see Section 4.2). Subsequently, the method was applied to mouse kidney samples provided by the Keith Sharkey Lab to evaluate its efficacy in measuring zinc isotopic composition in complex

biological matrices (see Section 4.3). It is important to note that the liver and kidney samples did not target specific regions within the organs. Instead, each organ was homogenized in its entirety to ensure a representative sample of the whole organ. This approach was chosen to mitigate variability that might arise from sampling specific regions, which could lead to inconsistent or non-representative isotopic data. This step is crucial to ensure that the isotopic measurements provide a comprehensive overview of the zinc distribution within the organs, rather than localized variations.

3.3 Sample Preparation

3.3.1 Reagents, Materials, and Cleaning Procedures

Biological samples typically exhibit a very narrow range of Zn isotopic compositions. For example, kidneys in various species, including mice, pigs, and sheep, generally show δ^{66}Zn values outside the typical range of -0.4‰ to +0.2‰. Based on the data provided, mice kidney tissues have δ^{66}Zn values ranging from -0.37‰ to 0.05‰, pig kidney tissues show δ^{66}Zn values from -0.73‰ to -0.55‰, and sheep kidney tissues exhibit δ^{66}Zn values from -0.37‰ to -0.02‰. The typical δ^{66}Zn values for liver tissues in species such as mice range from -0.27‰ to -0.05‰, indicating some variation in Zn isotopic compositions across different species and tissue types.[33; 37; 38].

Contamination can shift these delta values outside their expected ranges, leading to incorrect biological interpretations. Thus, controlling Zn contamination is essential throughout the digestion, ion exchange, and measurement processes. All sample preparation procedures were performed in a Class 1000 cleanroom on a lab bench equipped with a Clean-Cell fan filter module (HEPA filter station) to prevent contamination. This setup helps control contamination from airborne particles and other environmental factors. Cleanroom water, purified to 18.2 MΩ cm by a Milli-Q water polishing system, along with analytical reagent-grade nitric acid (65% w/w, Aristar Plus, VWR International), underwent single and double sub-boiling purification, using a DST-1000 Savillex sub-boiling distillation system (Eden Prairie, MN, US). Additionally, ultra-pure water, hydrochloric acid (Aristar Ultra, VWR International, Mississauga, Ontario, Canada), and double sub-boiled nitric acid were purified using the same sub-boiling distillation system. These reagents were utilized for sample preparation and Zn purification. Moreover, to further prevent contamination, all labware listed in Table 3.2 was soaked in 6 mol L^{-1} HCl for two weeks to remove any excess Zn and washed ten times with Milli-Q water before use. Specifically, the frits from Poly-Prep® Chromatography Columns underwent an extended soaking period in 6 mol L^{-1} HCl for four months to ensure thorough decontamination. AG MP-1 resin was also cleaned with 0.5 ml of 1 mol L^{-1} HCl for at least two weeks, achieving a Zn blank of less than 2 ng. The Sr-Spec resin was cleaned with 0.5 ml of 1 mol L^{-1} HCl for one week, resulting in a higher Zn blank of 76 ng. This detailed and specific cleaning of resins and frits highlights the importance of meticulous preparation in separation

and purification processes to prevent Zn contamination, ensuring that zinc and other metallic impurities do not skew isotope abundance measurements. Furthermore, acid baths for columns, pipette tips, and sample tubes must be replaced every three months. Over time, these baths can accumulate zinc and other contaminants. Regular replacement helps maintain a contamination-free environment. The cleanroom's bench, evaporation unit, and weighing scale were cleaned with cleanroom wipes (VWR International) and MQ water to minimize the risk of contaminating contaminants from previously digested or prepared samples and dust.

Table 3.2: Zn Blank Levels in Various Laboratory Materials and Reagents

Labware	**Brand**	**Zn blank (ng)**
AG MP-1 resin (washed)	Bio-Rad	0.13
50 ml tubes (acid washed and rinsed 10x with MQ water)	DigiTUBE, SCP Science	0.18
1.5 ml tubes (acid washed and rinsed 10x with MQ water)	SafeSeal Microcentrifuge	0.47
20 μl pipette tip (acid washed and rinsed 10x with MQ water)	Fisherbrand, Fisher Scientific, USA	0.46
1 ml pipette tip (acid washed and rinsed 10x with MQ water)	Fisherbrand, Fisher Scientific, USA	0.47
Polyethylene gloves	VWR International	30.45
Polyethylene gloves	Frutti Treasure	4.69
3% HNO_3	Aristar, VWR	0.37
0.1 mol L^{-1} HNO_3	Aristar Ultra, VWR International, Mississauga, Ontario, Canada	0.14
0.5 mol L^{-1} HCl	Aristar Ultra, VWR International, Mississauga, Ontario, Canada	1.5
6 mol L^{-1} HCl	Aristar Ultra, VWR International, Mississauga, Ontario, Canada	1.4
MQ water	-	0.06
1 mol L^{-1} HCl	Aristar Ultra, VWR International, Mississauga, Ontario, Canada	0.16
0.01 mol L^{-1} HCl	Aristar Ultra, VWR International, Mississauga, Ontario, Canada	0.16
Concentrated HCl	Aristar Ultra, VWR International, Mississauga, Ontario, Canada	0.05
Bath for columns (6mol L^{-1} HCl)	Aristar Ultra, VWR International, Mississauga, Ontario, Canada	2
Aristar Ultra water	Aristar Ultra, VWR International	0.06
Orange medical tube	-	3.57
Green medical tube	-	2
Poly-Prep® Chromatography Columns frits (washed)	Bio-Rad	2
Poly-Prep® Chromatography Columns frits (unwashed)	Bio-Rad	111

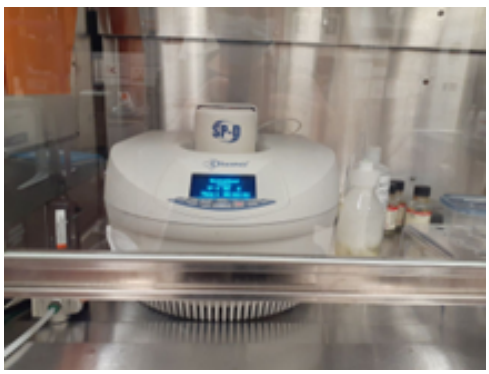
To track contamination, three sets of blanks were measured in each set of measurements:

- The digestion blank
- The Zn ion exchange blank
- The Ba^{2+} removing ion exchange blank

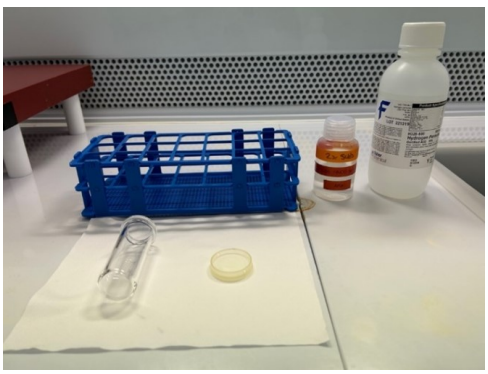
These blanks help determine the amount of Zn added to the sample during digestion and ion exchange, originating from frits, acids, sample tubes, and resin.

3.3.2 Sample Digestion Using Microwave Digestion

The initial step in measuring isotopic composition is digesting the sample, which is essential for effectively releasing zinc ions from biological matrices. Microwave digestion is preferred over traditional hotplate digestion due to its ability to complete digestion rapidly. It can reach full decomposition at 200°C in just four minutes—significantly faster than traditional hotplate methods, which can take up to 12 hours. Microwave digestion utilizes high-frequency electromagnetic waves to generate internal heat within the sample rather than through external heating of the container [39]. This technique provides uniform heat distribution and rapid temperature increase, leading to efficient breakdown of the sample matrix [40]. The generated heat causes the solvent in the sample to vaporize, creating pressure within the sealed vessel [41]. This pressure, combined with high temperatures, facilitates the rapid decomposition of organic and inorganic components in the biological matrices, thereby preventing partial digestion that could affect isotopic abundance ratios and yield inaccurate results[42]. This method is crucial for preventing sample contamination and loss, directly contributing to the accuracy of isotopic analysis. The sealed vessel setup reduces the risk of external contamination and sample loss, ensuring that the isotopic composition truly represents the sample's original concentration and isotopic composition. Such controlled conditions make microwave digestion particularly valuable for biological samples such as liver and kidney tissues, which require precise digestion to achieve accurate isotopic analysis [43],[44].



(a)



(b)

Figure 3.1: (a)Microwave Synthesizer Discover (CEM Corporation, Germany) and (b) 35 mL quartz vials

Samples, ranging from 100 to 500 mg based on their concentration, were also weighed and placed into 35 mL Quartz digestion vessels. Initially, 500 μl of sub-boiled concentrated nitric acid (w(HNO_3) = 67–70 %, Aristar Plus, VWR International) was added to each sample. After 10 minutes, 500 μl of hydrogen peroxide (w(H_2O_2) = 30 %, Fisher Scientific,122 Ottawa, Canada) has been added to the solution to further aid in the dissolution of organic materials and the breakdown of complex compounds, facilitating the release of zinc ions. Once prepared, the Quartz digestion vessels are securely sealed with caps provided by CEM Corp. (Kamp Lintfort, Germany), as shown in Figure 3.1(b). These caps and vessels undergo a rigorous cleaning process that includes a two-step acid cleaning with sub-boiled nitric acid, followed by leaching in hydrochloric acid (c(HCl) = 6 mol/L) for at least three days to ensure they are free from potential contaminants. After sealing, the samples were subjected to microwave digestion according to the specific conditions detailed in Table 3.3, as shown in Figure 3.1(a).

Table 3.3: Microwave Digestion Parameters

Power	**Temperature**	**Pressure**	**Ramp Time**	**Hold Time**
300 W	200°C	300 psi	4 min	3 min

After digestion, the solution was cooled and transferred to a 50 ml pre-cleaned sample tube (DigiTUBE, SCP Science) spiked with ^{64}Zn and ^{67}Zn isotopes and left to equilibrate for two hours to ensure even isotope distribution. It is crucial that during this period, the container remains sealed to prevent contamination or loss of sample. The solution was then dried in a closed DigiPREP unit (with a HEPA filter) to concentrate the zinc, resulting in a residue ready for ion exchange.

3.3.3 Ion Exchange for Zinc Isotope Analysis

The ion exchange process for zinc is a critical step in sample preparation for accurately measuring Zn isotopic compositions. This method isolated Zn from a complex matrix, resulting in a final solution that exclusively contained Zn isotopes. Such isolation significantly enhanced the precision of isotopic measurements by effectively removing isobaric interferences, which included ions from different elements that share the same mass-to-charge ratio as Zn, such as $^{138}Ba^{2+}$ and $^{64}Ni^{+}$, as demonstrated in Table 3.4. Eliminating these interferences ensures that the measured signal originated solely from Zn isotopes, thereby reducing potential inaccuracies in isotopic analysis. Cation ion exchange is specifically chosen over anion exchange due to zinc's properties in aqueous solutions, where it exists as a positively charged cation (Zn^{2+}), naturally suited for separation by cation exchange resins designed to attract and bind positively charged particles. To achieve this isolation, AG MP-1 Bio-Rad resins with a mesh size of 100-200 were utilized because of their ability to bind positively charged Zn^{2+} ions effectively. Before the resin is used for ion exchange, it is washed with 0.5 mol L^{-1} HCl and placed in a cleaned sample tube (DigiTUBE, SCP Science), which is fully covered with 0.5 mol L^{-1} HCl and allowed to sit for two days to

facilitate Zn leaching. After this period, the acid is drained, and the resin is washed three times with MQ water. This process, including the acid treatment and subsequent washings, is repeated over two weeks to minimize Zn contamination by reducing the amount of Zn in the resin. As depicted in Figure 3.2 and detailed in Table 3.4, the ion exchange setup involves several acid-cleaned glass micro-columns arranged on a laboratory bench. Each column is filled with 250 μL of the prepared resins. The digested and dried sample is then redissolved in 1 mol L^{-1} HCl and processed through these columns, ensuring accurate and precise measurement of zinc isotopic compositions by effectively removing potential contaminants and interferences. This setup not only guarantees the purity of the isolated zinc but also maintains the integrity of isotopic data. One essential quality control step is verifying the recovery of zinc after ion exchange. The zinc recovery involves assessing whether the recovered amount of zinc closely matches the amount added initially, which is crucial for confirming the accuracy of the recovery process. Ion exchange can lead to isotope fractionation, where zinc isotopes are separated or enriched at varying levels. To monitor this, a solution containing exactly 500 ng of a zinc standard (PCal, PlasmaCAL, SCP Science) is subjected to ion exchange, followed by spiking with an additional 500 ng of Double Spike. This spiking helps correct any fractionation effects, ensuring that the final isotopic ratios faithfully represent those of the original sample. Moreover, two column blanks—without any samples but processed similarly through ion exchange—are spiked with 500 ng of Double Spike to control for contamination. This method checks for any extraneous zinc introduced during the ion exchange process. Finally, all solutions are dried in a closed evaporation unit before undergoing a second ion exchange, further refining the precision of the zinc isotopic analysis.

