

DISSERTATION

DEVELOPMENT AND DEVICE INTEGRATION OF ELECTROCHEMICAL AND COLORIMETRIC
SENSORS TOWARDS IMPROVED LARGE-SCALE POINT-OF-NEED TESTING CAPABILITIES

Submitted by

Jason Boes

Department of Chemistry

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2026

Doctoral Committee:

Advisor: Alan Kennan

Charles S. Henry

Amber Krummel

Olve Peersen

Copyright by Jason Boes 2026

All Rights Reserved

ABSTRACT

DEVELOPMENT AND DEVICE INTEGRATION OF ELECTROCHEMICAL AND COLORIMETRIC SENSORS TOWARDS IMPROVED LARGE-SCALE POINT-OF-NEED TESTING CAPABILITIES

Safe and clean water is the most important resource in the modern world, and is not guaranteed, with around 40% of the world population living without access to a reliable clean water source. Without clean water, foodborne pathogens also become much more prevalent as sanitation practices become ineffective. Globally water access and safety is improving on average, primarily driven by infrastructure investment. However, as infrastructure ages and more people are introduced to on-demand clean water options, widespread monitoring is essential to ensure continued safety and progress. Domestically, water and food quality monitoring is becoming increasingly relevant in 2026 as climate change and policy decisions impact the availability and safety of water and food in the United States. Bacterial and heavy metals contaminants are common in older drinking water systems, and local contamination can be overlooked.

Chapter One examines this issue, the complexities that arise from the implementation of widespread monitoring, and recent technological and scientific developments towards point of need monitoring for these issues.

Chapter Two presents a bead-based electrochemical immunoassay for bacteria detection in food and water samples, allowing an end-user to test a liquid or solid sample for the presence of specific whole-cell bacteria. The assay was developed at Colorado State

University and characterized externally at the US Army Combat Capabilities Development Command (DEVCOM) to evaluate Limit of Detection (LoD), ease-of-use, and applicability towards food pathogen monitoring in the field. The assay is based on a traditional ELISA, allowing swapping of biorecognition elements for alternative targets, as shown in the adaptation of the assay between *Escherichia coli* and *Salmonella enterica*.

Chapter Three presents the design and optimization of a 3D-printed electroanalytical sensor for point of need detection of 3,4-Methylenedioxymethamphetamine (MDMA) in unknown tablet samples. The low-cost fabrication, ease-of-use, and disposable electrode design of the sensing system allows utilization at the point of need for unknown sample characterization. By 3D-printing the electrode, low-cost and disposable sensors are easily manufactured at scale in-lab, allowing extensive field deployment and testing. The electrode system was used for MDMA standardization, and subsequent detection of MDMA content in real-world seized unknown samples.

Chapter Four presents a 3D-printed preconcentration system in combination with a foldable reagent delivery and colorimetric readout card for instrumentation-minimal detection of heavy metals in water samples. By using specific chromogenic reagents for each heavy metal and pre-concentrating on paper, EPA-relevant limits of detection are achieved at a fraction of typical lab analysis cost. A smartphone is utilized for data analysis and readout, allowing usage of the device at the point of need within 30 minutes, as compared to the typical method of sample storage and transport to a central lab.

The development and implementation of pathogen, heavy metal, and drug compound sensors into point-of-need devices and testing programs has the potential to radically transform the availability and ease of collection of a wide range of critical data. By utilizing easily swappable and commercially available components, we found that our cost-effective electrochemical assay for bacterial pathogens could be transferred to an external lab and utilized in complex and varied samples for successful bacterial quantification and detection. The use of 3D-printed electrodes for MDMA detection allowed us to analyze real-world samples and easily determine drug dosage, an important step towards point-of-need testing for harm reduction. Finally, the use of a low-cost detection card and preconcentration system, and a smartphone-based quantification method allows an easier path to widespread environmental testing for heavy metal contaminants at relevant limits. Overall, the work in this dissertation demonstrates the power of simple and reliable engineering improvements to analytical systems for point of need testing. It is my hope that this work furthers the fields of analytical, environmental, and biochemistry by providing a small stepping stone towards widespread usage of low-cost analytical devices.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Alan Kennan for his unending support, trust, confidence, and guidance throughout my Ph.D. It has truly shaped my experience, and enabled me to learn, work, and collaborate daringly, always knowing Alan was in my corner through success and failure. Secondly, I would like to thank Dr. Chuck Henry for his support, guidance, and the incredible lab culture he has built on the fourth floor. I could never have dreamed of working in a lab that was as caring, inclusive, and supportive as the Henry lab. It is an inspiration to me in terms of science, people, possibilities, and collaboration that I hope to carry on in my work.

I would also like to thank my lab mates in the Henry group, both past and present. Dr. Thaisa Baldo, our outstanding post-doc throughout most of my Henry lab career, as well as Dr. Prakash Aryal, and Elisa Botelli, members who collaborated with me on the papers in this work. Additionally, I would like to thank the community members of Fort Collins, CSU, and the Chemistry Department who I have grown to know. Those that drop by the stockroom to chat, Janis at the front desk, the Dutch Bros coffee crew, my neighbors, acquaintances, and strangers who I have accosted about my research, willingly or not.

Finally, and most importantly, I would like to thank, and dedicate this work to, my family. To my partner, Charlie, for his eternal kindness, love, and ambition in life, and his tremendous strength and support throughout my Ph.D. To my brothers, Neil and Aaron, for aiming so high and achieving so much that I feel obligated to chase. To my parents, Ann and Greg Boes, NASA

engineers, incredible teachers, and the most kind and loving parents anyone could ask for. To the rest of my family, extended, found, and metaphorical, without who's support I would not have had the ability or courage to dream so big and fly so high. I love y'all. Thank you.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENTS	v
Chapter 1 – Introduction	1
1.1 Food and Waterborne Diseases	1
1.2 Point-of-Need Analysis in Food and Water	4
1.3 Passive Microfluidic Devices for Point of Need Testing	5
1.4 Electrochemical Point of Need Devices	6
1.5 Contributions	8
References	11
Chapter 2 – A Bead-Based Electrochemical Immunoassay For Pathogenic Bacteria Towards Point of Need Monitoring of Food and Water	15
2.1 Introduction	15
2.2 Materials and Methods	20
2.3 Results & Discussion	30
2.4 Conclusion	44
References	48
Chapter 3 – A Portable 3D-Printed System for In-Field Electrochemical Detection of MDMA in Unknown Tablets	51
3.1 Introduction	51
3.2 Materials and Methods	54
3.3 Results and Discussion	59
3.4 Conclusions.....	73
References	75
Chapter 4 – A Low-Cost 3D-Printed Preconcentration and Foldable Paper Card Detection System for Heavy Metals Analysis in River and Drinking Water	78
4.1 Introduction	78
4.2 Materials and Methods	82
4.3 Results and Discussion	86
4.4 Conclusions	101
References	103
Chapter 5 – Conclusions and Future Directions	108
Appendix A – Chapter Two Supplementary	110
Appendix B – Chapter Three Supplementary	120
Appendix C – Chapter Four Supplementary	123

CHAPTER ONE: INTRODUCTION

1.1 Food and Waterborne Diseases

Globally, food and waterborne pathogens are estimated to cause more than 400,000 deaths annually, with the majority in low and lower middle-income countries (LMIC).¹ These resource-limited areas are particularly vulnerable to pathogen outbreaks, often due to a systemic lack of access to reliable clean water sources.²⁻⁴ The systemic nature of the problem complicates mitigation efforts, and monitoring of new and ongoing outbreaks can be beyond the means of local health services.^{4,5}

Domestically, the annual cost of foodborne pathogens was estimated at 74 billion USD in 2023.⁶ While the pathogen detection and monitoring system in the US is strong in comparison to many parts of the world, the ubiquity of pathogenic bacteria means that the problem can never be truly erased.⁵ Additionally, recent transitions in the US Food and Drug Administration (FDA),¹¹ the US exit from the World Health Organization (WHO),¹² and the embracing of alternative health practices by the Department of Health and Human Services (HHS)^{13,14} may foreshadow an increase in domestic US foodborne bacteria concerns.

Escherichia coli, *Salmonella enterica* and *Listeria monocytogenes* make up a large portion of pathogenic bacteria found in food sources due to their hardiness and ease of spread. Approximately 45% of global deaths due to food pathogens are attributed to these bacteria.¹ Food is typically contaminated during preparation or packaging by human workers. While *Salmonella* and *Listeria* are typically only expelled during active infection, *E. coli* is ubiquitous in

the lower intestine of most mammals and is therefore used as an indicator organism for potential fecal contamination.

One of the major factors in foodborne illness and bacterial contamination is access to clean water and sanitation facilities.³ Great progress has been made on access to basic water sources, with the global population of people lacking an improved source of drinking water dropping from 18% in 2000 to 8.5% in 2024.³ However, much of the global population still lacks safely managed water. As infrastructure improvements age, weaknesses are being revealed in the form of bacterial or chemical contamination issues. Even if a practical and convenient source of water is available, bacterial contamination can make handwashing ineffective for food protection.^{2,4,7} Cooking with contaminated water is also in many cases hazardous, even if mitigation procedures such as boiling are utilized.^{4,7}

Some local water access improvements, such as rooftop tanks, can be susceptible to bacterial contamination, especially when maintenance is neglected.⁷ However, largescale bacterial or chemical contamination across a region is also prevalent, especially in the wake of mismanagement, environmental damage, or natural disaster.^{8,9} Finally, both outdated plumbing and incorrectly manufactured or installed new fixtures can cause dangerously increased levels of heavy metals in drinking water.¹⁰

Unfortunately, testing for both bacterial and metal contamination issues is extremely complicated due to the low relevant pathogen concentrations, widely prevalent background bacteria, and complex sample mediums. While 'off' flavor or smell may be reported, in many cases there may be no external indication that water is unsafe.¹⁵ Coliform analysis via agar

plating is the most common bacterial test on drinking water and must be done in a lab environment to accurately assess bacterial contamination.¹⁶ Singlehousehold sampling involves collection of 100 mL per sample for bacterial tests, often from multiple water sources. The logistics of properly collecting, shipping, and analyzing in a central facility are complicated and costly to implement on a large scale, which can hinder scientific research, regulatory monitoring, and personal testing.¹⁷

As recently highlighted by the COVID-19 pandemic, widespread analytical testing is often the only solution to tracking and understanding large-scale disease.¹⁸ Similarly, in environmental monitoring often the questions being studied necessitate large-scale testing. The traditional model of sample collection, storage, transportation, and eventual testing is inefficient and impractical in many situations, especially in LMIC where public health and laboratory resources are often severely limited.¹⁹ Timeliness of results and ease of use are two of the most important factors in large-scale data collection.¹⁸

To address these challenges, point-of-need testing devices have come into the forefront of health and environmental research in resource-limited settings.¹⁹ In 2003, the WHO Special Programme for Research and Training in Tropical Diseases published the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-free, Delivered) criteria, a set of real-world guidelines for developing ideal assays for use in the developing world at the point of care.²⁰ In the years since, advances in device materials, detection methodologies, and miniaturization have led to a wide variety of commercial point-of-need analytical devices for health and environmental testing, and great improvements in resource-limited testing availability.^{5,21}

However, point of need devices for monitoring bacteria in food and heavy metals at regulatory limits in drinking water remain relatively underdeveloped.

1.2 Point-of-Need Analysis in Food and Water

Bacteria are complicated targets for point of need assays. They typically require signal amplification in the form of antibody tagging with nanoparticles or enzymes to yield a detectable signal in a point of need device.²² While this enhances sensitivity, it can also lead to issues in the form of false positives when encountering complex samples. Most food samples are not 'clean' enough to run through typical point of need devices without prior sample preparation, which complicates the assay procedure, especially for untrained users.²³ Even in water samples, where the matrix is simple, lateral flow assays for bacteria tend to have very high limits of detection due to the need for bacterial concentration onto a test line to develop signal.^{22, 23}

Similarly, 'test strips' for heavy metals are readily available but are typically not capable of detecting down to guideline levels for drinking water safety. In this case, while the matrix is relatively clean, the limits for heavy metals in drinking water are very low, often ppb, which is typically lower than is practically detectable on a colorimetric test strip.²⁴ Portable heavy metal testing kits are available but are often economically unfeasible for personal use or widespread use in LMIC, and require sample prep work.^{21,25} Some work has been done in creating affordable point-of-need sensors for heavy metals, but most can

only detect a single ion, and are typically optimized for precise and targeted sampling of contaminated water sources, rather than widespread screening usage.²¹

Electrochemical sensors are an attractive option for point of need testing due to their sensitivity, multiplexing performance, and the ease of integration into both electronic and flow devices. However, their usage is often limited by a lack of specificity, or by surface fouling due to complex matrix compounds.²⁶ One mitigating technique is the use of enzymes for target tagging in an ELISA format, and the subsequent turnover of an electrochemically active substrate molecule. Recent developments in hollow-channel passively driven microfluidic ELISA systems,²⁷ as well as work in passive sample preconcentration from complex matrices utilizing magnetic beads²⁸ have helped to bring non-LFA ELISA closer to practicality in a point of need setting.

1.3 Passive Microfluidic Devices for Point of Need Testing

In contrast with typical microfluidics, which often utilize syringe pumps, external detection elements, and computer control, passive point of need microfluidic devices utilize a no-moving-parts approach to fluidic control. Gravity-driven²⁹, capillary-driven²⁷, and passive-pump devices^{27,30} provide opportunities to repeatably preconcentrate samples, mitigate complex matrices, and conduct lateral flow or bulk flow assays in the field. Common system variables, such as flow rate, reactant concentrations, and timing can be controlled by engineered design of the device, allowing assay optimization towards ease of use and reliability.³¹

However, the complexity of buffer optimization, biological recognition dynamics, enzyme kinetics, and fluid flow mean that the ‘sweet spot’ of optimization on a particular assay, device, or combination of the two is often extremely narrow. This can manifest as sample preparation sensitivity. Out-of-spec samples can cause complete system failure; clogging issues in lateral flow, deactivation of biorecognition elements, or elevation of background signal are all commonly seen failure modes.³¹ Additionally, point of need assays often necessitate direct sampling by the end-user, compounding the likelihood of issues with each sample preparation step.

In Chapter Four of this work, a novel passive microfluidic system is developed for preconcentration of heavy metal ions onto paper pads in preparation for colorimetric analysis, allowing the end user to prep sample by simply adding to a preconcentration tank and allowing it to drain through the detection card. This enables a single-step, no-movingparts 2500x preconcentration of the target, substantially lowering the limit of detection of the assay. The relatively clean intended matrix of drinking or river water combined with easily accessible large-volume samples allowed us to achieve simultaneous detection of 8 heavy metals at regulatory-relevant concentrations.