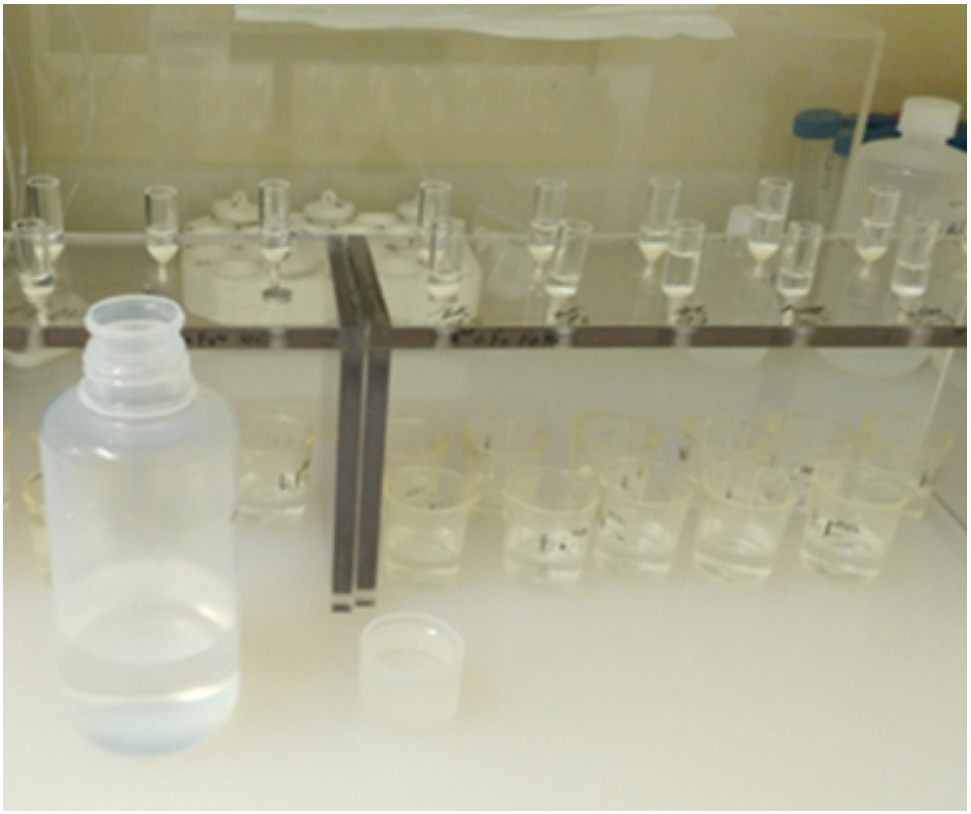


Figure 3.2: An ion exchange setup for sample preparation in isotopic analysis

Table 3.4: Zn ion exchange procedure

Step	**Volume**	**Eluant**
Load pre-cleaned AG MP-1	250 μl	-
Resin cleaning	2 ml	0.1 mol L^{-1} HNO_3
Resin cleaning	2 ml	MQ water
Column conditioning	1 ml	6 mol L^{-1} HCl
Equilibrating	2 ml	1 mol L^{-1} HCl
Sample loading	1 ml	1 mol L^{-1} HCl
Matrix elution	8 – 12 ml	1 mol L^{-1} HCl
Zn elution	6 ml	0.01 mol L^{-1} HCl

One of the significant challenges in zinc isotopic measurement is the presence of barium (Ba^{2+}) in the samples, often due to dust contamination during sample preparation. This contamination can occur during several stages, including ion exchange—when the sample is exposed to air for approximately eight hours during evaporation—and measurement processes conducted in a clean room environment. Samples are considered to have a high Ba content if they contain barium in quantities exceeding about 0.2 nanograms, equivalent to a 1 mV reading on the $^{138}Ba^{2+}$ signal during a Ba mass scan [45]. To ensure the reliability of the results, additional ion exchange processes are implemented to reduce the Ba content

to less than 2 ng. To achieve the removal of Ba^{2+}, 300 μL of Sr-Spec resin, previously cleaned with 0.5M HCl, was utilized. The cleaning protocol for the Sr-Spec resin is similar to that for the AG MP-1 resin, involving a comprehensive two-week pre-use cleaning process. This thorough preparation is crucial for effectively eliminating Ba^{2+} from the samples, thereby preventing any potential interference with the Zn isotopic measurements. The detailed steps for the Ba^{2+} removal ion exchange process are outlined in Table 3.5.

Table 3.5: Ba^{2+} ion exchange procedure

Step	**Volume**	**Eluant**
Load Sr-Spec resin	300 μl	-
Resin cleaning	two column volumes	3 mol L^{-1} HNO_3
Sample loading	0.5 ml	3 mol L^{-1} HNO_3
Zinc elution	250 μl	3 mol L^{-1} HNO_3

In addition, two column blanks are processed through the ion exchange and spiked with 500 ng of Double Spike to monitor for any zinc added to the samples during the Ba removal ion exchange. After the Ba ion exchange, the solutions are dried and capped, ensuring precise control over the sample preparation and analysis process.

3.4 MC-ICP-MS Operation and Calibration

3.4.1 Instrumentation

The Multi Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICP-MS) analysis in this study was conducted using the Thermo-Fisher Neptune system. This instrument, selected for its sensitivity and precision, is particularly effective at detecting zinc isotopes in samples with low concentrations. This effectiveness is due to its advanced features, such as high-resolution capabilities, low detection limits, and the ability to simultaneously measure multiple isotopes, which collectively enhance the accuracy and reliability of isotopic measurements. Regular calibration using certified reference zinc standard (PCal, PlasmaCAL, SCP Science) ensures that measurements are maintained within specified tolerances and are traceable to recognized standards. Such calibration is crucial for validating the reliability of isotopic ratio data.

3.4.2 MC-ICP-MS Configuration

The Thermo-Fisher Neptune MC-ICP-MS, depicted in Figure 3.3[46], is a state-of-the-art instrument for zinc isotopic analysis. Utilizing inductively coupled plasma (ICP) activated by argon gas and a radio frequency coil, it efficiently transforms aerosol-introduced samples into high-temperature plasma. The ions

are focused into a beam and sorted by their mass-to-charge ratio within the mass spectrometer. Equipped with nine ion collectors, the system excels at measuring various isotopes simultaneously, significantly enhancing the efficiency, precision, and accuracy of isotopic ratio determinations[47].

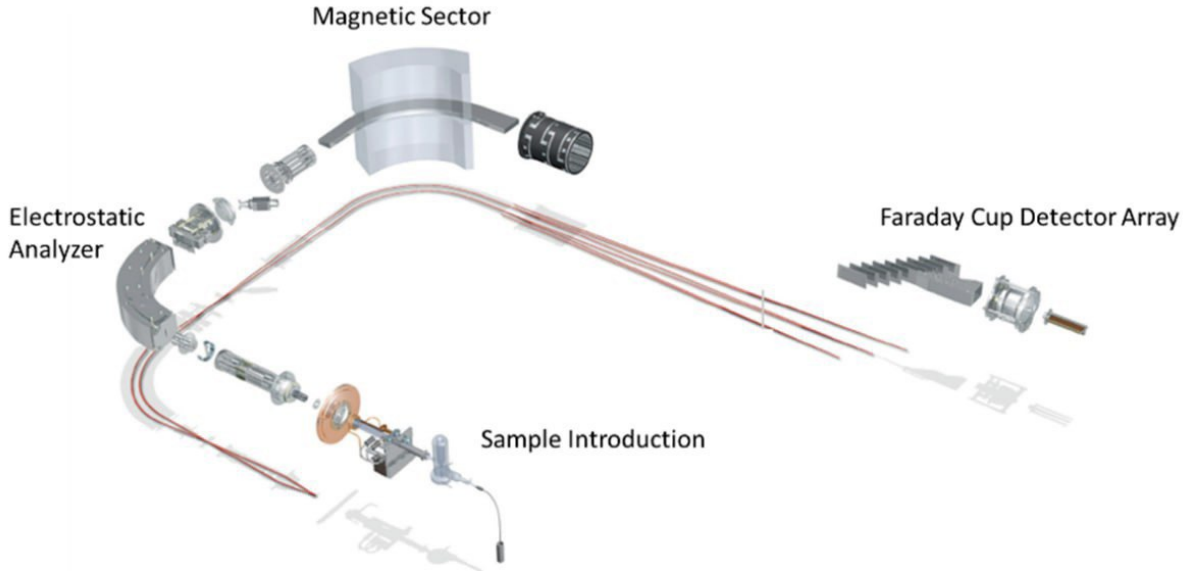
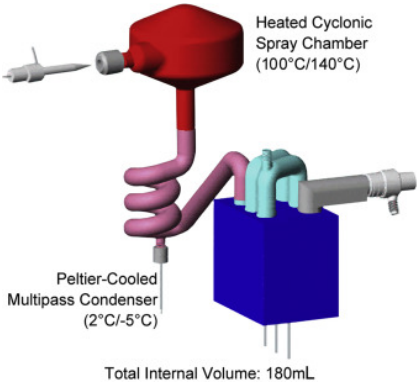
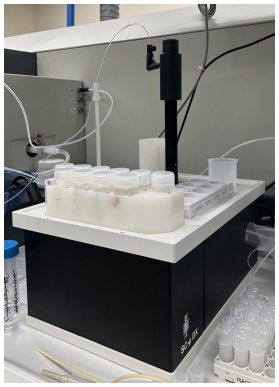


Figure 3.3: Thermo Fisher Scientific™ MC-ICP-MS Internal Components Schematic. Schematic representation of the internal components of the MC-ICP-MS, illustrating the path from the sample introduction system through the ionization process in the ICP source, followed by the magnet's separation of isotopes, and concluding with the multiple ion collectors for simultaneous measurement of isotopes.



(a) Heated cyclonic spray chamber and Peltier-cooled multipass condenser. From Elemental Scientific Inc[48]

(b) Apex desolvating nebulizer. From Elemental Scientific Inc. [49]



(c) ESI SC2-DX auto-sampler. From Elemental Scientific Inc.

Figure 3.4: Components involved in sample introduction in the Apex desolvating nebulizer. Here, a heated cyclonic spray chamber, a Peltier-cooled multipass condenser, and the ESI SC2-DX auto-sampler play crucial roles in ensuring sample nebulization and transportation into the plasma.