1.4 Electrochemical Point of Need Devices

The first successful electrochemical point of need testing device was the blood glucose strip, developed in the 1970s.³² The success of the device is due to the high concentration of available glucose in the bloodstream (700 – 2000 mg/L, 3.9 - 11 mM, in a typical healthy adult)

and the extremely stable and reliable performance of glucose oxidase and the 1:1 ferrocene-mediated electron transfer. However, commercial electrochemical biosensors are still relatively rare given the 50 year history. Simply, most analytes of interest in biosystems are not at electrochemically friendly concentrations in target matrices and are not directly measurable via enzyme-mediated reactions.²⁶

More typically, analytes of interest are in lower concentrations than most other matrix components and analyte recognition elements must be utilized for preconcentration onto a well plate, bead, or other matrix.²⁶ Labeling is often necessary for signal amplification, requiring the use of a calibration curve for quantification.³³ The loss of 1:1 electron transfer typically somewhat harms the ability of the assay to quantify accurately over a single run. However, electrochemical optimization, statistical data fitting, and replicate pooling can help make up for this. Electrochemical enzymatic assays performed in a well plate often perform as well as colorimetric plate-based enzymatic assays when provided a large pool of fitting data.³³

For enzymatic assays, electrochemical sensors perform particularly well due to the versatility of usage provided by even a basic potentiostat for data collection, allowing substantial method fine-tuning.³³ Interest in electroanalytical methodologies for use in LMIC has given rise to several low-cost alternatives to traditional instrumentation for analysis.³⁴ Standalone or phone-driven potentiostats can be acquired for < \$500 as of March 2026, low-cost NFC-driven potentiostats are available for \$50,³⁵ and open-source PCB designs are readily available,³⁶ drastically lowering the barrier for entry to electrochemical

analysis. Finally, the other main benefit offered by electrochemical detection modalities is ease of multiplexing. Many electroanalysis techniques are ready for integration into a multiplexed system, either by physical separation on discrete working electrodes, or by simultaneous multiple peak analysis.³⁷

In Chapter Two of this work, an electrochemical assay is developed for whole-cell bacteria in complex matrix samples. In this case, the electrochemical assay is designed for future implementation into a self-driven multiplexed flow assay system. Electrochemical signal analysis enables the future use of enzyme mixtures for multiplexing. In Chapter Three of this work, 3D-printed electrodes are developed for MDMA content analysis in seized pills. Electrochemical optimization allows specific targeting of the MDMA peak in the complex matrix, achieving sensitive and specific measurement of the target analyte utilizing a low-cost alternative electrode material.

1.5 Contributions

The bulk of this work (Chapters Two, Three, and Four) is comprised of research papers submitted for publication, in preparation, or published. Please see below, or corresponding title pages for information. All three papers were collaborative efforts with colleagues, with substantial contributions from all parties. Jason Boes directed and led the work in Chapter Two and equally collaborated with Prakash Aryal in developing the work presented in Chapter Four. Elisa Botelli led the work presented in Chapter Three, with Jason Boes designing the assay, manufacturing electrodes, conducting electrochemical analysis, and editing the paper.

The first paper presented in this work is in preparation for submission to *Analytical Chemistry* and appears as in Chapter Two. The paper was prepared and developed in collaboration with the US Army DEVCOM for external validation testing. The work was coauthored with Shannon McGraw-Manza, Charles S. Henry, and Alan Kennan. Jason Boes was responsible for conceptualization, design, engineering, data collection and analysis, drafting, and editing. Charles S. Henry was responsible for funding, conceptualization, editing, and advising. Alan Kennan was responsible for editing and advising. Shannon McGraw-Manza was responsible for external validation data collection, and editing.

The second paper presented, a 3D-printed electrochemical sensor for MDMA analysis in seized pill samples, has been submitted to *Analytical Chemistry* and appears as in Chapter Three. The work was coauthored with Elisa Botelli, Maxwell D. Bridges, Thaisa A. Baldo, Lucas F. de Lima, Larissa M. A. Melo, Wendell K. T. Coltro, Luciano C. Arantes, William R. de Araujo, and Charles S. Henry. Jason Boes was responsible for conceptualization, engineering and design, electrochemical analysis, and manufacturing of electrodes. Elisa Botelli was responsible for conceptualization, electrochemical analysis, manufacturing and activation of electrodes, data collection and analysis, drafting, and editing. Chuck Henry was responsible for conceptualization, funding acquisition, advising, and editing. Other authors were also responsible for drafting and editing, as well as supporting data collection, analysis, and experimental design.

The third paper presented has been published in ACS Sensors and appears in Chapter Four as published. The work was coauthored with Prakash Aryal, Chuck Henry,

Eric Brack, and Todd Alexander. Jason Boes was responsible for conceptualization, engineering and design, manufacturing, drafting, and editing. Prakash Aryal was responsible for conceptualization, engineering and design, manufacturing, data collection and analysis, drafting, and editing.

References

- 1) World Health Organization. Foodborne Diseases Estimates.
<https://www.who.int/data/gho/data/themes/who-estimates-of-the-global-burden-offoodborne-diseases> (accessed May 3 2026).
- 2) Allegranzi, B.; Tartari, E.; Kilpatrick, C.; Storr, J.; Bellare, N.; Bana, J.; Santos, A. F.; Charnaud, S.; Ross, A. L.; Schwaber, M. J.; Pittet, D. WHO Global Research Agenda for Hand Hygiene Improvement in Health Care: A Delphi Consensus Study.
Infect. Control Hosp. Epidemiol. **2025**, *46* (5), 449–464.
<https://doi.org/10.1017/ice.2025.32>.
- 3) Progress on household drinking water, sanitation and hygiene 2000–2024: special focus on inequalities. Geneva: World Health Organization (WHO) and the United Nations Children’s Fund (UNICEF), 2025. Licence: CC BY-NC-SA 3.0 IGO.
- 4) Bell, R. L.; Kase, J. A.; Harrison, L. M.; Balan, K. V.; Babu, U.; Chen, Y.; Macarisin, D.; Kwon, H. J.; Zheng, J.; Stevens, E. L.; Meng, J.; Brown, E. W. The Persistence of Bacterial Pathogens in Surface Water and Its Impact on Global Food Safety.
Pathogens **2021**, *10* (11), 1391. <https://doi.org/10.3390/pathogens10111391>.
- 5) Jayatilleke, K. Challenges in Implementing Surveillance Tools of High-Income Countries (HICs) in Low Middle Income Countries (LMICs). *Curr Treat Options Infect Dis* **2020**, *12* (3), 191–201. <https://doi.org/10.1007/s40506-020-00229-2>.
- 6) Hoffmann, S.; White, A. E.; McQueen, R. B.; Ahn, J.-W.; Gunn-Sandell, L. B.; Scallan Walter, E. J. Economic Burden of Foodborne Illnesses Acquired in the United States.
Foodborne Pathogens and Disease **2025**, *22* (1), 4–14.
<https://doi.org/10.1089/fpd.2023.0157>.
- 7) Rubino, F.; Corona, Y.; Jiménez Pérez, J. G.; Smith, C. Bacterial Contamination of Drinking Water in Guadalajara, Mexico. *IJERPH* **2018**, *16* (1), 67.
<https://doi.org/10.3390/ijerph16010067>.
- 8) Levin, R.; Villanueva, C. M.; Beene, D.; Cradock, A. L.; Donat-Vargas, C.; Lewis, J.; Martinez-Morata, I.; Minovi, D.; Nigra, A. E.; Olson, E. D.; Schaidler, L. A.; Ward, M. H.; Deziel, N. C. US Drinking Water Quality: Exposure Risk Profiles for Seven Legacy and Emerging Contaminants. *J Expo Sci Environ Epidemiol* **2024**, *34* (1), 3– 22.
<https://doi.org/10.1038/s41370-023-00597-z>.
- 9) Campbell, C.; Greenberg, R.; Mankikar, D.; Ross, R. A Case Study of Environmental Injustice: The Failure in Flint. *IJERPH* **2016**, *13* (10), 951.
<https://doi.org/10.3390/ijerph13100951>.
- 10) US Consumer Product Safety Commission. Lead in Water Faucets.
<https://www.cpsc.gov/Safety-Education/Safety-Education-Centers/Lead-in-WaterFaucets> (accessed May 3 2026).
- 11) Laurel Oldach, Chemical and Engineering News. How will FDA changes reshape drug approval in 2025 and beyond?
<https://cen.acs.org/pharmaceuticals/drugdevelopment/FDA-changes-reshape-drug-approval/103/web/2025/05> (accessed May 3, 2026).

- 12) Alice Park, TIME USA. The U.S. Has Pulled Out of the WHO. Here's What That Means for Public Health. <https://time.com/7357180/us-withdrawal-who-world-healthorganization/> (accessed May 3, 2026).
- 13) Lucey, J. A. Raw Milk Consumption: Risks and Benefits. *Nutrition Today* **2015**, 50 (4), 189–193. <https://doi.org/10.1097/NT.000000000000108>.
- 14) Madeleine Aggeler, The Guardian. The truth about raw milk and why experts are 'absolutely horrified' by the trend. <https://www.theguardian.com/wellness/2024/nov/26/what-is-unpasteurized-raw-milk> (accessed May 3, 2026).
- 15) Lin, L.; Yang, H.; Xu, X. Effects of Water Pollution on Human Health and Disease Heterogeneity: A Review. *Front. Environ. Sci.* **2022**, 10, 880246. <https://doi.org/10.3389/fenvs.2022.880246>.
- 16) Tambi, A.; Brighu, U.; Gupta, A. B. Methods for Detection and Enumeration of Coliforms in Drinking Water: A Review. *Water Supply* **2023**, 23 (10), 4047–4058. <https://doi.org/10.2166/ws.2023.247>.
- 17) Hansen, S.; Abd El Wahed, A. Point-Of-Care or Point-Of-Need Diagnostic Tests: Time to Change Outbreak Investigation and Pathogen Detection. *TropicalMed* **2020**, 5 (4), 151. <https://doi.org/10.3390/tropicalmed5040151>.
- 18) Farmer, S.; Razin, V.; Peagler, A. F.; Strickler, S.; Fain, W. B.; Damhorst, G. L.; Kempker, R. R.; Pollock, N. R.; Brand, O.; Seitter, B.; Heilman, S. S.; Nehl, E. J.; Levy, J. M.; Gottfried, D. S.; Martin, G. S.; Greenleaf, M.; Ku, D. N.; Waggoner, J. J.; Iffrig, E.; Mannino, R. G.; F. Wang, Y.; Ortlund, E.; Sullivan, J.; Rebolledo, P. A.; Clavería, V.; Roback, J. D.; Benoit, M.; Stone, C.; Esper, A.; Frank, F.; Lam, W. A. Don't Forget about Human Factors: Lessons Learned from COVID-19 Point-of-Care Testing. *Cell Reports Methods* **2022**, 2 (5), 100222. <https://doi.org/10.1016/j.crmeth.2022.100222>.
- 19) Heidt, B.; Siqueira, W.; Eersels, K.; Diliën, H.; Van Grinsven, B.; Fujiwara, R.; Cleij, T. Point of Care Diagnostics in Resource-Limited Settings: A Review of the Present and Future of PoC in Its Most Needed Environment. *Biosensors* **2020**, 10 (10), 133. <https://doi.org/10.3390/bios10100133>.
- 20) Land, K. J.; Boeras, D. I.; Chen, X.-S.; Ramsay, A. R.; Peeling, R. W. REASSURED Diagnostics to Inform Disease Control Strategies, Strengthen Health Systems and Improve Patient Outcomes. *Nat Microbiol* **2018**, 4 (1), 46–54. <https://doi.org/10.1038/s41564-018-0295-3>.
- 21) Nayan Kumar, H. N.; Nagaraju, D. H.; Yhobu, Z.; Shivakumar, P.; Manjunatha Kumara, K. S.; Budagumpi, S.; Praveen, B. M. Recent Advances in On-Site Monitoring of Heavy Metal Ions in the Environment. *Microchemical Journal* **2022**, 182, 107894. <https://doi.org/10.1016/j.microc.2022.107894>.
- 22) Clerc, O.; Greub, G. Routine Use of Point-of-Care Tests: Usefulness and Application in Clinical Microbiology. *Clinical Microbiology and Infection* **2010**, 16 (8), 1054– 1061. <https://doi.org/10.1111/j.1469-0691.2010.03281.x>.

- 23) Kakkar, S.; Gupta, P.; Singh Yadav, S. P.; Raj, D.; Singh, G.; Chauhan, S.; Mishra, M. K.; Martín-Ortega, E.; Chiussi, S.; Kant, K. Lateral Flow Assays: Progress and Evolution of Recent Trends in Point-of-Care Applications. *Materials Today Bio* **2024**, 28, 101188. <https://doi.org/10.1016/j.mtbio.2024.101188>.
- 24) Cui, W.; Ren, Z.; Song, Y.; Ren, C. L. Development and potential for point-of-care heavy metal sensing using microfluidic systems: A brief review. *Sensors and Actuators A: Physical*. **2022**, 344, 113733. <https://doi.org/10.1016/j.sna.2022.113733>.
- 25) Hach. CEL Advanced Drinking Water Laboratory. <https://www.hach.com/p-celadvanced-drinking-water-laboratory/251235> (accessed May 3, 2026).
- 26) Campuzano, S.; Pingarrón, J. M. Electrochemical Affinity Biosensors: Pervasive Devices with Exciting Alliances and Horizons Ahead. *ACS Sens.* **2023**, 8 (9), 3276–3293. <https://doi.org/10.1021/acssensors.3c01172>.
- 27) Carrell, C.; Jang, I.; Link, J.; Terry, J. S.; Call, Z.; Panraksa, Y.; Chailapakul, O.; Dandy, D. S.; Geiss, B. J.; Henry, C. S. Capillary Driven Microfluidic Sequential Flow Device for Point-of-Need ELISA: COVID-19 Serology Testing. *Anal. Methods* **2023**, 15 (22), 2721–2728. <https://doi.org/10.1039/D3AY00225J>.
- 28) Fortunati, S.; Giannetto, M.; Giliberti, C.; Mattarozzi, M.; Bertucci, A.; Careri, M. Magnetic Beads as Versatile Tools for Electrochemical Biosensing Platforms in Pointof-Care Testing. *Analysis & Sensing* **2024**, 4 (3), e202300062. <https://doi.org/10.1002/anse.202300062>.
- 29) Vahabi, H.; Liu, J.; Dai, Y.; Joh, D. Y.; Britton, R.; Heggstad, J.; Kinnamon, D.; Rajput, S.; Chilkoti, A. A Gravity-Driven Droplet Fluidic Point-of-Care Test. *Device* **2023**, 1 (1), 100009. <https://doi.org/10.1016/j.device.2023.100009>.
- 30) Pradela-Filho, L. A.; Veloso, W. B.; Arantes, I. V. S.; Gongoni, J. L. M.; De Farias, D. M.; Araujo, D. A. G.; Paixão, T. R. L. C. Paper-Based Analytical Devices for Pointof-Need Applications. *Microchim Acta* **2023**, 190 (5), 179. <https://doi.org/10.1007/s00604-023-05764-5>.
- 31) Chiu, D. T.; deMello, A. J.; Di Carlo, D.; Doyle, P. S.; Hansen, C.; Maceiczky, R. M.; Wootton, R. C. R. Small but Perfectly Formed? Successes, Challenges, and Opportunities for Microfluidics in the Chemical and Biological Sciences. *Chem* **2017**, 2 (2), 201–223. <https://doi.org/10.1016/j.chempr.2017.01.009>.
- 32) Waly, Y.; Hussain, A.; Al-Majmuei, A.; Alatoom, M.; Alaraibi, A. J.; Alaysereen, A.; Deen, G. R. Evolution of Biosensors and Current State-of-the-Art Applications in Diabetes Control. *Biosensors* **2026**, 16 (1), 39. <https://doi.org/10.3390/bios16010039>.
- 33) Arya, S. K.; Estrela, P. Recent Advances in Enhancement Strategies for Electrochemical ELISA-Based Immunoassays for Cancer Biomarker Detection. *Sensors* **2018**, 18 (7), 2010. <https://doi.org/10.3390/s18072010>.

- 34) Rainbow, J.; Sedlackova, E.; Jiang, S.; Maxted, G.; Moschou, D.; Richtera, L.; Estrela, P. Integrated Electrochemical Biosensors for Detection of Waterborne Pathogens in Low-Resource Settings. *Biosensors* **2020**, *10* (4), 36. <https://doi.org/10.3390/bios10040036>.
- 35) Beck, J. J.; Alenicheva, V.; Rahn, K. L.; Russo, M. J.; Baldo, T. A.; Henry, C. S. Evaluating the Performance of an Inexpensive, Commercially Available, NFCpowered and Smartphone Controlled Potentiostat for Electrochemical Sensing. *Electroanalysis* **2023**, *35* (6), e202200552. <https://doi.org/10.1002/elan.202200552>.
- 36) von Zuben, T. W.; Salles, A. G.; Bonacin, J. A. Low-cost open-source potentiostats: a comprehensive review of DIY solutions and fundamental concepts of electronics and its integration with electrochemistry. *Electrochimica Acta* **2024**, 498, 144619. <https://doi.org/10.1016/j.electacta.2024.144619>
- 37) Zupančič, U., Rainbow, J., Flynn, C., Aidoo-Brown, J., Estrela, P., Moschou, D. (2021). *Strategies for Multiplexed Electrochemical Sensor Development*. In: Dutta, G., Biswas, A., Chakrabarti, A. (eds) *Modern Techniques in Biosensors. Studies in Systems, Decision and Control*, vol 327. Springer, Singapore. https://doi.org/10.1007/978-981-15-9612-4_3

CHAPTER TWO: A BEAD-BASED ELECTROCHEMICAL IMMUNOASSAY FOR PATHOGENIC BACTERIA TOWARDS POINT OF NEED MONITORING OF FOOD AND WATER

1. Introduction

Foodborne pathogens are one of the global leading causes of disease, with more than 400,000 deaths yearly, mostly in young children and older adults; populations which are particularly susceptible due to lowered immune system response.¹⁻³ Low and lower middle income countries (LMICs) as well as rural communities are especially vulnerable to bacterial outbreaks, and often local public health infrastructures are not prepared for large-scale monitoring or response.⁴ *Escherichia Coli*, and *Salmonella Enterica* are among the most common foodborne illness-causing bacteria, and can cause nausea, diarrhea, systemic infection, and death. To combat this, many areas implement bacterial testing of food and water sources, but this is challenging in resource-limited settings.^{5,6}

Coliform analysis on agar, polymerase chain reaction (PCR) screening, and plate-based enzyme linked immunosorbent assay (ELISA) are all current commonly used techniques for pathogenic bacterial detection, but bring distinct disadvantages in assay complexity and cost, requiring dedicated scientists and lab environments to ensure accuracy.⁷ PCR and ELISA are also heavily dependent on instrumentation, making them challenging to implement in a resource-limited setting.⁸⁻¹⁰

Some point-of-need lateral flow assay (LFA) based detection methods have been developed, but often lack the sensitivity required for pathogen screening, especially in food samples.¹¹ Additionally, foodborne pathogens are found in a wide variety of complex sample

matrices, which can greatly complicate sample preparation and hinder detection efforts.^{12,13}

LFAs are particularly susceptible to physical matrix effects such as particles and fat globules due to their need for consistent flow behavior.^{11,13}

Due to the ubiquity of nonpathogenic bacteria in food samples, specificity in any pathogenic bacterial assay is paramount.^{14,15} Agar-colonization detection methodologies often require the use of selective agar materials to inhibit growth of non-targeted bacteria in the sample.¹⁴ Use of biorecognition elements such as the antibodies often used in ELISA methodologies can improve selectivity significantly. However, plate-based ELISA detection, while often effective for bacterial targets, typically necessitates extensive sample preparation steps for complex matrix removal.¹³ Additionally, manual ELISA performance is heavily influenced by operator skill and requires the use of microbiological lab facilities. Automated solutions are typically expensive and not practically deployable to a field setting.^{6,7}

Lateral flow assays for point-of-need bacterial detection have also recently come into usage, however they typically suffer a lack of sensitivity to whole-cell bacteria as well as interference from complex matrices such as those found in food samples.^{1,11,12} These issues are typically due to the fundamental working principles of the assay, where antibody-tagged gold nanoparticles must build up to a visually detectable level, and consistency in flow is important for repeatable bacteria-antibody interactions.¹¹ This limits their usage to clean highly concentrated bacterial samples, or, in the case of alternative targets such as DNA, necessitates extensive sample preparation involving bacterial lysing and removal of matrix material.^{13,16}

Antibody-functionalized magnetic microbeads have been used for bacterial separation from complex matrices extensively in the literature.¹⁷⁻²⁴ The small size of the beads allows them to disperse through a complex sample matrix efficiently, and they can be magnetically separated from matrices containing a large portion of solids, rather than relying on removal of solids during sample preparation.¹⁷ However, the processing of these beads is typically still completed manually by lab personnel, and actual detection is accomplished via colorimetric plate ELISA, PCR, or agar colonization, resulting in similar lab and personnel requirements as the aforementioned bacterial assays.¹⁸

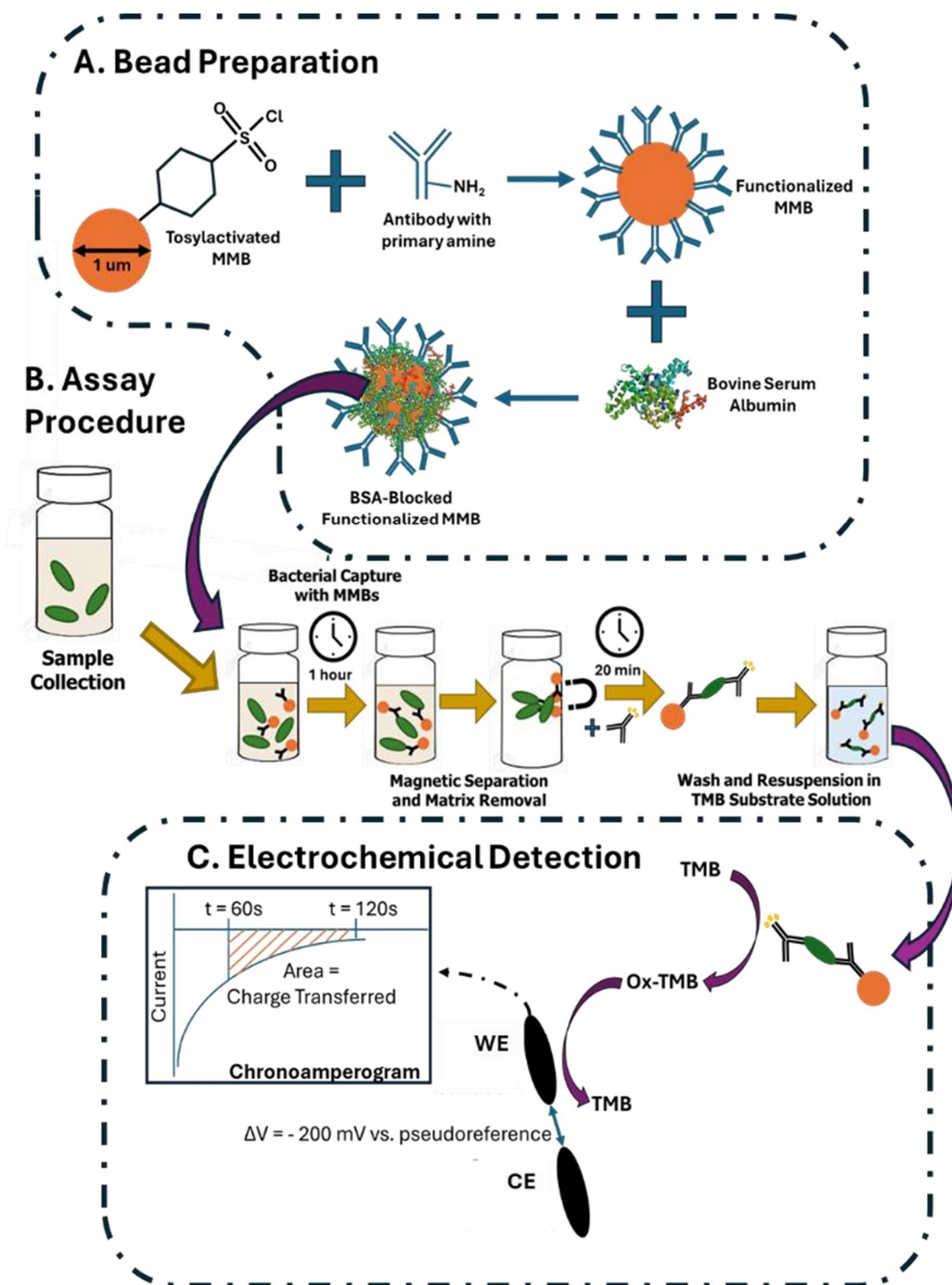
In order to help bridge this gap, we developed a magnetic bead-based electrochemical ELISA for bacterial pathogens to take advantage of the sensitivity, ease of miniaturization, and increasingly low cost of electrochemical measurement devices.^{25,26} The assay was specifically designed as a foundational, easily modified tool for bacterial detection, enabling simple target customization by swapping out antibodies without requiring protocol modification. This allows end-users to train for this system once, expanding the potential usage of the assay for alternative targets without expanding the technical knowledge required to generate high quality data. We aim to integrate this testing assay into a point-of-need (PoN) device, enabling in-field screening for bacterial pathogens.

The assay developed in this work is based around a magnetic microbead (MMB), conjugated with pathogen-specific antibodies to specifically capture bacteria from a complex matrix. After incubating the MMB with the sample (**Figure 1 A**), the beads are removed via magnetic separation, and the supernatant containing non-targeted bacteria and complex media components is discarded (**Figure 1 B**). The MMB-bacteria mixture is washed to further ensure

only targeted bacteria remain attached to beads and is then labeled with an enzyme-conjugated secondary antibody that is also specific for the target bacteria. After another wash to remove unbound antibody, the MMB-bacteria-enzyme sandwich is transferred to a solution containing enzyme substrate. Oxidized substrate is detected via chronoamperometry, with the total charge transfer correlating to bacterial concentration (**Figure 1 C**).

The sandwich ELISA format ensures excellent specificity in the assay, as both capture and labeling antibodies must remain bound through the wash processes to produce a positive signal. The use of an enzyme as the labeling component allows for signal amplification based on substrate incubation time as the substrate molecules are continually turned over by enzyme exposure, allowing tuning of the sensitivity of the assay.^{18,27,28} The use of a magnetic bead as the base for the sandwich ELISA allows improved capture dynamics when compared to a flat plate ELISA, since the beads can distribute throughout a complex matrix sample more efficiently, and have an increased chance of contacting and capturing each bacterium.¹⁷ These factors allowed us to effectively optimize the assay for whole-cell bacteria in complex matrices, achieving improved LoDs when compared to typical point-of-need bacterial assays, and allowing a minimally trained end user to successfully detect and quantify bacterial contamination in food.

The assay was developed in collaboration with the United States Army Combat Capabilities Development Command (DEVCOM), for deployment in resource-limited settings where traditional laboratory techniques for food pathogen analysis are logistically challenging. DEVCOM independently evaluated operator use and performance of the assay in their own lab environment for detection of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in pure culture dilutions as well as in multiple food types. DEVCOM evaluation was



performed directly from protocols, methodologies, and materials lists provided by CSU (**Appendix A**). Electrode arrays were provided by CSU, with the rest of the assay materials procured directly by DEVCOM.

2. Materials and Methods

To make the assay as accessible as possible, common commercially available materials were selected for use when possible. Electrodes were manufactured in-house at CSU, but the assay does not rely on the specific electrode utilized. Commercially available single-use electrodes such as Screen Printed Electrodes (SPEs) manufactured by Dropsens or others should also perform similarly in the assay format. Written protocols were prepared at CSU for assay validation by DEVCOM.

Assay Materials and Preparation

Phosphate buffered saline (PBS) tablets (11340439) and tetramethylbenzidine (TMB) substrate solution (N301) were purchased from Thermo Fisher Scientific (Waltham, MA). Tween-20, bovine serum albumin fraction V (BSA), sodium hydroxide, and hydrochloric acid were purchased from Sigma-Aldrich (St. Louis, MO). Microcentrifuge tubes (1.5mL) were purchased from VWR (Radnor, PA). PBS was prepared as instructed from tablet, adjusted to pH 7.4 with NaOH or HCl, sterilized via autoclaving, and stored at 4°C. Washing buffer (PBST) was prepared

from above PBS with the addition of 0.1% W/V Tween-20 and stored at 4°C. Full buffer preparation protocols are available in *Buffer & Solution Preparation (Appendix A)*.