Key features that enhance its reproducibility include a multiple collector array, which minimizes short-term instrument fluctuations, and high-precision mass analyzers that ensure measurement accuracy. A stable ion source provides consistent plasma generation, which is essential for reproducibility. Robust calibration procedures involve regularly using known standards, while advanced data processing software

corrects data set anomalies, ensuring measurement reliability. These capabilities are crucial for precisely determining isotopic ratios, even at low concentrations, and contribute to understanding zinc's intricate transport and distribution processes across various environmental settings. Exceptional reproducibility is evidenced by δ^{66}Zn values in soil reference materials showing minimal variability, better than 0.06‰ (2SD), and long-term reproducibility for δ^{66}Zn in geological certified reference materials reported as better than ±0.03‰ (2SD), underscoring the instrument's consistent performance over time [3]. Precisions of up to 0.05‰ have been achieved, demonstrating this instrument's high level of accuracy.

To further optimize isotopic analysis, particularly for samples prone to contamination, such as zinc isotopes, the ESI SC2-DX auto-sampler from Elemental Scientific is employed, depicted in Figure 3.4. This setup includes a PFA micro-flow nebulizer operating at a 50 μl/min flow rate, chosen to minimize the required sample volume and eliminate the need for N_2 add-gas, which could introduce interference. The incorporation of the ESI Apex-HF desolvation system significantly increases sensitivity by 3 to 10 times, which is invaluable for analyzing samples with lower concentrations. Additionally, to prevent the memory effect associated with the Double Spike, the spray chamber was rinsed for 5 minutes between each sample measurement, with the signal monitored to ensure there were no contamination or memory effects. The ESI Apex-HF's efficient rinse-out properties, combined with high sensitivity, ensure higher signal stability, minimize memory effects, and reduce solvent-based background noise due to its inert, o-ring-free flow path. These properties are crucial for achieving precise and accurate zinc isotopic measurements, mainly when dealing with limited volumes of zinc isotope samples.

In the context of zinc isotope analysis, MC-ICP-MS enables the determination of zinc isotopic composition by measuring the relative amounts of different isotopes (^{66}Zn/^{64}Zn, ^{67}Zn/^{64}Zn, ^{68}Zn/^{64}Zn, ^{70}Zn/^{64}Zn). During the analysis of Zn isotope abundance ratios, the MC ICP-MS typically operates under specific parameters: Power is set at 1200 W; Cool gas flow is maintained at 15 L/min; Auxiliary Gas flow ranges from 1.00 to 1.14 L/min; Sample Gas flow varies from 1.00 to 1.119 L/min; the Guard Electrode is kept on; Focus is adjusted between -600 to -800 V; Shape voltage is set between 200 to 220 V; X-position is set between -0.080 to -2.5 mm; Y-position is set from 2.14 to 2.46 mm; and Z-position is adjusted from -5.00 to -4.270 mm. To ensure the amount of Ba in our samples is under control, a cup is set at 69 mass, which is for $^{138}Ba^{2+}$, having the highest abundance of Ba isotopes. The signal intensity of $^{138}Ba^{2+}$ should be less than 1 mV, equivalent to 2 ng [45]. When $^{138}Ba^{2+}$ exceeds 1 mV, an observed shift in δ^{66}Zn values toward heavier δ^{66}Zn values, indicating a false enrichment in heavier isotopes.

Table 3.6: MC-ICP-MS Cup Configuration for Zinc and Barium Isotopes: The table lists cup assignments, natural isotope abundances, and typical ion beam intensities for each detected isotope.

Mass	**64**	**66**	**67**	**68**	**69**	**70**
Cup	**L2**	**C**	**H1**	**H2**	**H3**	**H4**
	^{64}Zn	^{66}Zn	^{67}Zn	^{68}Zn	^{138}Ba^{2+}	^{70}Zn
Abundance (%)	49.2(75)	27.7(98)	4.1(16)	18.5(63)	71.7(29)	0.61(10)
Typical ion beam intensity (V)	6.05	3.64	5.47	2.560	< 0.001	9.26e-002

The sample solution obtained after ion exchange or purification is introduced to the ICP, where it is atomized and ionized. The resulting zinc ions are then transported into the mass spectrometer, separated based on their mass-to-charge ratio. Multiple ion collectors are used to simultaneously measure the ion beams of different zinc isotopes. The measured ion beam intensities are converted into isotopic ratios, further corrected for instrumental and mass bias effects. Standard reference materials with known isotopic compositions are typically analyzed alongside the samples to ensure the accuracy and calibration of the measurements [50].

On measurement days, samples are treated with 100 μl of concentrated nitric acid, dried and redissolved in 1 ml of 3% nitric acid for at least two hours. They are then moved to pre-cleaned Teflon auto-sampler tubes for analysis.

3.4.3 Instrument Calibration

The calibration of the Thermo-Fisher Neptune MC-ICP-MS is a critical process, as it is the key to accurate zinc isotope ratio measurements. Mass fractionation, which can alter the measured zinc isotope ratios from their true values, is often caused by space charge effects—where positively charged ions within the plasma accumulate and repel each other, altering their trajectories and velocities and impacting measurement precision and accuracy. Additionally, supersonic gaseous expansion, which occurs when gases expand from a high-pressure to a low-pressure environment at speeds exceeding the speed of sound, can favor the transmission of heavier isotopes due to differential transmission efficiency based on mass.

Calibration begins by establishing the peak center using the IRMM-3702 zinc solution, ensuring that the mass spectrometer's detectors align precisely with the targeted ions' mass-to-charge (m/z) ratio. This alignment is crucial to avoid partial signal detection, which could skew isotopic ratios significantly. Figure 3.5 illustrates the effectiveness of the instrument's ion beam focusing, showing how a well-focused ion beam produces symmetrical peaks, minimizing spread that could lead to peak tailing or broadening. Well-defined peaks indicate sufficient resolution, which is essential for accurately differentiating between isotopes in this mass range.

Stable plasma, achieved by adjusting auxiliary and sample gas flows and optimizing the position of the torch, is essential in plasma-based systems like ICP-MS. This stability ensures consistent ionization rates and minimizes fluctuations that could affect peak symmetry. The cones, as shown in Figure of 3.6, which are components in the ICP-MS that help focus the ion beam into the mass spectrometer, must be cleaned regularly. This cleaning process is necessary and critical to remove any accumulated sample residues or contaminants, ensuring accurate and reliable isotopic measurements.

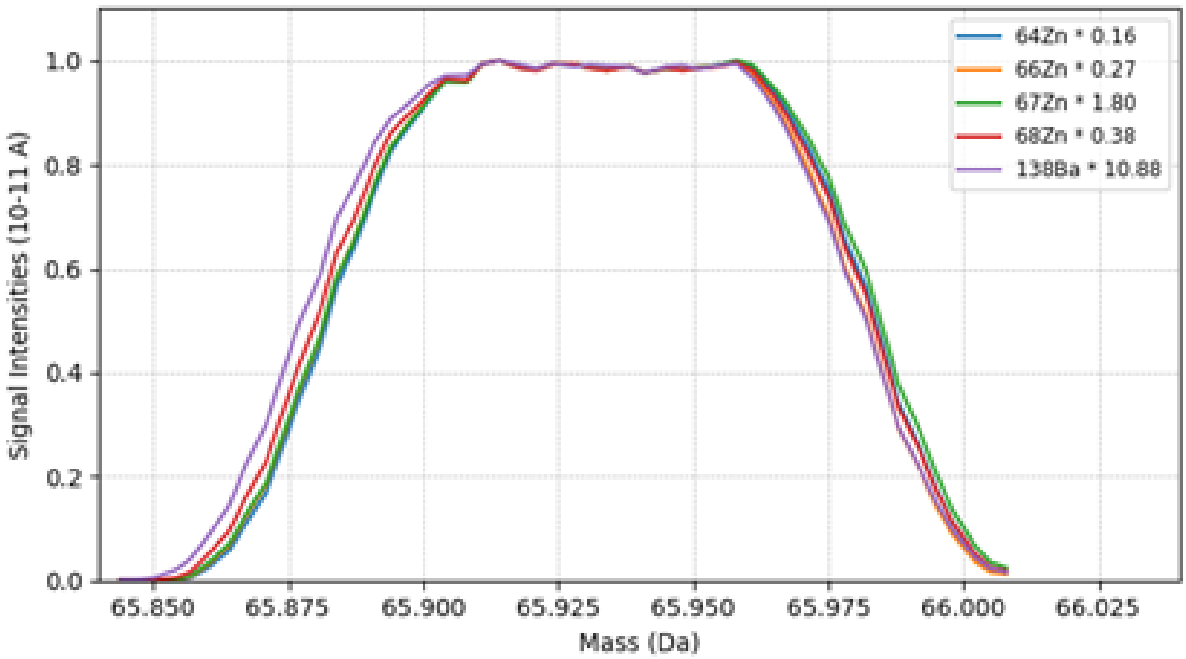


Figure 3.5: Peak shapes for zinc isotopes, showing flat peak tops confirming proper mass spectrometer tuning for accurate isotopic measurements.

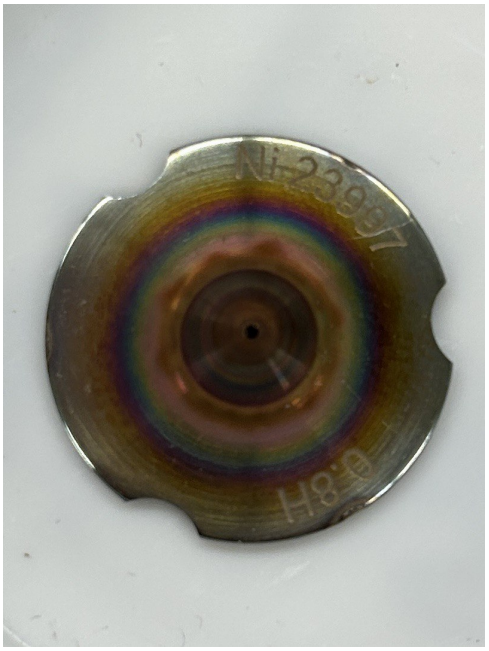


Figure 3.6: An MC-ICP-MS cone

Furthermore, monitoring the baseline signal, stability, and sensitivity of the samples during measurements is critical to ensure no clogs in the nebulizer, no memory effects in the spray chamber, and no build-up of materials in the cone. Consistent monitoring and maintenance of these parameters ensure that the instrument maintains peak characteristics and sensitivity throughout the analysis, supporting accurate and reliable zinc isotope measurements.

Ensuring the high precision required for reliable scientific investigations in MC-ICP-MS analysis, it is vital to emphasize the importance of peak center and resolution. A stable signal capture, less affected by instrumental drift or matrix effects, is crucial for reliable isotopic ratio assessments. Consistent calibration to these standards significantly improves the accuracy and reliability of measurements, supporting complex sample analyses.

After the peak center is determined and the peak shape established, the normalized isotope ratios of the in-house zinc standard, specifically n(^{66}Zn)/n(^{64}Zn), were measured and presented in Table 3.7. These were then compared to the normalized isotope ratios of certified reference materials to ensure the accuracy of the measured ratios. Subsequently, it's critical to monitor the stability and sensitivity of the signal and the normalized isotope ratios of the in-house zinc standard, comparing them to the calibration values to confirm the instrument's sensitivity and stability throughout the measurement process.