Bacterial Strains & Media

Bacteria strains of *E. coli* O157:H7 (ATCC 43895), *E. coli* DH5α (ATCC 47093), and *Salmonella Enterica* (ATCC TA 1535) were purchased from ATCC (Gaithersburg, MD). Dry LB broth (Miller), dry BHI broth and agar were purchased from Sigma-Aldrich and prepared as per manufacturer instructions. Agar plates were prepared using 1.5% agar and respective growth media and were stored at 4°C until use. Bacteria were grown to exponential phase, aliquoted, frozen, and stored at -20°C. Prior to use in assay, bacteria were thawed, spiked into 100 mL growth media in a 500 mL Erlenmeyer flask, and grown to exponential phase with continual mixing. Bacteria were diluted to working concentrations in PBS. Triplicate serial dilutions and subsequent agar plating post spiking were utilized to confirm whole cell bacterial concentration in the tested samples.

Antibodies and Conjugation Materials:

Primary and secondary antibodies for capture and labeling (See Table 1) were purchased from Bioss (Woburn, MA), LS Bio (Seattle, WA), Sino Biological (Beijing, China), Abcam (Cambridge, UK), Virostat (Westbrook, Maine), and Invitrogen (Washington, DC). Primary antibodies were conjugated with biotin Lightning-Link conjugation kits from Abcam (Cambridge, UK) as needed for streptavidin bead systems and remained unconjugated for tosylactivated beads; secondary antibodies were labeled with HRP as needed utilizing HRP Lightning-Link

conjugation kits from Abcam (Cambridge, UK). Manufacturer instructions were followed for lightning-link conjugations. Conjugated antibodies were diluted to working concentrations with PBS buffer.

Table 1: Antibodies Utilized for Capture & Labeling

Manufacturer	Bacterial Target	Manufacturer ID
Bioss	<i>E. coli</i>	bs-2033R
LS Bio	<i>E. coli</i>	LS-C507846-100
Sino	<i>E. coli</i>	13102-MM09
Sino	<i>E. coli</i>	40066-RP01
Abcam	<i>E. coli</i>	Ab20640
Abcam	<i>E. coli</i>	Ab35292
Abcam	<i>Salmonella</i>	Ab69255
Abcam	<i>Salmonella</i>	Ab35156
Abcam	<i>Salmonella</i>	Ab20506
Abcam	<i>Salmonella</i>	Ab8274
Bioss	<i>Salmonella</i>	bs-4581R
Abcam	<i>Listeria</i>	Ab20506
Abcam	<i>Listeria</i>	Ab20766
Abcam	<i>Listeria</i>	Ab35132
Bioss	<i>Listeria</i>	bs-4801R
Invitrogen	<i>Listeria</i>	MA5-33271
Sino	<i>Listeria</i>	40067-T16
Sino	<i>Listeria</i>	40067-MM06
Virostat	<i>Listeria</i>	4201
Virostat	<i>Listeria</i>	4204
Virostat	<i>Listeria</i>	4211
Virostat	<i>Listeria</i>	4212

Bead Functionalization Materials & Preparation

Two types of magnetic beads were utilized for functionalization with antibodies; streptavidin-coated and tosyl-activated. Streptavidin coated beads (SVM-05-10) were purchased from Spherotech (Green Oaks, IL). Tosylactivated beads (Dynabeads M280) and a Dynamag-2 bead separation magnet were purchased from Invitrogen (Waltham, MA). Borate buffer (0.1 M boric acid, 0.025 M sodium hydroxide, pH 8.5) was used for tosyl-activated bead functionalization with materials purchased from Thermo Fisher. Blocking buffers were prepared from PBS with the addition of 0.5% or 0.1% w/v BSA. Full buffer preparation protocols are available in *Buffer & Solution Preparation (Appendix A)*.

Tosyl-activated beads were prepared according to bead preparation protocols (**Appendix A**). Briefly, 30 mg/mL bead stock was vortexed to homogenize and 335 μ L of beads were transferred via micropipette to a microcentrifuge tube and magnetically separated from supernatant, which was discarded via aspiration. A 1 mL borate buffer wash with 10 second vortex mix was performed, and beads were again magnetically separated and the supernatant discarded. 50 μ L of 4 mg/mL antibody mixture was added to the beads, and total volume was adjusted to 250 μ L with borate buffer. The resulting solution was mixed on a rotator for 24 hours at room temperature. Beads were magnetically separated and resuspended to 1 mL in 0.5% BSA blocking solution for 3 hours. Beads were washed twice with 1 mL 0.1% BSA blocking solution with magnetic separation and discarding of supernatant. Beads were resuspended in 0.1% PBS to a concentration of 20 mg/mL and stored at 4°C until use.

Antibody Capture Screening

To screen antibodies for capture performance, beads were prepared with each tested antibody, and 100 µg beads were used to capture bacteria from a 1 mL sample at 10³ CFU/mL for 1 hour at room temperature with rotator mixing. After capture, three 1 mL PBST washes with magnetic separation were completed to remove unbound bacteria. Beads were sampled and plated in triplicate on agar to quantify bacterial carryover utilizing the following equation

$$\text{Equation 1: } \% \text{ Capture} = 100\% * \frac{\text{Bead Mixture Total CFU}}{\text{Original Sample Total CFU}} \quad (n = 3)$$

Nonspecific Binding Analysis

Functionalized streptavidin and tosyl-activated beads were tested for nonspecific binding to HRP-conjugated secondary antibodies. Six wash steps were completed with 1 mL PBST, with 50 µL supernatant from each wash sampled and tested for HRP with 150 µL TMB solution in a microplate to track wash effectiveness in removing conjugated antibody. After these six wash steps, beads were again separated, resuspended to 100 µL in PBS, and a 10 µL aliquot was tested with TMB solution in a microplate. This was repeated for a range of bead dosages between 0.5 µg and 500 µg of beads.

In order to verify that TMB turnover was due to bound HRP, background measurements of 50 µL aliquots of PBS buffer, 0.1 µg tosyl-activated beads in PBS, and 5% W/V Tween-20 in PBS were taken in a wellplate with 150 µL TMB, incubated for 30 minutes. All showed response within standard deviation of TMB solution alone.

Electrode and Device Materials

TC303 Graphite was purchased from Asbury Carbons (Asbury, NJ). Polystyrene and dichloromethane (DCM) were purchased from Sigma-Aldrich (St. Louis, MO). Conductive paint was purchased from SPI Supplies (West Chester, PA). 3D printed electrode shells were manufactured in-house, utilizing a Formlabs Form 3 SLA Printer with Grey V4 and Clear V4 resins from Formlabs (Somerville, MA). Wells were manufactured in-house from double-sided adhesive (468MP) and diagnostic film (9962), purchased from 3M (Saint Paul, MN). Polymethyl methacrylate (PMMA) ¼" sheets and 2-part epoxy were purchased from Grainger (Lake Forest, IL). DSA, film, and PMMA templates were cut using an OMTech AF2028-60 laser cutter, with parameters optimized to ensure clean cutting performance with minimal deformation of materials and no scorching. Full details can be found in *Electrode and Channel Technical Specifications (Appendix A)*.

Electrode Manufacturing

Electrodes were prepared as previously reported.²⁹ Briefly, to prepare electrode material, 2.0 g of polystyrene was dissolved in 10 mL DCM and combined with 4.0 g of TC303 graphite. Solution was mixed on a silicon wafer while DCM evaporated until a gummy PS/graphite composite resulted, which was molded into a ball and kneaded until dry and fragile to the touch. The composite was dried at room temperature overnight to remove residual solvent, then pressed into a laser-cut PMMA electrode mold at 200°C and 2000 PSI utilizing a dual-platen heat press. Excess TPE material was removed with 150 grit sandpaper mounted to a flat glass surface, and electrode was smoothed with 600 grit sandpaper on a flat glass surface. Copper wire was attached to electrodes using conductive paint and coated with 2-part epoxy after

drying overnight. After setting epoxy overnight, electrodes were sealed into 3D printed casings with 2part epoxy. Electrodes were tested with multimeter to ensure resistance < 100 ohms from wire to TPE surface.

TPE Methodology

Thermoplastic electrodes were used with a TPE pseudoreference.³⁰ Electrodes were polished between use on 600 grit sandpaper in a 'figure-8' motion to remove the top layer of electrode material and fouling compounds. When working with live bacterial samples, electrodes were sterilized with a 2% chlorine bleach solution before polishing to prevent contamination and bacterial exposure. After polishing, electrodes were rinsed in deionized water and stored in a sealed container until use. To complete electrochemical analysis, a DSA and hydrophobic diagnostic film well was placed on the device surface around the electrode pads, and a 40 μ L droplet of sample after incubation with TMB was pipetted onto the electrode surface to cover all 3 electrodes. Electrochemical analysis was controlled with a Palmsens 4 potentiostat. Cyclic voltammetry was used for setting of initial electrochemical parameters. Chronoamperometry (CA) was tested for use in the assay against known HRP concentrations in TMB solution and was used for detection in the final assay.

Optimization of Electrochemical Analysis

Initial CA parameters were set based on CV results of HRP-reacted TMB. Measurement potential was set at -200 mV versus pseudo reference, interval of 0.1 seconds, and run length of 120 seconds with no initial equilibration. Data points were extracted from each run,

representing total charge over time. Data points were generated for time intervals from 0 – 120 seconds, and SNR for each increment was utilized to determine optimal sampling interval.

For evaluation of capture and labeling performance of live bacteria in buffer, wellplate detection of 50 μL of final bead solution in 150 μL of TMB with a 30 minute incubation was completed. Samples from $2.9 \times 10^3 \text{ CFU/mL}$ to $3.0 \times 10^4 \text{ CFU/mL}$ were utilized to achieve a linear dose-response fit.

On-Bead Electrochemical Assay Methodology

Full protocols for the assay can be found in **Appendix A**. Briefly, 1000 μL samples spiked with targeted bacteria were taken and added to microcentrifuge tubes. 100 μg antibodyfunctionalized beads were added to each tube, and they were incubated for 1 hour at room temperature with constant rotation. Beads were magnetically separated for 1 minute on the magnet and supernatants were discarded. Two 30 second vortex rinses in 1 mL PBST with magnetic separation were completed to remove unbound bacteria. To label bacteria, beads were resuspended in 50 μL HRP-conjugated secondary antibody solution at an antibody concentration of 25 $\mu\text{g/mL}$. Tubes were vortexed for 10 seconds to mix and incubated for 20 minutes at room temperature with constant rotation. Beads were magnetically separated for 1 minute, excess secondary antibody solution was discarded, and three 30 second vortex washes with 1 mL PBST were completed, with 1 minute magnetic separation between each. Labeled beads were resuspended in 1 mL PBST, vortexing to mix. To measure HRP concentration, 10 μL of bead mixture was added to 40 μL TMB in a microcentrifuge tube. This was allowed to incubate

15 minutes, pipetted into the well on the electrode, making sure to fully cover all 3 electrode contacts, and measured via chronoamperometry at -200 mV for 120 seconds.

Complex Matrix Materials and Preparation

Ground beef, collard greens, whole milk, and apple juice were purchased from King Soopers (Denver, CO). Sterile Whirl-Pak filter membrane homogenizer bags (4 oz) were purchased from Sigma-Aldrich (St. Louis, MO). To prepare complex matrix samples, 10 g complex matrix material was placed into the homogenizer bag and 100 μ L aliquots of bacterial solution were spiked directly onto the matrix material. 90 mL of extraction buffer was then added to the bag, the bag was sealed and massaged vigorously by hand for 5 minutes. After preparation, samples were withdrawn from the filtered side of the sampling bag for analysis.

Assay Optimization Methodology

Secondary antibody concentration, bead dosage, and capture time were selected for optimization to maximize signal to noise ratio and repeatability. Secondary antibody labeling concentration was tested in triplicate at zero and 28000 CFU/mL with secondary antibody concentrations from 0.1 – 200 μ g/mL with a 20 minute labeling time. Other than antibody concentration, assay was performed as per protocol. Bead dosage optimization was completed at 0, 10^3 CFU/mL, and 10^6 CFU/mL with a 1 hour capture time. Signal to noise ratio was determined via comparison with triplicate results at 0 CFU/mL (**Equation 2**) for dosages of 20 – 500 μ g beads in a 1 mL sample. Capture time was investigated at 2800 CFU/mL with 100 μ g

beads, from 30 minutes to 6 hours. Result was quantified by plating beads on agar after secondary antibody labeling step.

Data Analysis Optimization

Collected 120 second chronoamperometry curves were analyzed for optimal data processing method via signal to noise ratio over the entire collected dataset for live bacterial analysis results in buffer. Sections of data were selected for total charge transfer analysis, and SNR was computed by the following equation for each increment:

Equation 2:

$$SNR = \frac{\sigma_{\text{Avg}(\text{Sample})}}{\text{Avg}(\text{Blank}) + 3 * \sigma_{\text{Blank}}}$$

Salmonella and Listeria Assay

To utilize assay for *Salmonella* and *Listeria* detection, the same protocols as for *E. coli* detection were followed. Capture and labeling antibodies specific to *Salmonella* or *Listeria* were utilized, and beads were conjugated using bead conjugation protocols. Capture antibodies were screened using the same methodology as *E. Coli*, with plating on LB broth (*Salmonella*) or BHI broth (*Listeria*) agar plates for concentration and capture efficiency characterization.

DEVCOM Evaluation Materials & Methods

DEVCOM evaluation used materials from *Consumables & Equipment Purchasing List (Appendix A)*, in addition to electrodes supplied by the team at CSU. For complex matrix testing,

EPA General Test Water (GTW), apple juice, raw ground beef, and fresh romaine were utilized. Whirl-pak bags (1627 mL) were used for matrix preparation.

DEVCOM evaluation was completed from *Bead Preparation Protocol V3* and *Singleplex Assay Protocol V4.1 (Appendix A)*. A QUANTOM Tx Microbial Cell Counter system was used to determine bacterial concentration in the stock solution, which was then serially diluted to working concentrations. Concentrations were not quantified post-dilution. In *E. Coli* LOD testing, aliquots from this serial dilution were tested in triplicate. In *Salmonella* LOD testing, testing of aliquots from the serial dilution was not replicated. For complex matrix testing, a 'standard cutoff' was implemented for the *E. coli* assay utilizing all measurements taken in complex matrices and buffer, setting the cutoff 3 standard deviations above the blank average.

3. Results & Discussion

Antibody Screening Results

Antibody screening in bead-sandwich format was completed with *E. coli* DH5α using streptavidin coated magnetic beads, using SNR of the assay at 10³ CFU/mL as the performance indicator. Initial screening showed the limiting factor of assay performance was the ability of the antibody to keep bacteria bound to the bead through the final three wash steps of the assay, which was confirmed by agar plating of final bead mixtures. This enabled a higher throughput screening for *E. coli* O157:H7, *Salmonella*, and *Listeria* antibody screening, whereupon post capture and wash bead samples were serially diluted and plated, and bacterial retention was quantified by direct counting of resultant bacterial colonies. *Salmonella* and *Listeria* were

screened on tosyl-activated beads only, due to high nonspecific secondary antibody binding found with streptavidin beads.