Table 3.7: Comparison of normalized ratios, showing precision and 2SD values from MC-ICP-MS calibration and operation

Isotope ratio	**IRMM-3702 (by IRMM)[51]**	**IRMM-3702 (by Moeller)[52]**	**IRMM-3702 (by Mohamed)[45]**	**This study (n=22)**
n(^{64}Zn)/n(^{66}Zn)	0.56397 (30)	0.56728 (41)	0.56730 (50)	0.56722 (13)
n(^{67}Zn)/n(^{66}Zn)	0.082166 (35)	0.082753 (44)	0.082753 (38)	0.082756 (17)
n(^{68}Zn)/n(^{66}Zn)	0.37519 (16)	0.37739 (54)	0.37739 (33)	0.37734 (8.7)
n(^{70}Zn)/n(^{64}Zn)	0.012418 (23)	0.012417 (28)	0.012566 (94)	0.012562 (15)

One factor potentially leading to differences between measured normalized ratios and those of reference materials is the memory effect, which occurs when the double spike isn't fully cleared from the spray chamber. To address this, the MC-ICP-MS protocol includes a cleaning step involving baseline measurements of a pure 3% HNO_3 solution. This adjustment compensates for any background signals linked to the memory effect, ensuring the accuracy of signals originating from the sample. Furthermore, to thoroughly eliminate any residual double spike from prior analyses, the protocol requires a 1-minute rinse with 3% HNO_3 after each measurement cycle. This step is crucial for purging any lingering zinc residues in the spray chamber and is essential for maintaining the precision of subsequent measurements. Additionally, careful monitoring of all zinc isotopes during the rinse confirms complete removal from the system. This ensures precise control over the zinc concentration within the 3% HNO_3 solution, necessary for subsequent calibration and corrections. In cases where the memory effect persists despite

using a 3% HNO_3 solution, a more rigorous approach involves a one-hour wash of the spray chamber with 1M HCl. Following the HCl wash, the normalized isotope ratios of the zinc standard experienced changes of approximately 0.00882%, 0.00846%, and 0.00795%, which brings the ratios closer to the values reported in Table 3.6, highlighting the impact of the memory effect on the isotopic composition. The HCl wash effectively removed all traces of the memory effect from the double spike in the spray chamber, significantly reducing background signals and cross-sample contamination. This rigorous cleaning ensures that subsequent measurements are not influenced by residues from previous samples, thereby enhancing the precision and reproducibility of the isotopic data.

Table 3.8: Changes in Normalized Zinc Isotope Ratios Before and After HCl Wash

Isotope ratio	**Before wash**	**After wash**
$n(^{64}Zn)/n(^{66}Zn)$	0.56716	0.56721
$n(^{67}Zn)/n(^{66}Zn)$	0.082727	0.082734
$n(^{68}Zn)/n(^{66}Zn)$	0.37730	0.37733
$n(^{70}Zn)/n(^{64}Zn)$	0.012418	0.012562

3.5 Isobaric Interferences

In the analysis of zinc isotopes using Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICP-MS), isobaric interferences from elements such as nickel (Ni) and barium (Ba) pose significant challenges. These interferences, often referred to as Isobaric Elemental Interference, occur when nickel isotopes (^{60}Ni, ^{61}Ni, ^{62}Ni) and doubly charged barium isotopes ($^{137}Ba^{2+}$ interfering with ^{68}Zn, $^{135}Ba^{2+}$ with ^{67}Zn) contribute to measurement complexities. To address the spectral interference from Ba^{2+} in samples containing more than 0.2 ng, which corresponds to a 1 mV signal of $^{138}Ba^{2+}$, a second ion exchange is performed. This measure is critical to achieving accurate isotopic measurements, as observed shifts in $\delta^{66}Zn$ values of up to +0.3‰ in these samples confirm the need for barium removal.

Figure 3.7 displays the mass-to-charge ratio (Da) versus the signal intensity (V) for isotopes of zinc (Zn) and doubly charged barium (Ba^{2+}). The highest peak, corresponding to ^{64}Zn, demonstrates the predominant isotopic presence in the sample. Lower intensity peaks at mass numbers 135 and 137 for Ba^{2+} could potentially overlap with the masses of ^{67}Zn and ^{68}Zn, highlighting the importance of correcting these interferences. The figure shows that the Ba^{2+} signal can overlap with zinc signals, which necessitates corrective measures to ensure accurate isotopic analysis.

Additionally, isobaric molecular interference further complicates the analysis when a molecular ion formed from argon and oxygen ($^{40}Ar^{16}O^{+}$) interferes with an elemental ion like ^{56}Fe. The complexity of biological matrices, which include a mix of organic and inorganic substances, leads to the formation of molecular ions or polyatomic species, complicating zinc isotopic measurement. To remove the ^{56}Fe interference

during ion exchange steps, the matrix wash volume has been increased to 12 ml of 1 mol L^{-1} HCl from 8 ml, as detailed in Table 3.4.

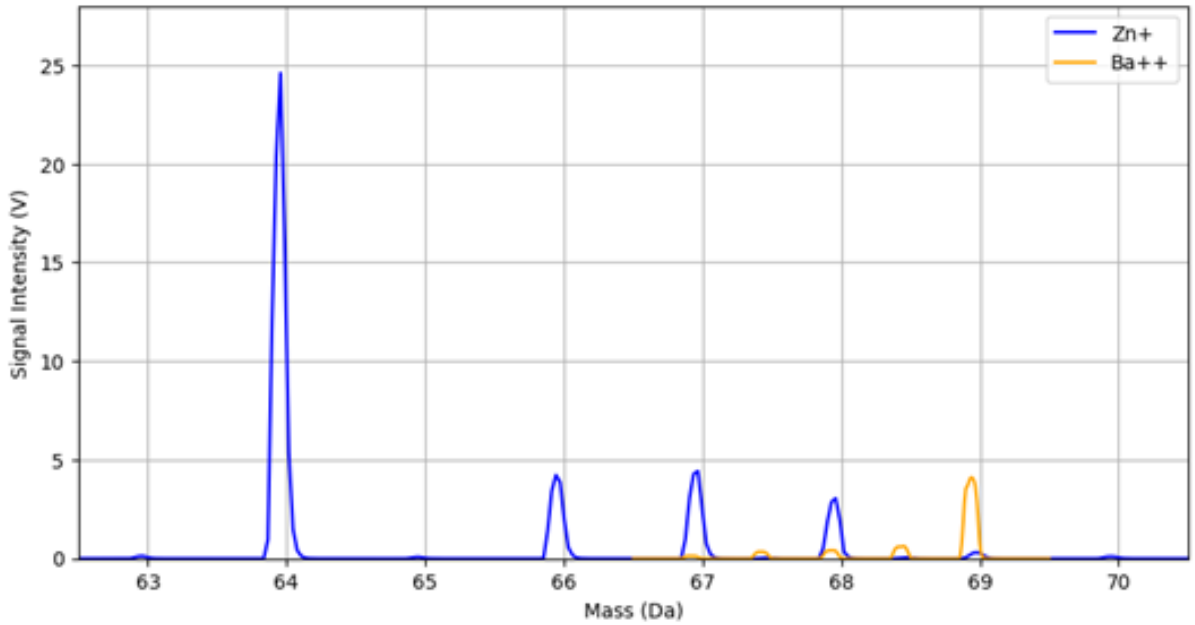


Figure 3.7: Mass Spectrometry Profile for Zinc and Doubly Charged Barium Ions: This graph displays the signal intensity (V) for singly ionized zinc (Zn^{+}) and doubly ionized barium (Ba^{2+}) isotopes over a mass range of 63 to 70 Daltons (Da).

Table 3.9: Isobaric Interference of Zinc

Isotope	**Elemental Ion**	**Polyatomic Molecular Ion**
$^{64}Zn^{+}$	$^{127}I^{++}$, $^{64}Ni^{+}$, $^{128}Xe^{++}$, $^{129}Xe^{++}$, $^{128}Te^{++}$	$^{48}Ti^{16}O$, $^{28}Si^{36}Ar$, $^{24}Mg^{40}Ar$, $^{48}Ca^{16}O$, $^{14}N^{18}O^{16}O^{16}O$, $^{23}Na^{23}Na^{16}O^{2}H$
(49.17%)		
$^{66}Zn^{+}$	$^{131}Xe^{++}$, $^{132}Xe^{++}$, $^{132}Ba^{++}$, $^{133}Cs^{++}$	$^{26}Mg^{40}Ar$, $^{48}Ti^{18}O$, $^{50}Ti^{16}O$, $^{36}Ar^{14}N^{16}O$, $^{50}Cr^{16}O$
(27.73%)		
$^{67}Zn^{+}$	$^{133}Cs^{++}$, $^{134}Ba^{++}$, $^{134}Xe^{++}$, $^{135}Ba^{++}$	$^{17}Al^{40}Ar$, $^{31}P^{36}Ar$
(4.04%)		
$^{68}Zn^{+}$	$^{135}Ba^{++}$, $^{137}Ba^{++}$, $^{136}Ba^{++}$, $^{136}Ce^{++}$, $^{136}Xe^{++}$	$^{32}S^{36}Ar$, $^{28}Si^{40}Ar$, $^{52}Cr^{16}O$
(18.45%)		
$^{70}Zn^{+}$	$^{70}Ge^{+}$, $^{139}La^{++}$, $^{140}Ce^{++}$, $^{141}Pr^{++}$	$^{54}Fe^{16}O$, $^{54}Cr^{16}O$, $^{40}Ar^{14}N^{16}O$, $^{30}Si^{40}Ar$
(0.6%)		

3.6 Internal Calibration Method

Calibrating the instrument to address mass-dependent effects is crucial in the analysis of zinc isotopes using Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICP-MS). Various methods exist to correct instrumental mass bias, including Standard-Sample Bracketing (SSB), Elemental Doping, and the Double Spike (DS) technique.

Standard Sample Bracketing (SSB) is widely used for its simplicity and effectiveness in compensating for instrumental drift over time. By analyzing known standards immediately before and after the sample, the isotopic ratios from these standards are used as a reference to adjust the sample's measured isotopic ratios. This approach ensures that the measured isotopic ratios are not skewed due to temporal variations in the instrument's performance, making it particularly useful in long analytical sessions [53],[29].

Elemental Doping involves adding a known quantity of a dopant, such as copper, to the sample. The isotopic composition of the dopant, which acts as an internal standard, is already well-characterized. During the analysis, any deviations in the isotopic measurements of the doping element indicate instrumental bias, which can then be corrected in the analysis of the primary element. This method is beneficial when the sample matrix might cause specific interferences or when the instrument's sensitivity varies across different mass ranges, providing a continuous check against these variations [54]. Due to its precision, the Double Spike (DS) method was chosen for this study, and its outcomes were compared with those obtained through SSB [55]. The Double Spike (DS) Technique, detailed in Section 2.4, was chosen for this study due to its precision and robustness in correcting instrumental and matrix-induced fractionation.

3.6.1 Double Spike Preparation and Calibration

The stock double spike solution used in this thesis was prepared using a method described by [45]. This solution was created from single spikes obtained from Oak Ridge National Laboratory, USA, known for their high purity and consistent isotopic composition. The isotopic concentrations of the solution, which are crucial for this study, are detailed in Table 3.10[45]. The n(^{64}Zn)/n(^{67}Zn) ratio was approximately 4.5, closely aligning with the optimal ratio suggested by Rudge (2009) to minimize measurement uncertainties.

Table 3.10: The isotopic abundances in the Zinc DS stock solution

Isotope	^{64}Zn (%)	^{66}Zn (%)	^{67}Zn (%)	^{68}Zn (%)	^{70}Zn (%)
Zinc DS	79.15	0.70	19.43	0.69	0.01

The double spike solution was carefully diluted to achieve the target concentration of 1μg/mL. This step is crucial for minimizing measurement errors. Three mixtures of the zinc standard solution and the double spike, each with a sample-to-spike ratio of 1:1, were prepared precisely. The known concentration of the zinc standard (PCal, PlasmaCAL, SCP Science) allowed for the accurate addition of the double spike

to each mixture. Once prepared, the isotope ratio of each mixture was measured. To ensure accuracy, background signals from 3% HNO_3 were subtracted to correct signal errors. The concentration of the zinc double spike in these mixtures was calculated using established double spike equations, detailed in Chapter 2. This calculation is essential for refining the data reduction algorithms used in the isotopic analysis, ensuring reliable data on the isotopic composition of zinc (Zn). Table 3.11 presents the isotope composition of zinc for the mixtures, the zinc standard (PCal, PlasmaCAL, SCP Science), and the double spike ratio. This data is crucial for using the software developed by Alex Tennant, which facilitates the analysis of these measurements, providing enhanced insights into zinc isotopic variations and their scientific implications.

Table 3.11: Isotope Ratios of the Double Spike, Standard, and Mixture. With $n = 3$, which is the number of measurements taken, and $k = 1$ represents the uncertainty factor applied to the standard deviation of the measurements.

Isotope Amount Ratio	**Mixture Ratios**	**Standard Ratios**	**Spike Ratios**
$^{64}Zn/^{66}Zn$	4.3965(249)	1.6709(1632)	111.25(105)
$^{67}Zn/^{66}Zn$	0.8351(23)	0.1492(148)	27.99(22)
$^{68}Zn/^{66}Zn$	0.7082(106)	0.7008(700)	1.0039(13)
$^{70}Zn/^{66}Zn$	0.0233(4)	0.0236(24)	0.0128(43)

3.7 Method Validation with Reference Materials

Validating the methodology using reference materials is crucial for ensuring the accuracy and precision of isotopic measurements. This process confirms the consistency and reliability of isotopic ratios across various sample preparations and analytical runs, such as δ^{66}Zn values. Critical steps in this validation include verifying spiked standards within the ±0.05‰ range, assessing duplicate sample δ^{66}Zn consistency (reproducibility), which is expected to have values within 0.08‰ of each other, maintaining zinc blanks below 2 ng, and aligning measured isotope ratios with certified values during instrument calibration. If any of the quality control measures do not meet the specified criteria, the entire samples undergo ion exchange and are analyzed a second time.

3.7.1 Validation of Zinc Isotope Composition Measurement Method using δ^{66}Zn and ICP-MS Double Spike Technique

This study utilized the double spike (DS) technique combined with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to quantify zinc isotopes accurately. To ensure an optimal isotopic ratio for measurement, 500 ng of the double spike and 500 ng of Certified Reference Material (CRM) were precisely weighed and added to each mixture, maintaining a 1:1 spike-to-sample mass ratio. This specific ratio

is crucial for accurately tracking isotopic fractionation and enhancing the robustness of the isotopic measurement. The analytical method was validated by measuring reference biological materials with well-established zinc isotopic compositions. All measurements were reported using the delta notation δ^{66}Zn.

To ensure the validity of the measured isotopic composition, it is essential to keep track of the δ^{66}Zn values in the double-spiked ICP-DS (Zn single element solution (PCal, PlasmaCAL, SCP Science)) mixtures, which must remain within a narrow tolerance of 0.05‰ to assure measurement accuracy in each set of measurements. Figure 3.8 shows the δ^{66}Zn value of the Double Spike and Zn single element mixtures for each set of measurements. Values outside this range may suggest potential fractionation during the ion exchange process, instrumental drift, contamination, poor stability, buildup of materials in the cone, memory effect in the spray chamber, or presence of Ba^{2+} in the samples.

As seen from the data, the accuracy and precision of the δ^{66}Zn improved over time and have been within the expected range by controlling these potential factors. The δ^{66}Zn value of the measured CRM and mouse organ was not considered valid if the δ^{66}Zn value of the ICP-DS mixture was outside this range.

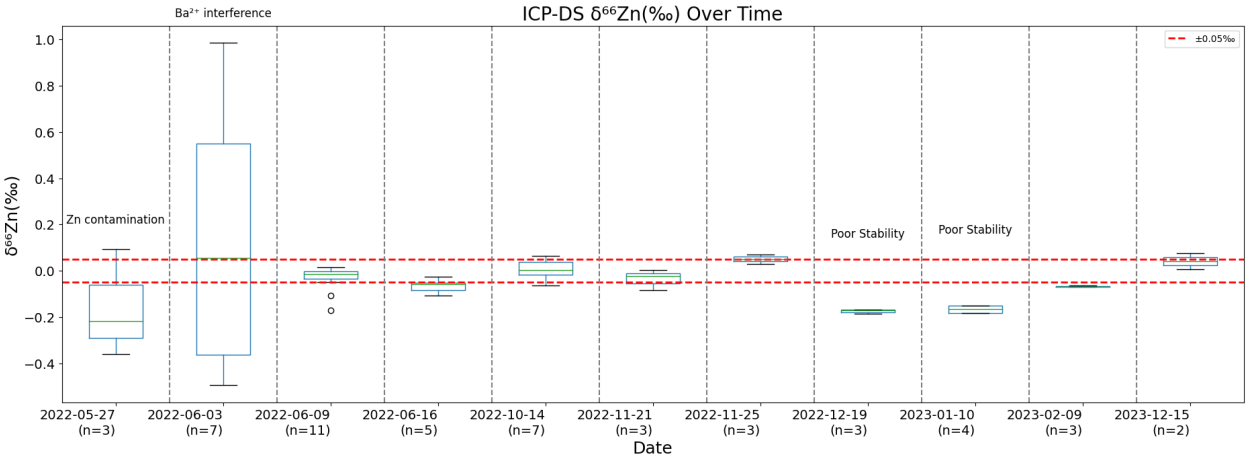


Figure 3.8: Variability of δ^{66}Zn isotope ratios Over Time as Measured by ICP-MS with and without ion exchange: This box plot series illustrates the distribution and stability of δ^{66}Zn values across various measurement dates, with the number of samples (n)

In addition, δ^{66}Zn values of the ICP-DS mixture with and without ion exchange have been measured to ensure no isotopic fractionation occurred. The δ^{66}Zn value with ion exchange should be within a range of 0.07‰, essential for monitoring and controlling mass fractionation effects inherent to the ion exchange methodology. The collected data, represented in Figure 3.8, consistently showed that the δ^{66}Zn values fell within the expected precision range of 0.05‰, confirming the method's accuracy.

Also, it was necessary to track the amount of Zn added at each step to identify the source of contamination and ensure that the blank remained under control. To prevent any shift in the delta value due to the blank, it needed to be less than 10% of the zinc amount in the sample, which, in this case, amounted to less than 5 ng. Following all cleaning procedures, the digestion and column blanks consistently remained

below 5 ng as shown in Table 3.12, meeting the requirement of being less than 10% of the 500 ng of zinc present in the sample, thereby avoiding a shift in δ^{66}Zn. The Ba^{2+} ion exchange blank measured 7 ng and 74 ng. This blank's source could be attributed to inadequately cleaned resin (despite a cleaning period of a week and a half) or contamination from the bench during sample transfer to the autosampler tube for measurement.

Table 3.12: Measured values for zinc ion exchange and digestion blanks (in ng) throughout the study period.

Date	Zn Ion Exchange Blank (ng)	Digestion Blank (ng)
2022-05-27	1.09, 0.71, 0.70	0.76
2022-06-03	2.21, 232.11	0.83
2022-06-09	1.57, 1.74	0.84
2022-06-16	0.94, 0.99, 0.77	2.10
2022-10-14	0.41, 1.78	2.00
2022-11-25	2.74	1.20
2022-12-19	6.60, 4.60	0.14
2023-01-10	1.80	None
2023-01-11	1.81	None
2023-02-09	1.75	None
2023-02-10	0.42	None
2023-02-11	0.53	None
2023-03-10	113, 356	None
2023-12-16	0.42, 7.00	None
2024-01-26	0.53, 74.00	None

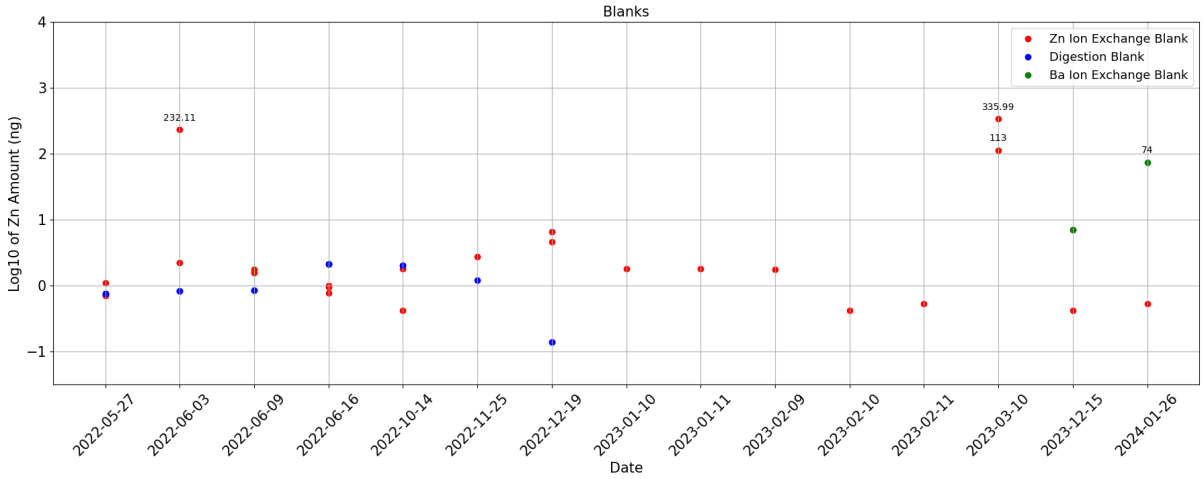


Figure 3.9: Trends in Zinc Contamination Measured in Column Blanks and Digestion Blanks Over Time: The graph tracks the recorded amount of zinc (ng) present in column blanks and digestion blanks on various dates, with the number of samples (n) indicating the variability and possible sources of contamination in the analytical process.

3.8 Evaluation of Uncertainty Sources in Zinc Isotopic Measurements

Accurate determination of zinc isotopic composition using Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC ICP-MS) combined with the double spike method necessitates an in-depth understanding of various sources of uncertainty. These uncertainties distinctly impact concentration measurements and isotopic ratios ($\delta^{66}Zn$), influencing the overall accuracy and precision of the analysis.

Sources of Uncertainty in Concentration Measurements

DS Algorithm: The uncertainty from the DS Algorithm primarily arises from computational errors in calibrating the sample with the double spike. These errors affect the calculated isotopic ratios by potentially misestimating spike-sample proportions.

Weighing Scale: Uncertainty from the weighing scale contributes significantly, stemming from inaccuracies in measuring the precise quantities of spikes and samples. This can alter the intended ratios in the sample preparation, impacting the resulting isotopic analysis.

Blank Measurements: These involve potential contamination from labware and reagents, which can introduce unwanted isotopes into the sample, thereby affecting the measured concentrations.

Repeatability: Repeatability is defined as the standard deviation of concentration measurements of the double spike solution used in calibration.

Sources of Uncertainty in $\delta^{66}Zn$ Measurements

Repeatability: For $\delta^{66}Zn$ measurements, repeatability is crucial and predominantly encompasses the standard deviation of the $\delta^{66}Zn$ values obtained from both processed and unprocessed mixtures of Zn standard and double spike solution. This metric reflects the consistency and reliability of isotopic ratios under variable experimental conditions, indicating the method's ability to reproduce consistent results regardless of processing.

Blank Measurements: Contamination impacts are also critical here, as they can skew the isotopic purity and accuracy of the $\delta^{66}Zn$ values.

Spike-to-Sample Ratio: Errors in maintaining the correct spike-to-sample ratio directly influence the isotopic equilibrium, crucial for accurate $\delta^{66}Zn$ determinations.