This procedure was able to identify optimal antibodies for *E. coli* O157:H7 as well as *Salmonella*. It was unable to identify a working antibody for *Listeria*, which we hypothesize to be due to the gram-positive nature of the bacterium requiring the antibody to target pili rather than cell wall epitopes, which may not provide enough stability to retain whole cell bacteria through the washing sequence. This issue may be able to be overcome with a larger screening set of antibodies. Beads without antibodies did not carry over any nonspecifically bound whole cell bacteria, shown by lack of growth on agar. Additionally, in the cases of *E. coli* antibodies Ab20640 and Ab35292, optimal antibody was chosen based on percentage of bacteria retained through the assay.

Nonspecific Binding Results

Microwell plating of wash supernatant with TMB showed that Ab-HRP was no longer being removed from beads after three washes (**Figure 2**). The fourth through the sixth wash supernatant showed no statistically significant difference in HRP concentration from blank. Additionally, there was no statistical difference between 0.1% and 5% Tween-20 after the 3rd wash. Based on this, three washes utilizing PBST at 0.1% Tween-20 was deemed sufficient for removal of unbound Ab-HRP and was implemented in assay protocol.

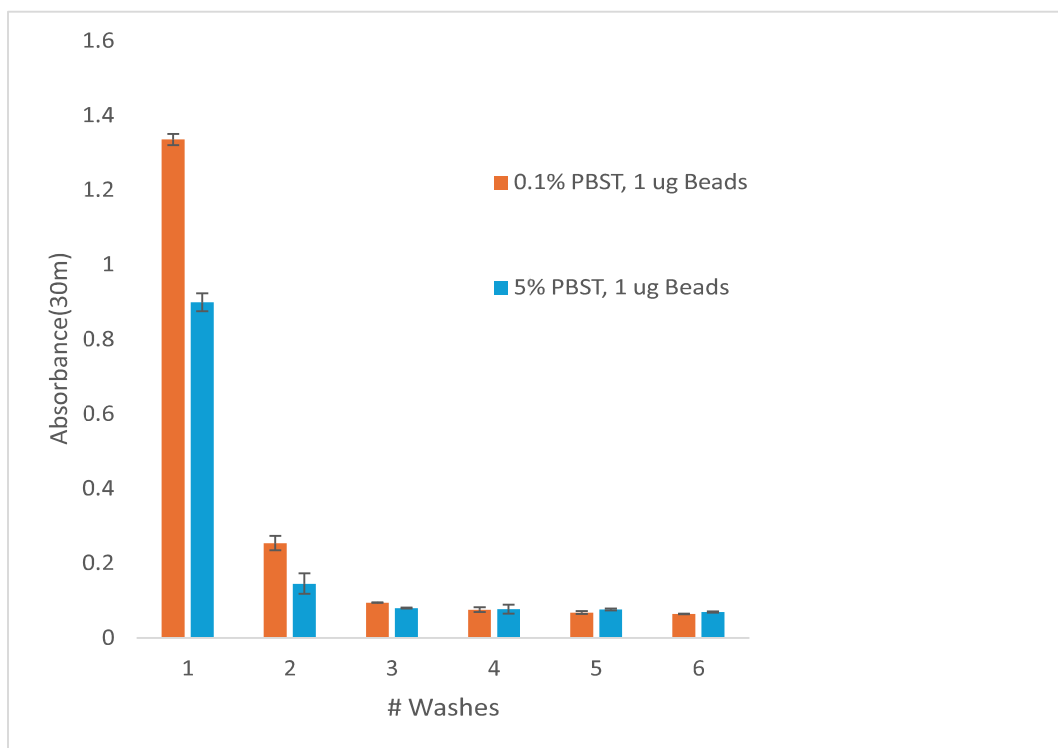


Figure 2: Microwell TMB Analysis of HRP in Supernatant Following PBST Washes

Microwell plating of the beads after 6 washes showed marked difference between streptavidin-coated and tosyl-activated beads (**Figure 3**). This drastic difference in nonspecific binding led us to choose tosyl-activated beads for the assay going forward in order to minimize background signal while still using enough beads in the assay for efficient capture performance.

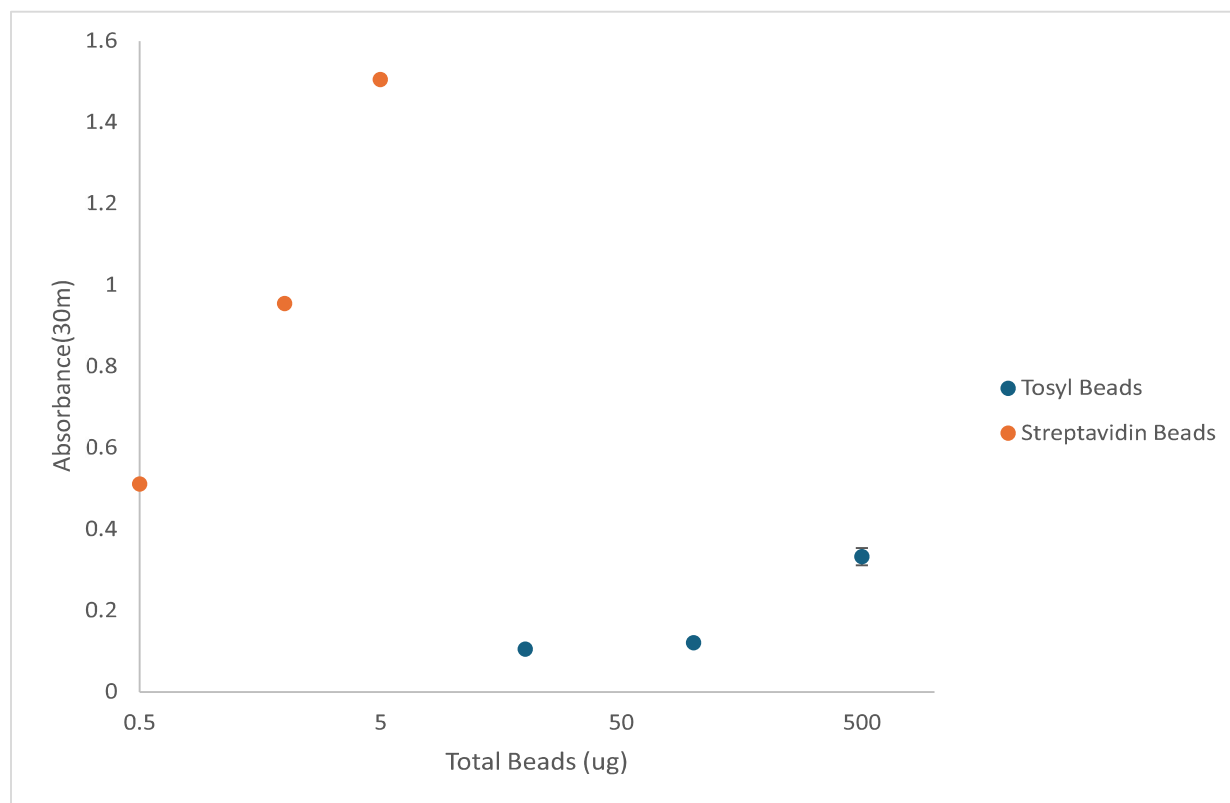


Figure 3: Microwell TMB analysis of HRP bound to MMBs following PBST washes

HRP Detection on TPE Electrodes

Cyclic voltammetry (CV) was utilized to determine CA parameters for initial testing. CV analysis in unreacted TMB solution showed mixed results, with potential shifts of up to 75 mV between runs on separate electrodes (**Figure 4**). At the most negative potentials, peaks were located at – 190 mV vs pseudo reference. Peak current repeatability was also somewhat inconsistent, with differences of up to 1 μ A (30 % of peak height) between electrodes in TMB solutions. Due to the potential shifting, CA was selected for assay use, eliminating the need for identification of multiple shifting peaks within the dataset.

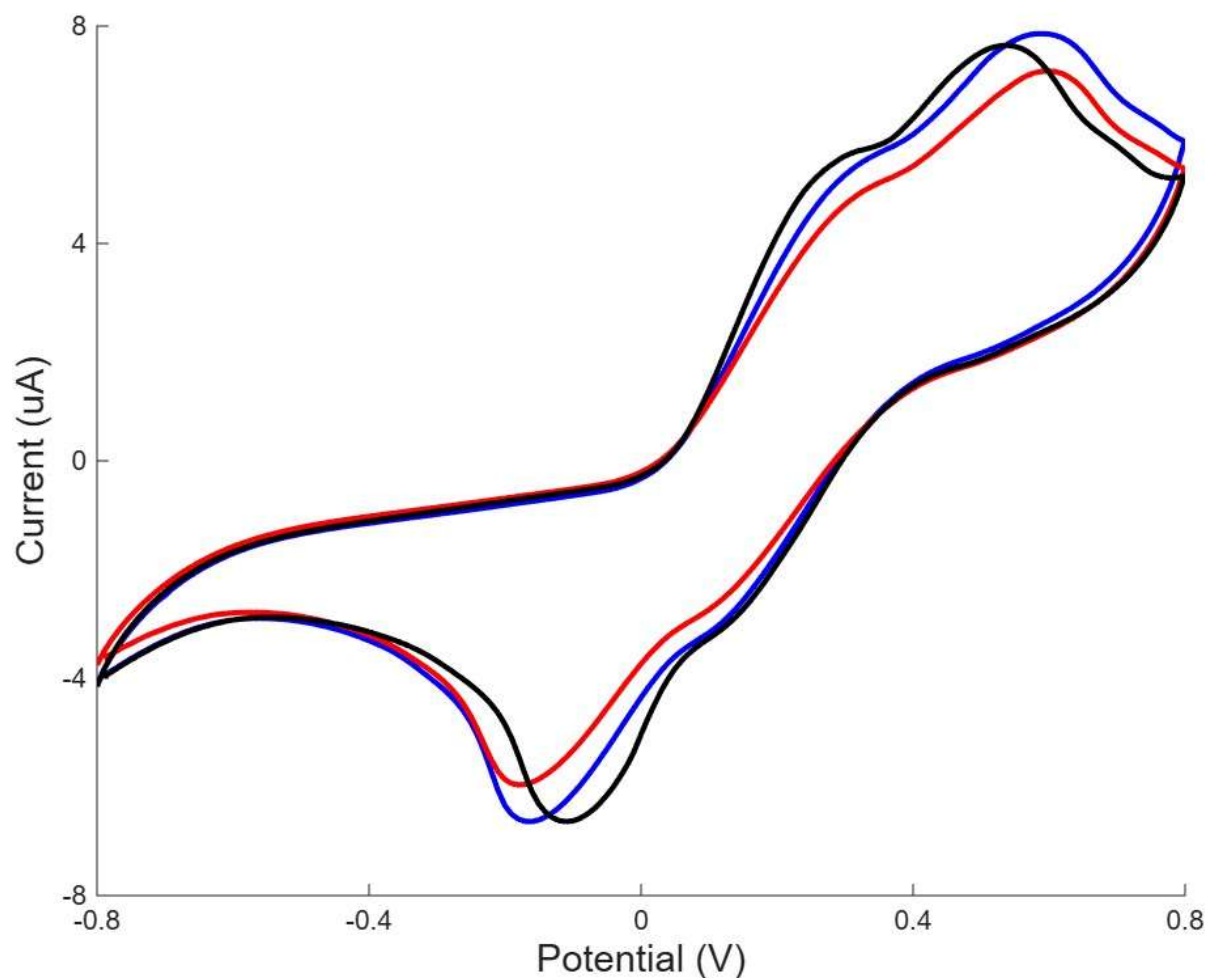


Figure 4: Representative Cyclic Voltammograms of TMB redox process on 3 separate TPEs in TMB reagent solution.

Based on CV results, the potential for CA measurement was set at - 200 mV vs. pseudo reference, to ensure that any oxidized TMB would be reduced regardless of shifting reference potential. Analysis of SNR throughout the range of the assay showed that signal from total charge transfer from 60 - 120 seconds was optimal, eliminating the early timepoints that could cause instability and increased background signal, and collecting data over a large range to maximize sensitivity (**Figure 5**).

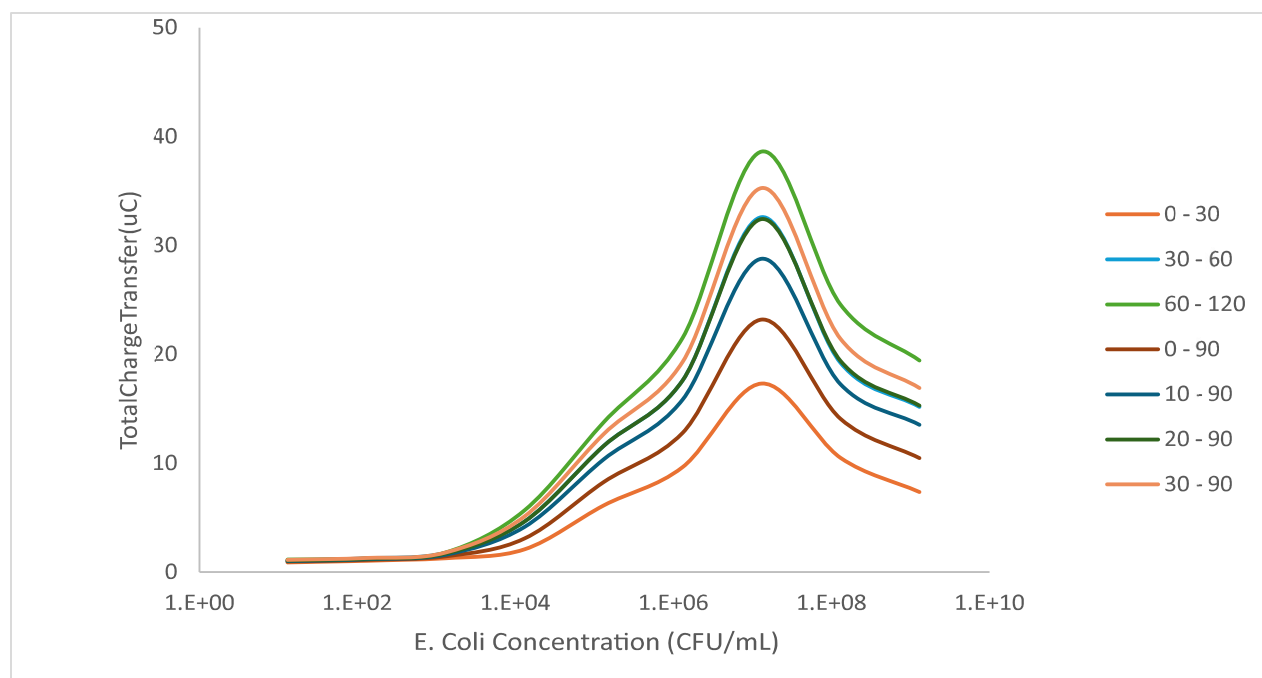


Figure 5: Analysis of total charge transfer over different time periods

Assay Optimization Results

At high concentration of bacteria (10^6 CFU/mL), SNR was inversely proportional to bead dosage, indicating that the limiting factor was background signal from nonspecific binding, and that the increased capture efficiency at high bead concentrations did not outweigh the negative effects of increased background (**Figure 6**). At moderate bacterial concentration (10^3 CFU/mL), SNR peaked at 100 μ g beads per sample. At 20 μ g beads per sample, capture efficiency was low (< 5% by agar plating of beads and initial sample), which we hypothesize was due to a lowered number of bead-bacteria interactions during the capture step. Accordingly, SNR was poor. At 500 μ g beads, SNR was slightly reduced compared to the 100 μ g datapoint, due to an increase in background signal driven by nonspecific binding that was unable to be overcome by the increased capture efficiency at the high bead concentration. 100 μ g beads per sample was

determined to be optimal to keep capture efficiency high at low bacterial concentrations without limiting the assay due to increased background signal.

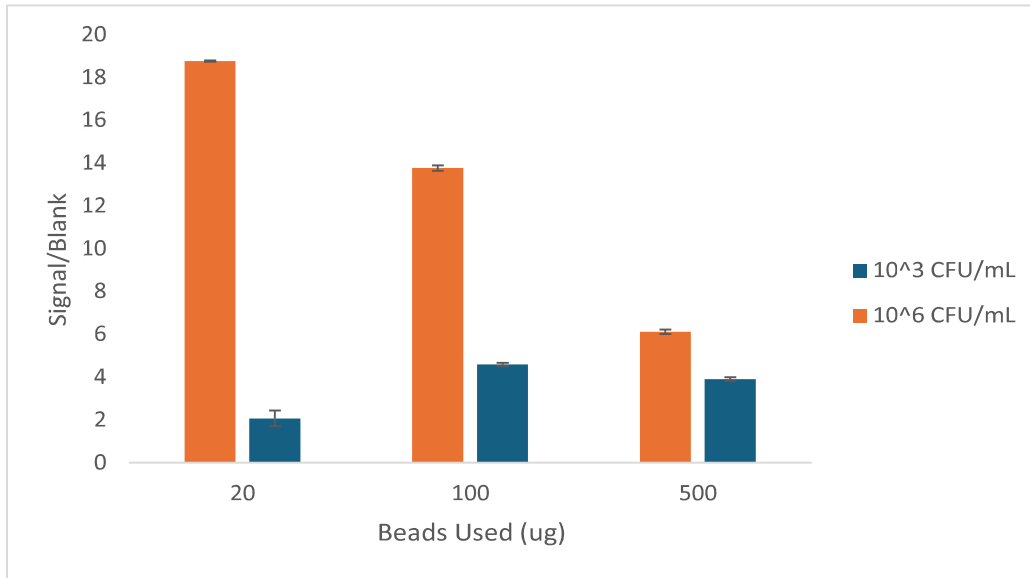


Figure 6: Bead dosage optimization results utilizing tosyl-activated beads.

Capture timing optimization revealed that capture efficiency increased linearly with incubation time between 15 minutes and two hours at a bacterial concentration of 10^3 CFU/mL (**Figure 7**). Beyond the two-hour mark, increase in incubation time provided no significant benefit. In order to manage total assay timeframe, a one-hour capture was utilized, which provided an increased capture efficiency compared to a 20-minute incubation, while still keeping the total assay time to under 2 hours.

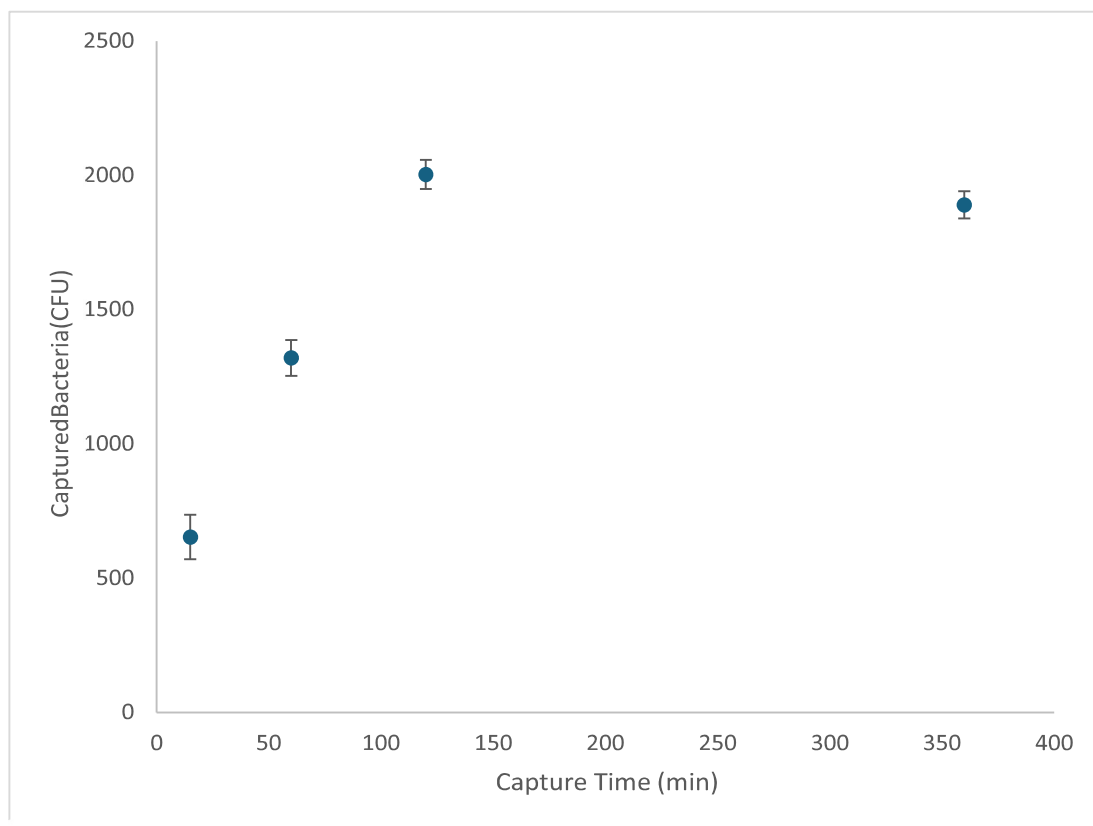


Figure 7: Capture time optimization results in a 10^3 CFU/mL *e. Coli* solution

Secondary antibody labeling concentration optimization showed that at low antibody concentration (0.1 & 1 μ g/mL), large deviations were seen from run to run (**Figure 8**). Additionally, SNR averaged ~ 4 at both these concentrations and at 200 μ g/mL, the highest concentration tested, indicating that the low concentrations were unable to fully label all bound bacteria, and the highest concentration could not overcome increased background signal from nonspecifically bound secondary antibody. The two moderate concentrations (10 & 25 μ g/mL) had an average SNR of 11.26 and 12.85 respectively, and 25 μ g/mL was chosen to utilize in the assay based on the increased SNR and highly repeatable results.

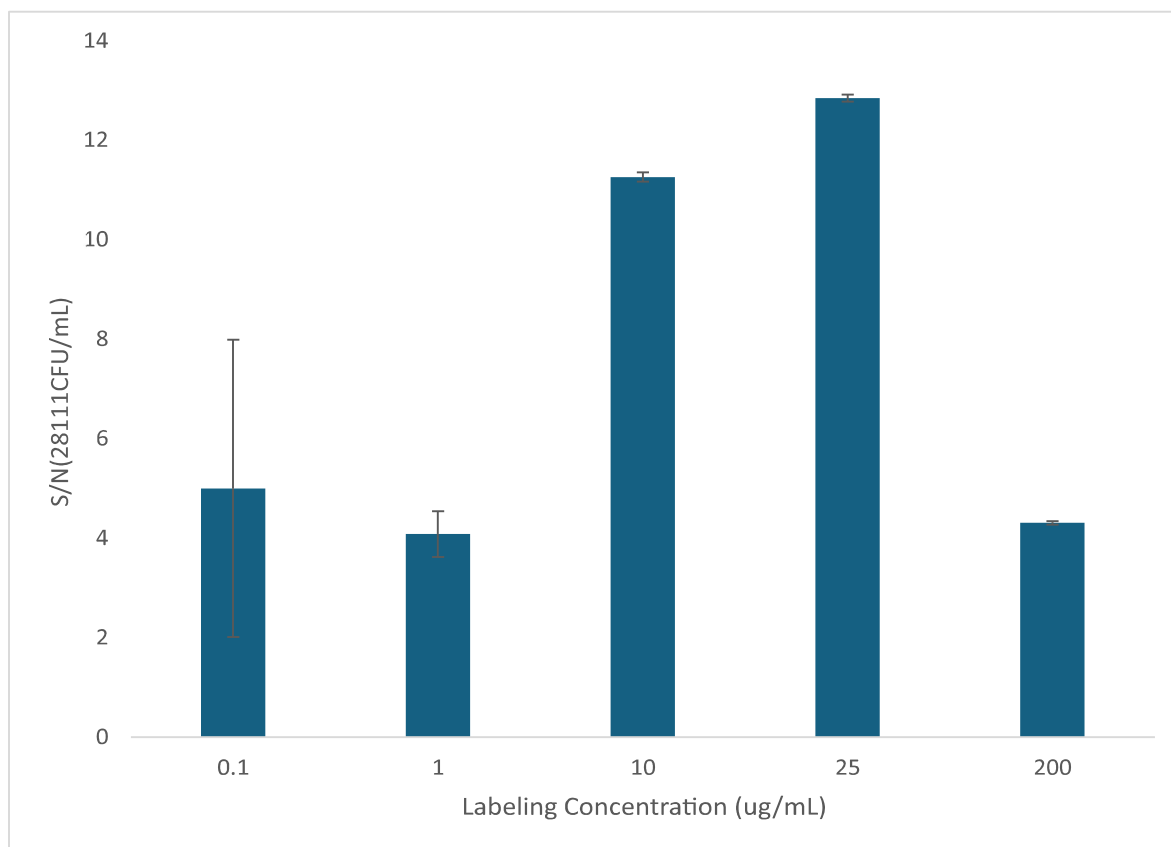


Figure 8: Secondary Antibody Concentration Optimization Results in a 2.8×10^4 CFU/mL e. Coli solution

Limit of Detection Estimation

Live bacterial testing of assay performance found that the assay gave a linear response in well plate between 0 and 3×10^4 CFU/mL, with the lowest tested concentration at 2.9×10^3 CFU/mL (**Figure 9**). At 0 CFU/mL, response was within standard deviation of blank TMB sample with no beads. LoB and LoD of the assay were estimated from this data to be 8 CFU/mL and 180 CFU/mL, respectively.