Kragten Method for Quantifying Combined Uncertainty

The Kragten method[56] systematically quantifies the combined uncertainty from these sources. It employs a quadratic sum of individual uncertainties, assuming they are independent and normally distributed. The formula used is:

$$\sigma_{\text{Combined}} = \sqrt{\sigma^2_{\text{Spike-to-Sample Ratio}} + \sigma^2_{\text{Repeatability}} + \sigma^2_{\text{Blank}} + \sigma^2_{\text{Weighing Scale}}} \tag{3.1}$$

The expanded uncertainty (U), with a coverage factor $k = 2$, is:

$$U_{\text{combined}} = 2 \times \sigma_{\text{combined}} \tag{3.2}$$

To demonstrate the impact of each source of uncertainty on the total uncertainty, the percentage contribution is calculated as follows:

$$\text{Percentage Contribution of Each Source} = \left(\frac{\sigma^2_{\text{source}}}{\sigma^2_{\text{combined}}} \right) \times 100 \tag{3.3}$$

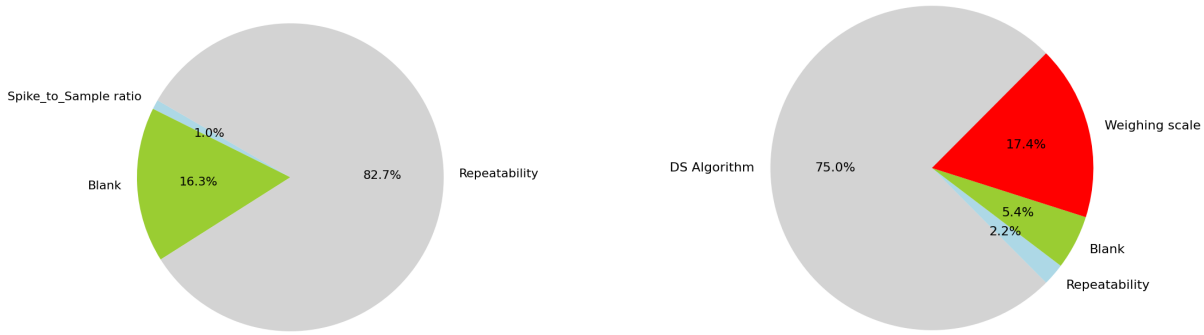
The relative contributions of each source of uncertainty are shown in Figure 3.10.

- **Concentration Uncertainty:** The DS Algorithm is the largest contributor at 75.0%, followed by the Weighing Scale at 17.4%, indicating that computational accuracy and weighing precision are pivotal. Blank measurements and repeatability, though lesser, still pose significant roles at 5.4% and 2.2% respectively.

- $\delta^{66}Zn$ **Uncertainty:** Repeatability dominates with 82.7%, underscoring the importance of consistent methodological execution. Blanks contribute 16.3%, reflecting the need for stringent contamination controls. The minor yet impactful 1.0% from the Spike-to-Sample Ratio highlights the need for precise sample handling.

$$U_{\text{combined}} = 2 \times \sigma_{\text{combined}} \tag{3.4}$$

This analysis not only clarifies the individual and combined impacts of each uncertainty source but also guides where methodological improvements could enhance the reliability and accuracy of zinc isotopic measurements in biological matrices.



(a) δ^{66}Zn(‰) Uncertainty

(b) Concentration Uncertainty

Figure 3.10: Distribution of Uncertainty Sources in Zinc Isotopic Analysis. This pie chart quantifies the contributions from the ICP-DS mixture, blank contributions, the weighing scale, and the double spike method to the total measurement uncertainty.

Chapter 4

Results

This chapter analyzes the experimental data derived from the isotopic analysis of certified biological materials and additional samples, including mouse kidneys and chicken liver purchased from a local store. The certified materials allow us to validate the reliability of the measured isotopic compositions.

4.1 Method Validation with Chicken Liver Samples

The isotopic composition of zinc (Zn) in chicken liver samples purchased from a local store has been analyzed to develop the method. The liver typically has a high concentration of iron (Fe), ranging from approximately 56.97 to 411 mg/kg [57], [58]. While Fe does not have direct isobaric interference with Zn, its high concentration can cause matrix effects that complicate the accurate measurement of Zn isotopes. Therefore, these samples were ideal for demonstrating the ion exchange method in removed Fe interference and testing the sample digestion and ion exchange processes before working with Certified Reference Materials and mouse kidney samples.

The results of the measured isotopic composition are displayed in Figure 4.1 and detailed in Table 4.1. During the first measurement, the spike ratio was outside the optimal range (45% to 55%), skewing δ^{66}Zn value to a positive. The blank for both days of measurement was under 3 ng (1.1 $\pm$ 1.9 ng (2 σ), n = 2, on 2022-10-14 and 2.74 $\pm$ 0.22 ng (2 σ), n = 1, on 2022-11-25), and the signal intensity of $^{138}Ba^{2+}$ was under 1 mV. The higher δ^{66}Zn values emphasize the effect of the spike ratio on the δ^{66}Zn, as discussed in Section 2.4, which increases the uncertainty of the measurement's not maintained at $\sim$ 50%.

The spike ratio plays a significant role in determining the accuracy of the δ^{66}Zn values. On October 14, 2022, measurements had spike ratios significantly above the optimal range (77.7% - 81.6%), leading to skewed δ^{66}Zn values. In contrast, measurements on November 25, 2022, had spike ratios closer to the optimal range (38.7% - 46%), providing more accurate and reliable δ^{66}Zn values by having the lower uncertainty. Thus, the latter measurements are more likely to be correct due to better adherence to the

optimal spike ratio range.

Furthermore, as mentioned in Section 3.3.1, the δ^{66}Zn values for liver tissues in various species, including cows, mice, and sheep, range from -0.75‰ to +0.5‰ [33; 37; 59]. Although specific δ^{66}Zn values for chicken liver are not available in the literature, it is reasonable to estimate that they fall within this general range. Consequently, the data from both measurement days is likely within the expected range despite the differing spike ratios.

Assessing duplicate sample δ^{66}Zn consistency (reproducibility), expected to have values within 0.08‰ of each other, is crucial for validating the reliability of the measurements. The consistent Zn recovery percentages and δ^{66}Zn values between duplicates demonstrate the method's reliability and repeatability. For example, the difference between the δ^{66}Zn values for sample L-1 and its duplicate is 0.01‰ (October 14, 2022) and 0.07‰ (November 25, 2022), both well within the acceptable range.

Table 4.1: Detailed measurements of isotope ratios for chicken liver samples, including sample ID, spike ratio (%), δ^{66}Zn (‰), zinc concentration (μg/g), zinc recovery (%), and the date of analysis. Uncertainties are provided with a coverage factor of k = 2.

Sample ID	**Spike Ratio (%)**	**δ^{66}Zn(‰)**	**Zn mass fraction (μg/g)**	**Zn recovery (%)**	**Date**
L-1	78	-0.66 ± 0.09	-	90 ± 4 %	14 Oct 2022
L-1-Dup	78	-0.67 ± 0.09	-	90 ± 4 %	14 Oct 2022
L-2	78	-0.52 ± 0.09	-	90 ± 4 %	14 Oct 2022
L-2-Dup	78	-0.55 ± 0.09	-	90 ± 4 %	14 Oct 2022
L-3-Dup	82	-0.68 ± 0.09	-	90 ± 4 %	14 Oct 2022
L-1	46	-0.77 ± 0.06	3.82 ± 0.02	88 ± 2.8 %	25 Nov 2022
L-1-Dup	46	-0.84 ± 0.06	3.82 ± 0.02	88 ± 2.8 %	25 Nov 2022
L-2	44	-0.67 ± 0.06	2.86 ± 0.012	88 ± 2.8 %	25 Nov 2022
L-2-Dup	44	-0.71 ± 0.06	2.86 ± 0.012	88 ± 2.8 %	25 Nov 2022
L-3	39	-0.82 ± 0.06	3.79 ± 0.02	88 ± 2.8 %	25 Nov 2022
L-3-Dup	39	-0.87 ± 0.06	3.79 ± 0.02	88 ± 2.8 %	25 Nov 2022

Note: "Dup" denotes duplicate samples. Data for the original L-3 from 14 Oct 2022 is missing due to sample loss during transfer.

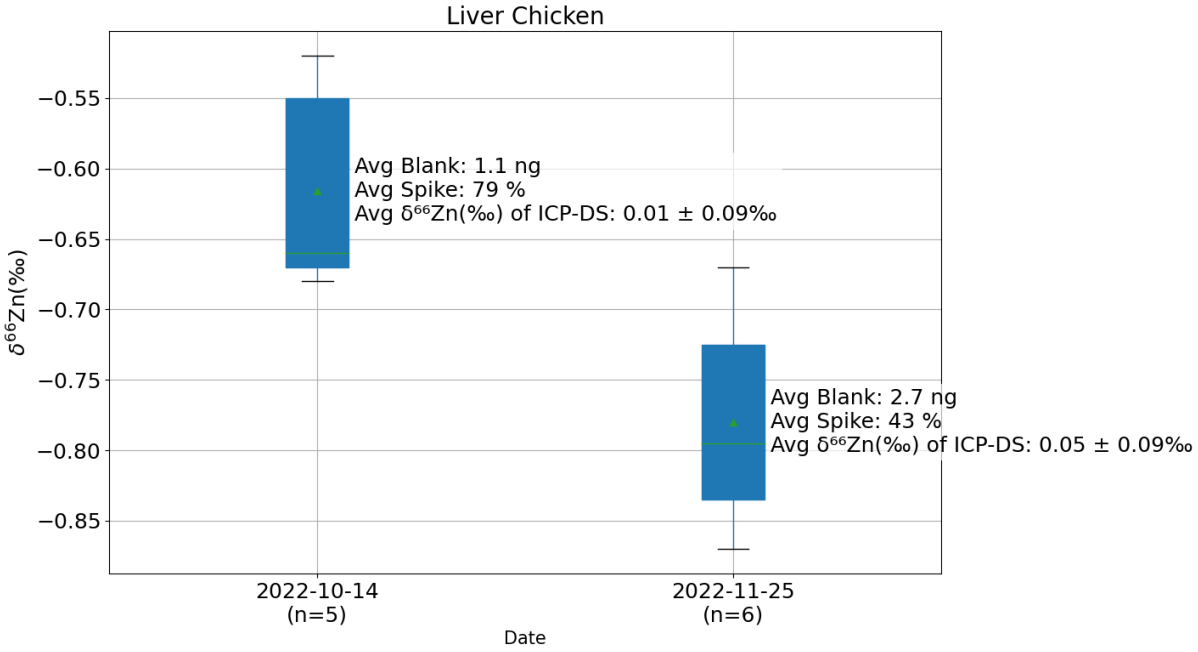


Figure 4.1: δ^{66}Zn values for the chicken liver samples with average blank, spike ratio, and δ^{66}Zn of ICP-DS. The errors for individual Zn isotopic measurements represent expanded uncertainty (k = 2).

Consistent zinc recovery percentages and δ^{66}Zn values between duplicates indicate the method's reliability and repeatability, as discussed in Section 3.7. The measured δ^{66}Zn values in duplicate samples are expected to be within 0.08‰ of each other, demonstrating the method's precision. For example, the differences in δ^{66}Zn values for sample L-1 and its duplicate were 0.01‰ on October 14, 2022, and 0.07‰ on November 25, 2022, both well within the acceptable range.

The initially developed analytical method, which includes sample digestion, effective ion exchange, accurate calibration, and precise measurement, has demonstrated its effectiveness in measuring the isotopic composition of biological samples. This effectiveness was evidenced by consistently low blank levels, effective removal of contaminants, and reproducibility of isotopic ratios. The high concentration of Fe in chicken liver samples tested the ion exchange method's ability to remove Fe and other potential contaminants that could skew the results.

The next steps in validating methods involve testing samples with known isotopic compositions. This direct comparison against established benchmarks will provide a clearer assessment of the method's accuracy and reliability. By aligning these results with known standards, the method's performance can be more confidently evaluated, allowing for any necessary adjustments to enhance its robustness and applicability to a wider range of complex biological samples.

4.2 Zinc Isotopic Composition in Certified Biological Materials

The results for δ^{66}Zn isotopic composition, depicted in Figure 4.2 and detailed in Table 4.2,display the performance of the analytical method developed in this study using the Double Spike (DS) method compared to a prepFAST method and established literature values.The prepFAST system automates dual-column purification by sequentially using different resins to isolate zinc from other matrix elements and interferences, thereby enhancing precision and minimizing contamination and human error.[55].

Figure 4.2 shows that the results for δ^{66}Zn isotopic composition using the DS method align closely with literature values for reference materials such as G090101 and G07601. The median values for these materials show minimal deviation from the known literature values, indicating that the measured isotopic compositions are very close to the published values within the uncertainties. For example, the measured value for G07601 using the DS method is 0.06 ± 0.09‰, close to the published value of 0.05 ± 0.10‰ [55], using a coverage factor of k = 2.