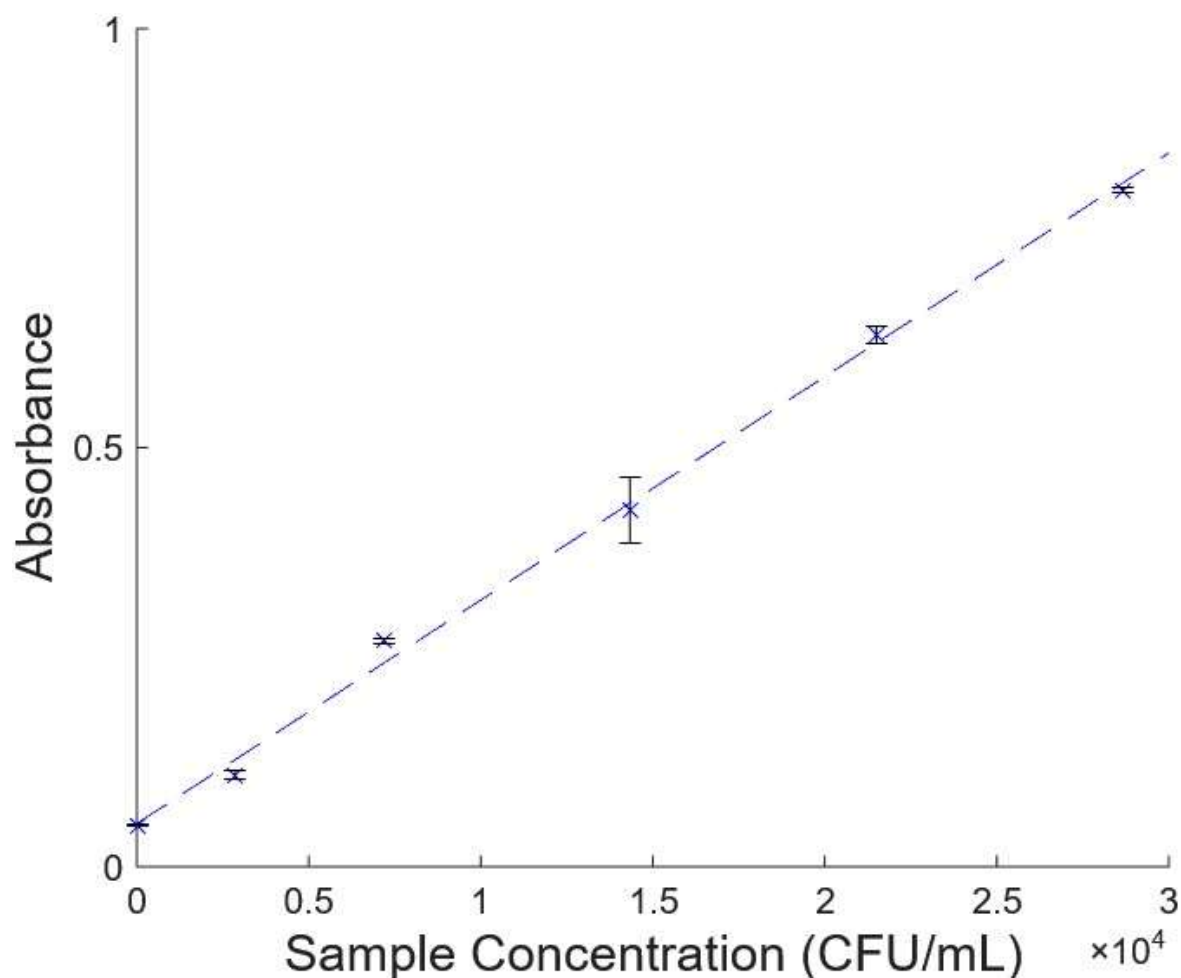
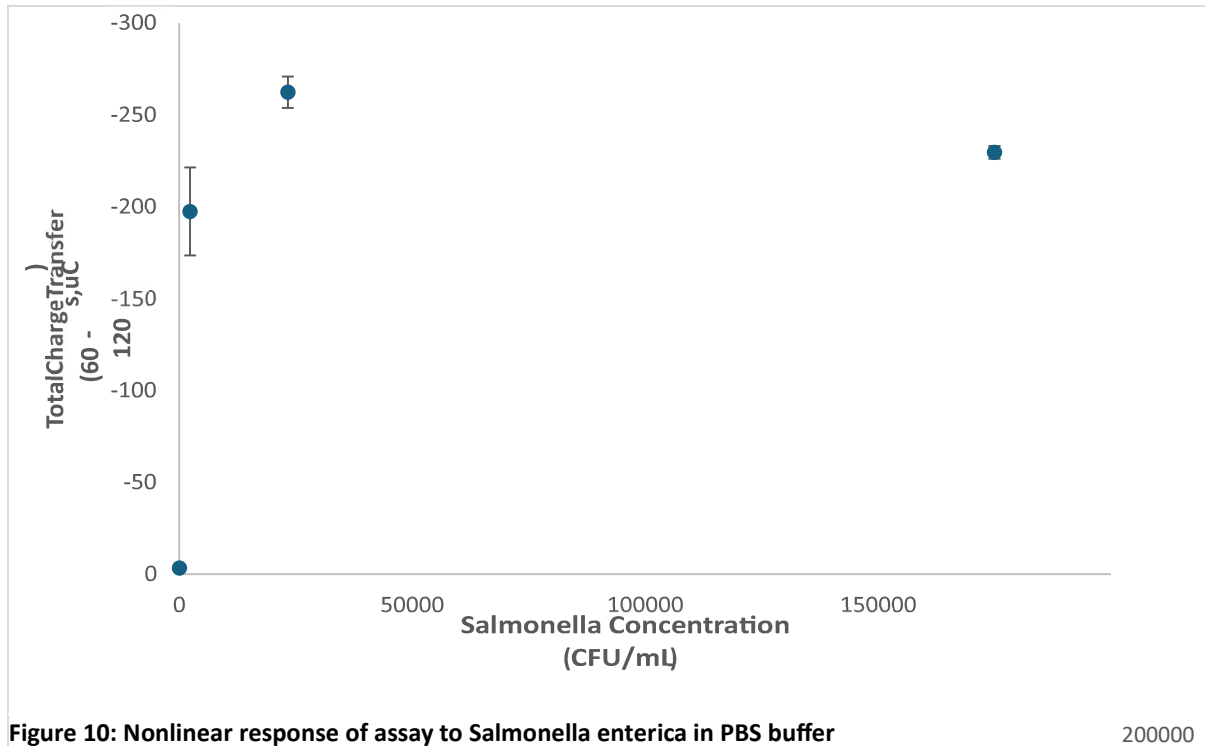


Figure 9: Linear response of Assay to *E. coli* from 0 to 3×10^4 CFU/mL.

Salmonella Study Results

Modifying the assay for *Salmonella* utilizing the optimal antibody identified in Antibody Screening Results, above, yielded a similar working assay for whole cell *Salmonella* detection using identical protocols, procedures, and detection modality to the *E. coli* assay. The assay was tested at 0, 10^3 10^4 , and 10^5 CFU/mL, all showing very positive results (SNR of 32 at 2,300 CFU/mL, **Figure 10**). LoD was estimated to be the lowest of these results, 2300 CFU/mL, due to

nonlinearity of the collected data. At this lowest tested datapoint, increased SD was seen, likely due to a steep dose-response curve at this concentration.



DEVCOM Results

Total charge transfer evaluation was used for quantification of live bacterial concentration in Butterfield's buffer (**Figure 11**). Analysis of assay results from 60 – 120 seconds was completed in triplicate at zero and between 10^{-10} - 10^9 CFU/mL in a 10x dilution series. At 13 and 130 CFU/mL, the assay gave a response above the standard cutoff in only 1 out of 3 cases. At 1300 CFU/mL and above, the assay responded above cutoff in 100% of cases, indicating a positive sample. At 10^6 CFU/mL, deviation in assay response increased when compared to previous points. Above 10^7 CFU/mL, average assay response decreased moderately, indicating that at extremely high

concentrations secondary antibody labeling was insufficient to provide a quantitatively accurate result. Other than the decrease seen at high bacterial concentrations, the data followed the expected sinusoidal result. Based on the 100% success at the 10^3 datapoint, the LOD of the assay was estimated to be 1,300 CFU/mL.

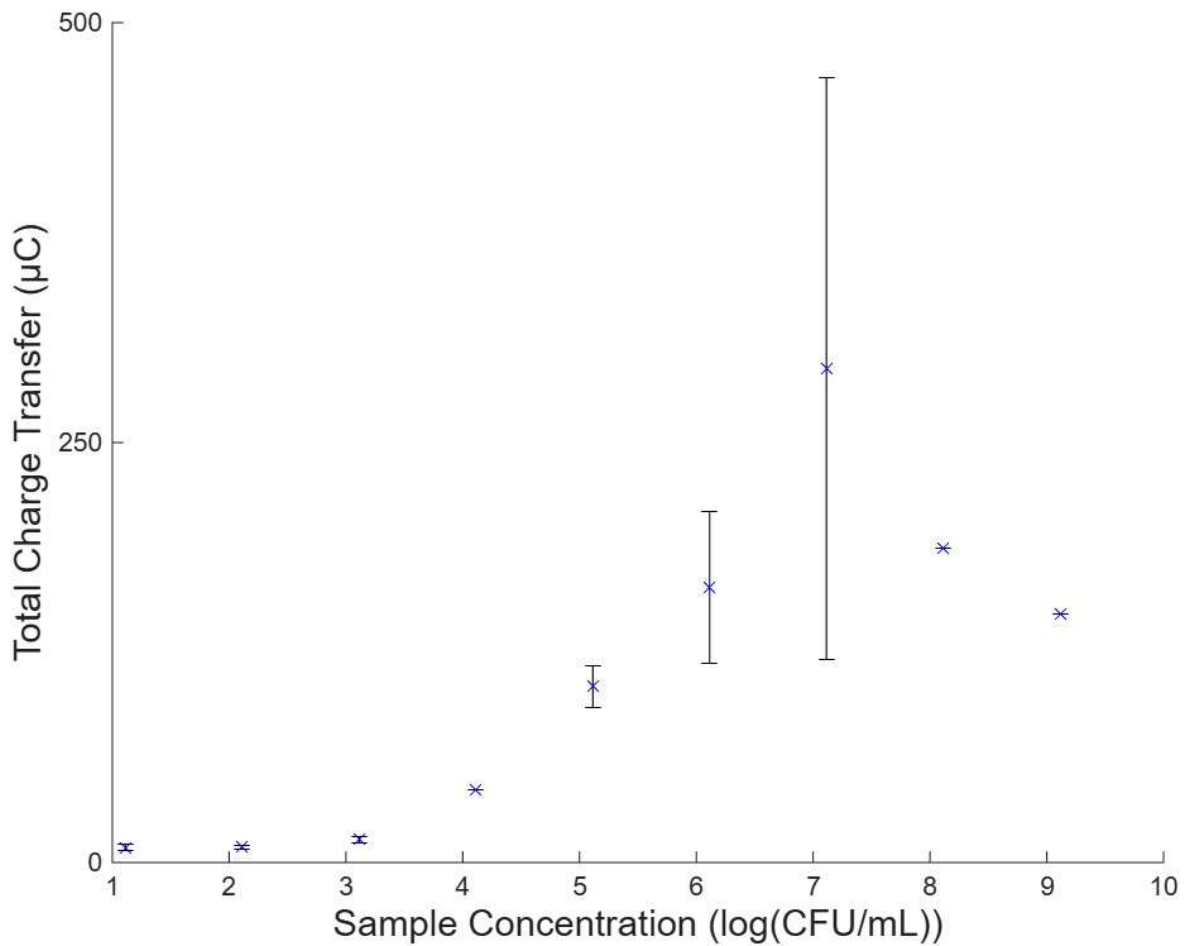


Figure 11: Assay dose response in Butterfields buffer on electrodes collected by DEVCOM

Live *E. Coli* bacterial testing in complex matrices was evaluated via total charge transfer from 60 – 120 seconds at zero, 10^4 , 10^5 , and 10^6 CFU/mL (**Figure 12**). In leafy green washings and ground beef washings the assay was successfully able to identify a positive sample in 100%

of cases at 1.3×10^4 CFU/mL, the lowest tested concentration. General test water was not identified as positive at 10^4 CFU/mL but had a strong positive response in 100% of cases at 10^5 CFU/mL and above. Apple juice was identified as positive in 50% of cases at 10^4 CFU/mL and 100% of cases at 10^5 CFU/mL and above.

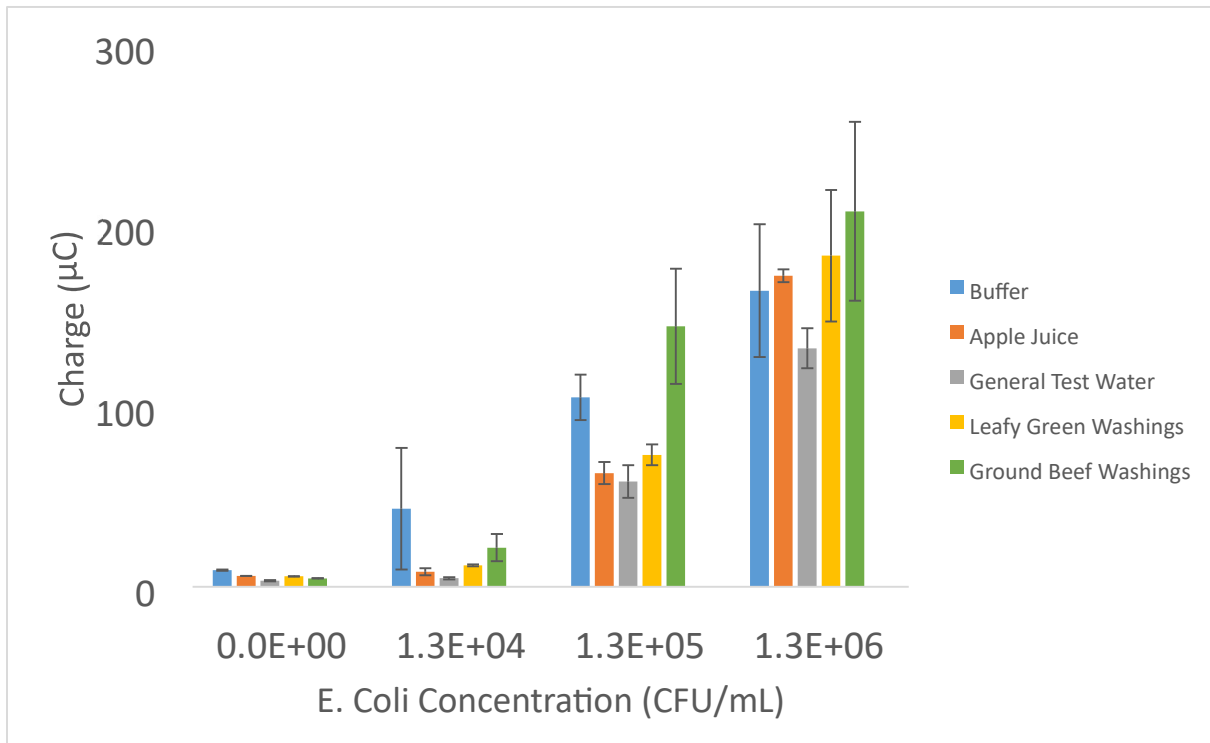


Figure 12: Assay results for complex matrices collected by DEVCOM

Based on this data, the LoD of the assay at DEVCOM was estimated to be 10^5 CFU/mL for all complex media samples, or 10^4 CFU/mL in solid media samples. The discrepancy between solid samples and GTW or apple juice could be due to the presence of complex media components such as proteins in the sample, which may further buffer sample solutions and provide a better environment for antibody recognition of targeted bacteria.

Analysis of the blanks collected for each of the complex matrices showed that

Butterfields buffer showed the highest blank of all the tested samples (**Figure 13**). For LoD estimations, all tested blanks were pooled for averaging and standard deviation analysis to provide the most accurate estimation of overall assay performance.

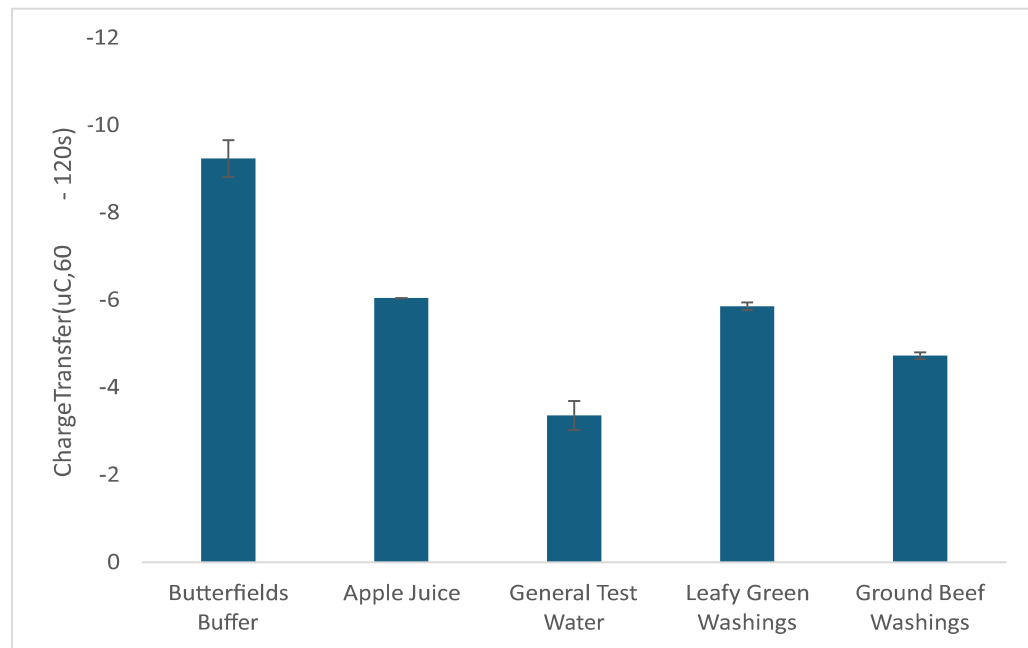


Figure 13: E. Coli assay blanks collected for each complex matrix

Salmonella system analysis was performed identically to the *E. coli* assay in buffer, with *E. Coli* antibodies swapped out for identified optimal *Salmonella* antibodies (**Figure 14**). The assay showed similar results, with positive detection of *Salmonella* at 10^5 CFU/mL and above, and blanks averaging -4.3 μ C, within the range of typical blank results as shown above in Figure 11. It should be noted that the hook effect for *Salmonella* was much more intense than for *E.coli*, with a 50% drop in signal at 10^8 CFU/mL and above. At 10^7 CFU/mL and below, the data followed the expected sinusoidal curve.