The close alignment between the measured and published values for G07601 suggests that the DS method can achieve high accuracy in reproducing known isotopic compositions. However, further measurements are needed to validate the method's reliability fully. Accurate matching with literature values, such as for G090101 and G07601, demonstrates the DS method's potential for broader application in isotopic studies, as it can provide consistent and precise measurements.

In contrast, the prepFAST method, while precise, shows a tighter interquartile range for samples like SWB L-3 and USGS42. The interquartile range (IQR) represents the middle 50% of the data, indicating data spread. For example, the IQR for USGS42 with the prepFAST method is approximately -0.1 to 0.1‰, indicating high precision with a small data spread.

The DS method exhibits a more pronounced range for reference materials ERM-DB001 and NIST SRM 1577c, with boxplot whiskers extending from approximately -0.2‰ to -0.5‰ for ERM-DB001 and from -0.1‰ to -0.3‰ for NIST SRM 1577c. While these observations suggest heightened sensitivity to isotopic variations, it is essential to note that these conclusions are based on limited sample comparisons, and further studies are required to confirm these findings across a broader set of samples. For instance, the DS method's value for NIST SRM 1577c is -0.31 ± 0.09‰, compared to the literature value of -0.46 ± 0.07‰ [60].

The blank from the Ba removal ion exchange was 76 ng for the last set of measurements. As mentioned in Section 3.3, it is essential to keep the blank below 5 ng, and the data detailed in Table 4.3 indicate how the blank can skew the δ^{66}Zn value for the NIST SRM 1577c sample from -0.31 ± 0.09‰ to 0.009 ± 0.09‰. This highlights the need to keep the blank levels low to get the accurate δ^{66}Zn values. These detailed results underscore the DS method's reliability, demonstrating accuracy in line with the literature and sensitivity to isotopic variations. The DS method data thus provides a strong foundation for isotopic analysis in biological samples, which is necessary for tracing metabolic pathways and environmental zinc

cycling. The method's ability to detect subtle isotopic differences highlights its potential utility in forensic and environmental studies where precision is paramount.

Table 4.2: Comparison of zinc mass fraction (in μg/g), zinc recovery rates (in %), and $\delta^{66}Zn_{IRMM\text{-}3702}$ values from this study against published values for a variety of certified reference materials (CRMs). Uncertainties are presented with a coverage factor of k = 2.

CRM	**Zn mass fraction (µg/g)**	**Zn recovery (%)**	δ^{66}**Zn/**$^{\textbf{IRMM-3702}}$ **(‰) this study**	$\delta^{66}Zn_{IRMM\text{-}3702}$ **(‰) Published**	**Reference**
NIST SRM 1577c	164 ± 30	92 ± 13	-0.31 ± 0.09	-0.46 ± 0.07	[60]
				-0.48 ± 0.04	[61], [62]
				-0.42 ± 0.05	[55]
SeroNorm Whole Blood L-3	8.3 ± 0.1	91 ± 1	-0.10 ± 0.02	0.02 ± 0.05	[63], [64]
				-0.02 ± 0.05	[65], [66]
				-0.15 ± 0.06	[55]
GBW07601	177 ± 5	93 ± 3	0.06 ± 0.09	-0.22 ± 0.06	[63]
				0.05 ± 0.10	[55]
GBW09101	166 ± 17	88 ± 10	-0.12± 0.09	-	-
ERM-DB001	188 ± 19	90 ± 10	0.12± 0.09	-	-
USGS42	173 ± 17	99 ± 10	-0.07± 0.09	-	-
USGS43	202 ± 20	99 ± 10	-0.07± 0.09	-	-

Table 4.3: Comparison of zinc mass fraction (in μg/g), zinc recovery rates (in %), and $\delta^{66}Zn_{\text{IRMM-3702}}$ values from this study with published values for various certified reference materials (CRMs). Measurements include a blank of 76 ng, and uncertainties are expressed with a coverage factor (k = 2).

CRM	**Zn mass fraction (μg/g)**	**Zn recovery (%)**	**δ^{66}Zn/$^{\text{IRMM-3702}}$ (‰) this study**	**$\delta^{66}Zn_{\text{IRMM-3702}}$ (‰) Published**	**Reference**
NIST SRM 1400	171 ± 7	95 ± 4	0.74 ± 0.02	0.68 ± 0.07	[67]
				0.71 ± 0.05	[68]
NIST SRM 1486	119 ± 12	85 ± 9	0.98 ± 0.02	0.90 ± 0.08	[60]
				0.87 ± 0.07	[60]
NIST SRM 1577c	164 ± 30	92 ± 13	0.09 ± 0.09	-0.46 ± 0.07	[60]
				-0.48 ± 0.04	[62] , [69]
				-0.42 ± 0.05	[55]

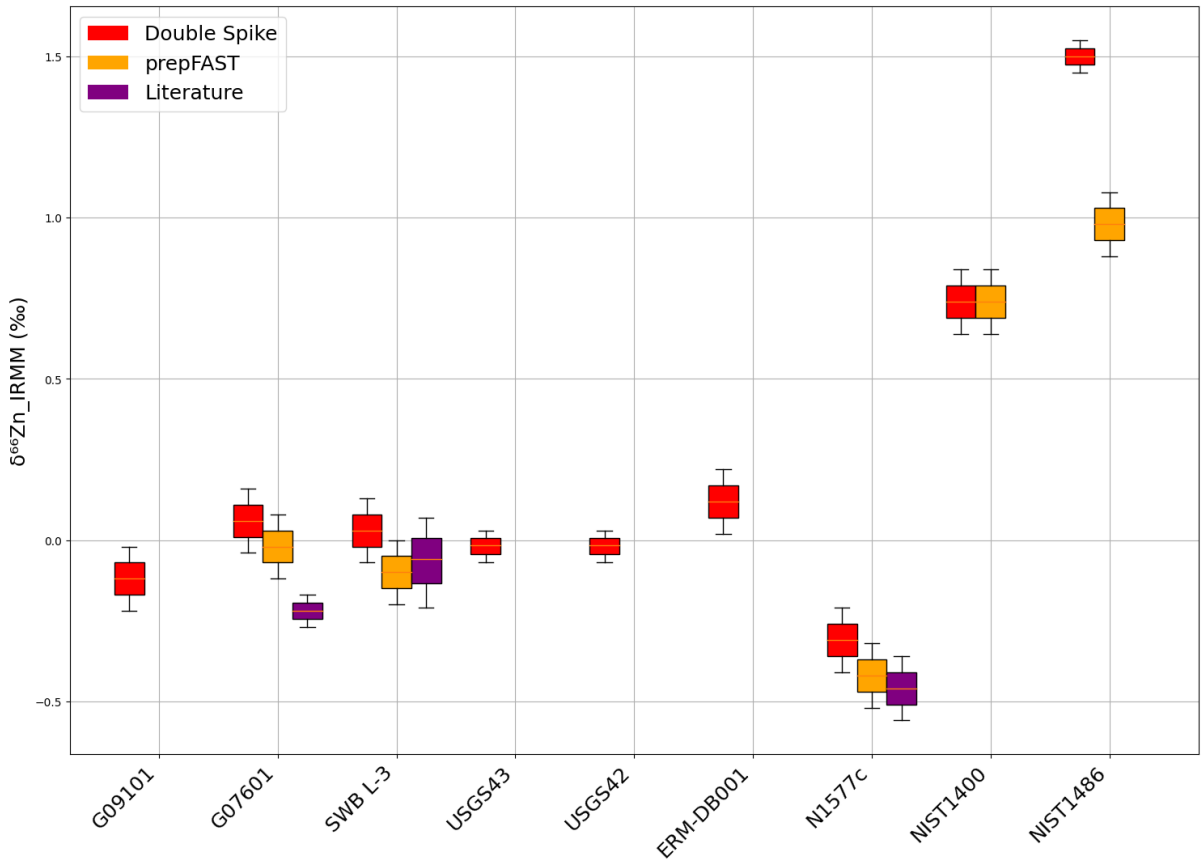


Figure 4.2: Comparative δ^{66}Zn Isotope Variability in Reference Materials. The box plot displays δ^{66}Zn ratios measured by Double Spiking (DS), prepFAST automated sample preparation, and values reported in the literature for various geological and biological reference materials.

4.3 Zinc Isotopic Composition and Concentration in Mice Organs

As discussed in Section 3.2, the mouse kidney samples were utilized to develop reliable and precise analytical methods for biological samples. These methods are crucial for ensuring accurate isotopic measurements across various biological matrices. All experiments involving mice were performed by trained personnel under Dr. Keith Sharkey's Animal Use Protocol #AC19-01214, which was approved by the University of Calgary Health Sciences Animal Care Committee according to the guidelines established by the Canadian Council on Animal Care. All mice were born and housed at the International Microbiome Centre at the University of Calgary. After 12 weeks, the mice were anesthetized using isoflurane and euthanized by cervical dislocation, after which the left kidneys were collected and stored in blue and yellow 2 mL centrifuge tubes. [70].

Maintaining an optimized spike-to-sample ratio is essential for accurate zinc isotopic composition measurements, as suggested by Rudge et al. [31]. This parameter ensures that the zinc within the sample remains representative of its original biological context. To achieve this, measuring the precise zinc concentration

in each sample and correctly adding the appropriate amount of double spike, maintaining a 1:1 ratio to ensure reliable δ^{66}Zn measurements.

However, the mouse kidney samples analyzed in this study were digested 8 months before measurement and stored in Teflon beakers immersed in 3% HNO_3 to prevent zinc leaching. Due to the samples being stored in low molarity HNO_3, their concentrations decreased, causing the spike ratios of the first six samples to fall out of the desired range, making the results unreliable (Table 4.4). Additionally, a measured digestion blank with a zinc concentration of -0.114 ng. As it is impossible to get a negative concentration, it shows that the concentration was close to zero and below the detection limit.

A study from Bornhorst et al. corroborates this issue of stability [71], which found that nitric acid at a concentration of 0.12 mol L^{-1} generally maintained the stability of most trace elements, including zinc. However, there was a slight reduction in concentration from an initial ratio of 1.00 to 0.95 after 65 days. This aligns with our findings and emphasizes the critical importance of the type and concentration of acid used in sample preservation. Such parallels suggest that even minor deviations in molarity can significantly impact the integrity of trace element measurements, reinforcing the need for careful control of storage conditions.

To address this issue, 50 µL of concentrated HNO_3 were added to each sample the day before spiking and preparing them for ion exchange. This technique was designed to stabilize the zinc concentration, ensuring that it remained constant between concentration assessments and isotopic composition.

Consequently, the last three samples maintained consistent concentrations, and their spike ratios fell within the optimized range, yielding reliable results4.4. For this set of measurements, the blank was 0.9 ± 0.5 ng, and the reproducibility was -0.067 ± 0.02‰. So, this set met the quality control criteria, and the measured values are reliable.

The isotopic analysis of mouse kidney samples successfully showcased the capabilities and limitations of the methods employed. It revealed variations in zinc concentrations, ranging from 22.23 ppm to 70.29 ppm. The study effectively detected and quantified these variations across samples, which varied in age and body weight, demonstrating the methods' precision. Despite this, the limited sample size restricts our ability to draw broader conclusions about zinc metabolism. Future research, utilizing a larger cohort and leveraging the detailed cohort data provided in Table 4.4, is essential to validate and expand upon these preliminary findings.

The analysis also highlighted the critical impact of spike ratios on the precision of isotopic measurements. Samples KL-18, KL-19, and KL-22, which maintained spike ratios within the ideal range of 45% to 55%, displayed more precise δ^{66}Zn measurements with lower uncertainties, such as 0.16 ± 0.02‰ for KL-18 and 0.42 ± 0.02‰ for KL-19. Conversely, samples with spike ratios outside this optimal range, including KL-1 through KL-6, showed increased uncertainties in their δ^{66}Zn values.

Table 4.4: Zinc isotopic data and recovery rates in mouse kidney samples. Includes sample ID, matrix, spike ratio, δ^{66}Zn (‰), zinc mass fraction (μg/g), sample type, and zinc recovery (%). Uncertainties are provided with a coverage factor of k = 2.

Sample ID	**Matrix**	**Spike Ratio**	δ^{66}**Zn ‰**	**Zn mass fraction (µg/g)**	**Zn recovery (%)**
KL-1	Mouse Kidney	73%	0.50 ± 0.03	22	95%
KL-2	Mouse Kidney	74%	0.44 ± 0.03	33	95%
KL-3	Mouse Kidney	76%	0.50 ± 0.03	42	95%
KL-5	Mouse Kidney	71%	0.45 ± 0.03	58	95%
KL-6	Mouse Kidney	68%	0.41 ± 0.03	49	95%
KL-18	Mouse Kidney	55%	0.16 ± 0.02	55	98%
KL-19	Mouse Kidney	56%	0.42 ± 0.02	70	98%
KL-22	Mouse Kidney	55%	0.47 ± 0.02	49	98%

As shown in Section 4.2, maintaining the spike ratio within the expected range is essential to obtain valid δ^{66}Zn values for the samples. Figure 4.3 compares the spike ratio and δ^{66}Zn. As the spike ratio increases, the δ^{66}Zn values also tend to increase. The samples exhibit a general trend where higher spike ratios correspond to higher δ^{66}Zn values, suggesting that the spike ratio significantly influences the δ^{66}Zn values. Maintaining an optimal spike ratio is crucial for accurate isotopic analysis.

The variability in δ^{66}Zn values across different spike ratios demonstrates the diverse responses within the sample set. Some samples show a steady increase in δ^{66}Zn values with increasing spike ratios ranging from 0.3‰ to 0.5‰. Others exhibit more dispersed δ^{66}Zn values, indicating variability in response to different spike ratios. Some samples display the lowest δ^{66}Zn values among the dataset for the given spike ratios, ranging from approximately 0.2‰ to 0.5‰, showing less variance but generally similar trends at higher spike ratios.

This analysis underscores the importance of maintaining an appropriate spike ratio to obtain reliable δ^{66}Zn measurements. The data highlight the critical role of controlled experimental conditions in ensuring the accuracy of isotopic composition measurements.

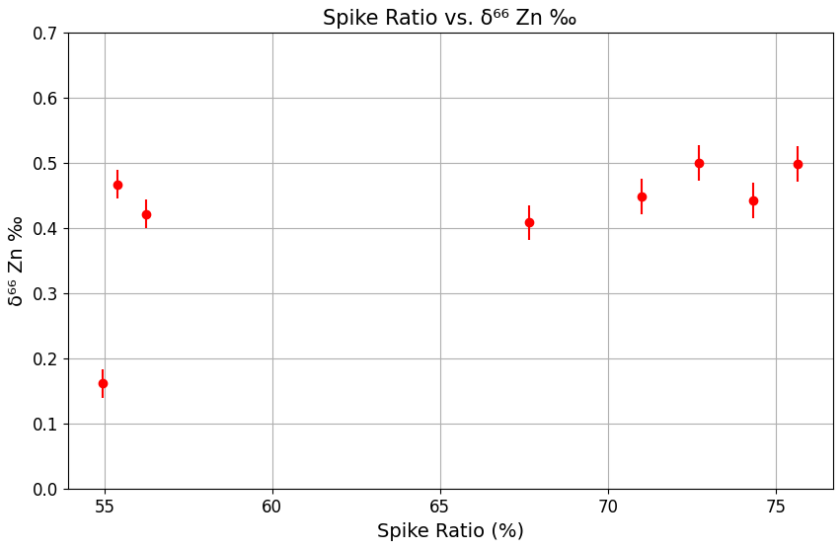


Figure 4.3: Correlation between Spike Ratios and δ^{66}Zn in the mouse kidney samples. This scatter plot compares the δ^{66}Zn‰ values against spike ratios

The observed differences in zinc isotopic compositions across various mouse samples offer preliminary insights into understanding zinc metabolism. However, the limited sample size makes these observations doubtful and should not be used to draw definitive conclusions. The primary emphasis of this study remains on validating and enhancing the robustness of the analytical methods employed.

As mentioned in Section 3.7, it must meet established quality control criteria for the measured data to be considered valid. Table 4.5 presents zinc isotopic data for mouse kidney samples, which unfortunately do not meet these criteria. The measured blank during the ion exchange averaged 234.5 ± 1.6 ng, significantly exceeding the acceptable threshold of less than 5 ng. Additionally, the reproducibility was 0.233‰, which

is beyond the acceptable range of ± 0.05‰. Furthermore, the average spike-to-sample ratio was 80.45%, well outside the recommended range of 45% to 55%. Given these deviations from the quality control standards, the data cannot be reliably used to draw definitive conclusions about zinc isotopic variations.

Table 4.5: Zinc isotopic data and recovery rates in mouse kidney samples. Includes sample ID, matrix, spike ratio, δ^{66}Zn (‰), zinc mass fraction (μg/g), sample type, zinc recovery (%), and Blank of 235.5 ng. Uncertainties are provided with a coverage factor of k = 2.

Sample ID	**Matrix**	**Spike Ratio**	δ^{66}**Zn (‰)**	**Zn recovery (%)**	**Blank (ng)**	**Reproducibility** δ^{66}**Zn (‰)**
KL-14	Mouse Kidney	70%	1.08 ± 1.56	98.5%	234.5 ± 1.6	0.22 ± 1.56
KL-17	Mouse Kidney	86%	1.28 ± 1.56	98.5%	234.5 ± 1.6	0.22 ± 1.56
KL-20	Mouse Kidney	80%	1.30 ± 1.56	98.5%	234.5 ± 1.6	0.216 ± 1.56
KL-23	Mouse Kidney	85.8%	1.33 ± 1.56	98.5%	234.5 ± 1.6	0.22 ± 1.56

Chapter 5

Summary and Outlook

5.1 Overview

This chapter presents the main findings from the isotopic measurements and methodological advancements. It evaluates the methods' efficacy, discusses the biological implications where applicable, and suggests future research directions.

5.2 Goals and Methodological Challenges

The primary objective of this study was to enhance the precision and accuracy of zinc isotopic composition measurements in biological samples, mainly focusing on liver and kidney tissues. This required ensuring the accuracy of isotopic measurements despite the complex nature of biological samples.
Challenges were abundant and varied:

- **Narrow Range of Isotopic Fractionation:** Zinc isotopes exhibit a limited natural mass-dependent fractionation, making high precision critical.
- **Complexity of Biological Matrices:** The diverse chemical composition of biological samples can interfere with ionization and lead to inaccurate readings. Careful sample preparation is essential to isolate zinc and avoid contamination.
- **Controlling Ba Signal Concentration:** Managing barium signals was crucial as its interference could skew zinc measurements, affecting the accuracy of the isotopic data.
- **Instability in Signal Strength of ICP-MS:** Fluctuations in signal strength during analyses posed challenges in maintaining consistent data quality, impacting the reliability of results.
- **Accumulation of Oil or Grease on Chromatography Columns:** This issue could take up to

a month to resolve, reducing sample processing efficiency and delaying analyses.

- **Double Spike Accumulation in the Spray Chamber:** Accumulation affected sample ionization consistency due to the memory effect, leading to potential errors.
- **Accumulation of Other Elements on the Cone:** This could affect the system's signal stability and calibration, leading to inaccurate measurement.

5.3 Methodological Summary

5.3.1 Quality Control Measures

Quality control criteria were established based on experimental validation and previous research findings[45]. These measures were essential in achieving reliable isotopic measurements, as detailed in Table 5.1.

Table 5.1: Quality Control Criteria for Isotopic Measurements

Criterion	**Standard**
Blank Measurements	≤ 5 ng
Spike-to-Sample Ratio	45% to 55%
Reproducibility of δ^{66}Zn	± 0.05‰
Consistency of Duplicate Samples	± 0.08‰

5.4 Summary of Findings

5.4.1 Validation and Methodology

The isotopic analysis of certified biological materials and chicken liver samples offered significant insights into zinc isotopic compositions. The use of Certified Reference Materials (CRMs) such as Bovine Liver (NIST1577c), Human Hair (GBW07601), Whole Blood (SeroNorm), Bone Ash (NIST1400), and Bone Meal (NIST1486) verified the precision of the analytical methods. The δ^{66}Zn values obtained consistently aligned within 0.05‰ of the values reported in the literature, demonstrating the accuracy of the methods. Notably, the validation with chicken liver samples was crucial for showing the robustness of the isotopic measurements in the presence of high Fe concentrations through effective ion exchange methods. The comparison of the Double Spike (DS) method with the prepFAST MC™ ion exchange method underscored the DS method's flexibility, as it does not require 100% zinc recovery, making it a reliable option for complex biological matrices.

5.4.2 Sample Preparation and Analytical Steps

The following steps outline the meticulous procedures employed to prepare and analyze samples, ensuring the accuracy of the isotopic measurements:

- **Sample Digestion:** Microwave digestion was employed to ensure the complete decomposition of biological matrices. This method facilitated uniform heat distribution and rapid temperature increase, using specific parameters (300 W, 200°C, 300 psi, 4 minutes ramp time, 3 minutes hold time).
- **Ion Exchange:** Zinc isolation and contaminant removal were achieved using AG MP-1 and Sr-Spec resins, minimizing interferences and enabling precise δ^{66}Zn measurements. The ion exchange process involved conditioning columns with 6 M HCl, equilibrating with 1 M HCl, loading the sample, and eluting the zinc with 0.01 M HCl.
- **Cleaning Procedures:** Rigorous cleaning protocols were implemented for all labware and resins to prevent contamination. This included soaking labware in 6 M HCl for two weeks and washing it with Milli-Q water. Resins were cleaned with 1 M HCl for two weeks to achieve low blank levels.
- **Spike Ratios:** Maintaining optimal spike ratios (45% to 55%) was crucial for accurate isotopic measurements. This helped minimize uncertainties in the δ^{66}Zn values. Samples with spike ratios within this range showed lower uncertainties, emphasizing the importance of correct spike ratios for precise analysis.

5.5 Methodological Developments

While the sample size was insufficient to comprehensively explore zinc isotopes in the mouse model, the refinements in methodology have prepared a foundation for future research in this area. The methods used have been thoroughly tested and validated, proving their effectiveness for complex biological samples.

5.6 Future Research Directions

Given the limitations of the sample size and the preliminary nature of the study, future efforts should apply these refined methodologies to more extensive and more diverse sample sets. This approach would facilitate a more thorough understanding of zinc isotopic variation and its biological implications. Furthermore, adapting these methods for other trace elements might offer insights into the dynamics of nutrients within biological systems, potentially impacting areas such as nutritional science, medicine, and environmental health.

5.7 Conclusions

The study has successfully designed and validated a method suitable for precisely and accurately measuring zinc isotopic composition in biological tissues. Future studies using these methodologies could broaden the understanding of zinc's roles in biological systems and refine the techniques for broader applications.

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Appendix

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Figure 3.3 on page 25:

Thermo Fisher Scientific™ MC-ICP-MS Internal Components Schematic. Schematic representation of the internal components of the MC-ICP-MS, illustrating the path from the sample introduction system through the ionization process in the ICP source, followed by the magnet's separation of isotopes, and concluding with the multiple ion collectors for simultaneous measurement of isotopes.

Citation

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Figure 3.4 (a), (b), and (c), page 25:

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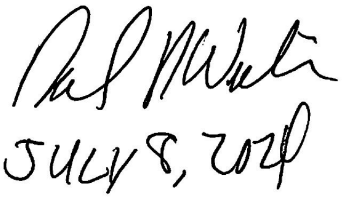
Signature: 

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