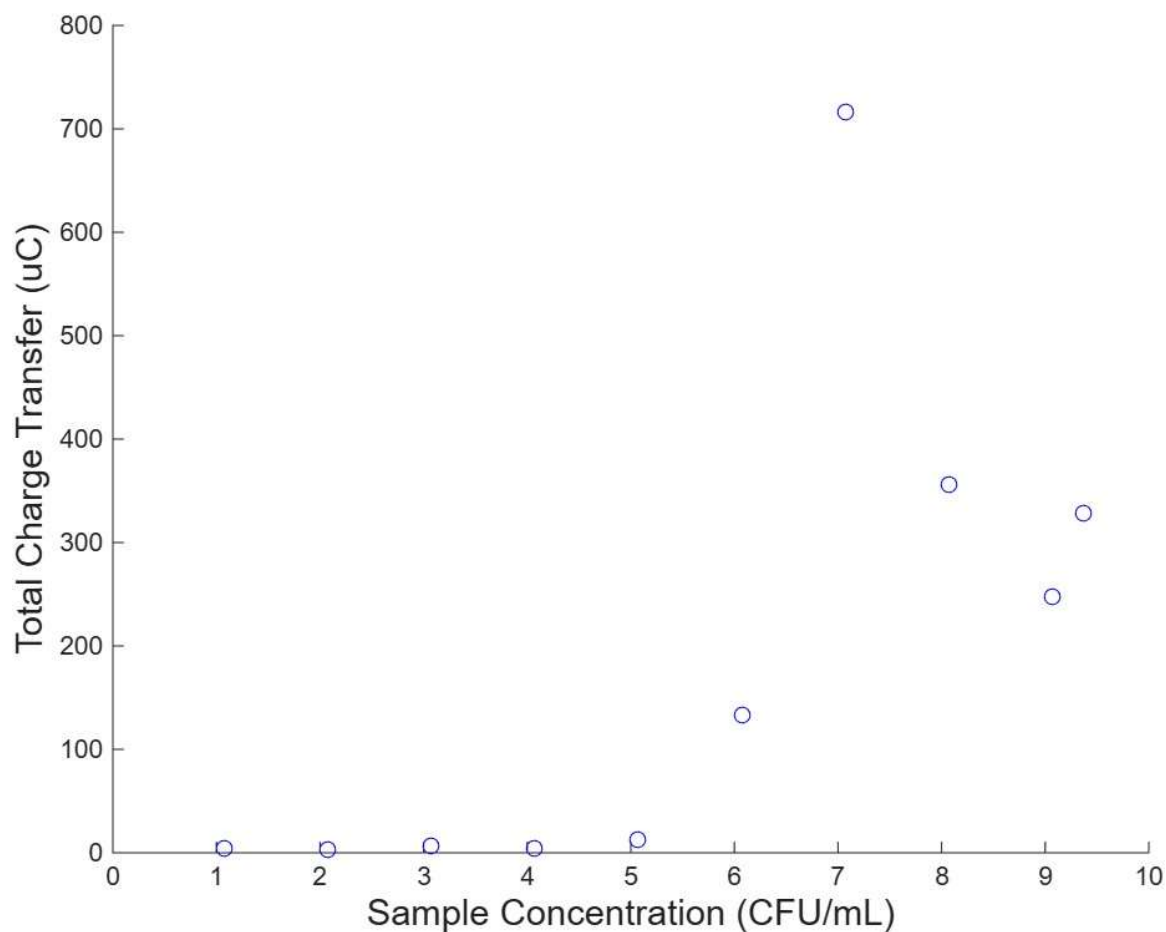


Figure 14: Salmonella Dose-Response in Butterfields buffer (n = 1).

4. Conclusion

Performance of the assay at CSU was exemplary, with an estimated LoD of 180 CFU/mL. This puts it on the same magnitude for quantification as agar plating, which is most accurate from 200 – 2000 CFU/mL with a 100 μ L tested aliquot, and is much more sensitive than a typical plate ELISA.^{13,31} The specificity of the assay was excellent, showing no positive response to non-targeted bacteria or components of the typical food matrices tested. As shown by the linear response from 0 to 3×10^4 CFU/mL, the assay can be used for quantification and bacterial

content estimation at moderate concentrations and could be used to monitor bacterial growth in media or food samples. Additionally, the assay is much faster than typical culture or ELISA procedures, taking less than 3 hours from test to result.¹³

Overall performance of the assay at DEVCOM was successful, achieving estimated LoDs of 1300 CFU/mL in typical buffer, 10^4 CFU/mL in leafy greens and beef washings and 10^5 CFU/mL in GTW or apple juice. While this represents an order of magnitude increase from the results found in the CSU lab, this discrepancy could be caused by a number of factors. Primarily, we hypothesize the differing concentration analysis methods may provide fundamentally different results. Plating of samples directly before analysis with the bead assay shows the total whole cell bacteria concentration at the time of analysis. Testing of an aliquot of growth media before dilution as done at DEVCOM should provide an accurate cell count but does not account for cell degradation during and after dilution and resuspension in matrix media. Additionally, the low buffer strength of Butterfields buffer (0.31 mM phosphate) and GTW (1.47 mM bicarbonate) versus PBS (12 mM phosphate) may have contributed both to bacterial degradation as well as the binding dynamics of the bead system during the capture step.³² Washed leafy greens and ground beef release various compounds that may buffer the solution in the same way that the high phosphate concentration does.^{15,33} Finally, further training and study on the electrochemical system may reveal other important technique differences between the CSU and DEVCOM procedures that could contribute to the discrepancy. Large-scale testing involving both methods would reveal this difference and could help improve the LoD of the assay as a whole.³⁴

The ability of the assay to be converted to *Salmonella* detection easily is a major benefit, as many testing methodologies require extensive reworking to be suitable for alternative bacterial targets.¹² Ease of use is one of the primary hinderances towards widespread adoption of testing methodologies in resource-limited settings, and a 'universal' solution such as this has the potential to overcome the initial barriers in implementing effective monitoring programs.³⁵

Automation of the assay via microfluidic flow driving and automated magnetic separation could be promising for use in a point-of-need setting, and would reduce the need for manual washing currently present in the assay.¹² Use of automatic pipetting methods could also increase the repeatability and therefore sensitivity of the assay, but would require initial investment in engineering and fluid handling solutions to replace the manual pipetting currently required. This could be especially helpful for initial screening of antibodies for assay usage, as this is one of the most time and labor-intensive processes required for the assay development.

Finally, if increased sensitivity is required for the assay, it could be combined with a sample preparation step of spiking into growth media. This would allow bacteria some time to equilibrate and multiply, allowing detection at lower sample concentrations.³⁶ We do not believe that the assay currently would be greatly effected by increases in substrate incubation time, as turnover is already significant across all positive concentrations.

In summary, we believe that this approach and assay contribute greatly to the ability of labs and researchers to monitor and analyze bacterial contamination in food and water samples at the point of need, as well as in a lab setting. The fact that no specialized training was required to get positive and specific results for *E. Coli* and *Salmonella* showed that the engineering of this

assay goes a great way towards simplifying and bringing electrochemical bead-based ELISA bacterial detection into a universally achievable technique.

Finally, we believe that the tunability of the assay represents a step forward in modular lab tools for bacterial detection. The assay could be expanded to multiplex with the integration of multiple capture antibodies onto the bead, or a mixture of activated beads, and labeling with multiple enzymes could enable a direct single-step multiplexed bacterial assay. The integration of the assay with a point-of-need capable low cost electrochemical potentiostat such as those controlled via NFC by smartphone or on-board potentiostats such as the Sensit Wearable from Palmsens would be the next step forward in a truly point-of-need bacterial assay, and would open the door to near real-time monitoring of bacterial contamination on a large scale, such as across multiple drinking water sources in a geographical area, or tracking of point sources of contamination in a river or stream.

5. References

- (1) Shafi, Z.; Shahid, M.; Singh, R. Food-Borne Bacterial Pathogens: Emerging Approaches in Detection and Prevention. *Arch Microbiol* **2026**, *208* (2), 109. <https://doi.org/10.1007/s00203025-04675-8>.
- (2) Bell, R. L.; Kase, J. A.; Harrison, L. M.; Balan, K. V.; Babu, U.; Chen, Y.; Macarisin, D.; Kwon, H. J.; Zheng, J.; Stevens, E. L.; Meng, J.; Brown, E. W. The Persistence of Bacterial Pathogens in Surface Water and Its Impact on Global Food Safety. *Pathogens* **2021**, *10* (11), 1391. <https://doi.org/10.3390/pathogens10111391>.
- (3) Bryce, J.; Boschi-Pinto, C.; Shibuya, K.; Black, R. E. WHO Estimates of the Causes of Death in Children. *The Lancet* **2005**, *365* (9465), 1147–1152. [https://doi.org/10.1016/S01406736\(05\)71877-8](https://doi.org/10.1016/S01406736(05)71877-8).
- (4) Almaary, K. S. Food-Borne Diseases and Their Impact on Health. *Biosci., Biotech. Res. Asia* **2023**, *20* (3), 745–755. <https://doi.org/10.13005/bbra/3129>.
- (5) Alegre, J. C.; Sharma, S.; Cleghorn, F.; Avila, C. Strengthening Primary Health Care in Low- and Middle-Income Countries: Furthering Structural Changes in the Post-Pandemic Era. *Front. Public Health* **2024**, *11*, 1270510. <https://doi.org/10.3389/fpubh.2023.1270510>.
- (6) Grace, D. Burden of Foodborne Disease in Low-Income and Middle-Income Countries and Opportunities for Scaling Food Safety Interventions. *Food Sec.* **2023**, *15* (6), 1475–1488. <https://doi.org/10.1007/s12571-023-01391-3>.
- (7) Kabiraz, M. P.; Majumdar, P. R.; Mahmud, M. M. C.; Bhowmik, S.; Ali, A. Conventional and Advanced Detection Techniques of Foodborne Pathogens: A Comprehensive Review. *Heliyon* **2023**, *9* (4), e15482. <https://doi.org/10.1016/j.heliyon.2023.e15482>.
- (8) Nnachi, R. C.; Sui, N.; Ke, B.; Luo, Z.; Bhalla, N.; He, D.; Yang, Z. Biosensors for Rapid Detection of Bacterial Pathogens in Water, Food and Environment. *Environment International* **2022**, *166*, 107357. <https://doi.org/10.1016/j.envint.2022.107357>.
- (9) Wu, Y.; Wang, C.-W.; Wang, D.; Wei, N. A Whole-Cell Biosensor for Point-of-Care Detection of Waterborne Bacterial Pathogens. *ACS Synth. Biol.* **2021**, *10* (2), 333–344. <https://doi.org/10.1021/acssynbio.0c00491>.
- (10) Reali, S.; Najib, E. Y.; Treuerné Balázs, K. E.; Chern Hui Tan, A.; Váradi, L.; Hibbs, D. E.; Groundwater, P. W. Novel Diagnostics for Point-of-Care Bacterial Detection and Identification. *RSC Adv.* **2019**, *9* (37), 21486–21497. <https://doi.org/10.1039/C9RA03118A>.
- (11) Sohrabi, H.; Majidi, M. R.; Khaki, P.; Jahanban-Esfahlan, A.; De La Guardia, M.; Mokhtarzadeh, A. State of the Art: Lateral Flow Assays toward the Point-of-care Foodborne Pathogenic Bacteria Detection in Food Samples. *Comp Rev Food Sci Food Safe* **2022**, *21* (2), 1868–1912. <https://doi.org/10.1111/1541-4337.12913>.
- (12) Jiang, Y.; Zou, S.; Cao, X. Rapid and Ultra-Sensitive Detection of Foodborne Pathogens by Using Miniaturized Microfluidic Devices: A Review. *Anal. Methods* **2016**, *8* (37), 6668–6681. <https://doi.org/10.1039/C6AY01512C>.
- (13) Zhao, X.; Bhat, A.; O'Connor, C.; Curtin, J.; Singh, B.; Tian, F. Review of Detection Limits for Various Techniques for Bacterial Detection in Food Samples. *Nanomaterials* **2024**, *14* (10), 855. <https://doi.org/10.3390/nano14100855>.

- (14) Den Besten, H. M.; Mentani, J.; Zwietering, M. H. Sampling and Testing for Pathogens in Food: Finding the Needle in a Haystack and the Impact of the Food Microbiome. *Current Opinion in Food Science* **2025**, *65*, 101332. <https://doi.org/10.1016/j.cofs.2025.101332>.
- (15) Eisel, W. G.; Linton, R. H.; Muriana, P. M. A Survey of Microbial Levels for Incoming Raw Beef, Environmental Sources, and Ground Beef in a Red Meat Processing Plant. *Food Microbiology* **1997**, *14* (3), 273–282. <https://doi.org/10.1006/fmic.1996.0094>.
- (16) Vidic, J.; Vizzini, P.; Manzano, M.; Kavanaugh, D.; Ramarao, N.; Zivkovic, M.; Radonic, V.; Knezevic, N.; Giouroudi, I.; Gadjanski, I. Point-of-Need DNA Testing for Detection of Foodborne Pathogenic Bacteria. *Sensors* **2019**, *19* (5), 1100. <https://doi.org/10.3390/s19051100>.
- (17) Tu, S.-I.; Reed, S.; Gehring, A.; He, Y.; Paoli, G. Capture of Escherichia Coli O157:H7 Using Immunomagnetic Beads of Different Size and Antibody Conjugating Chemistry. *Sensors* **2009**, *9* (2), 717–730. <https://doi.org/10.3390/s90200717>.
- (18) Delibato, E.; Volpe, G.; Romanazzo, D.; De Medici, D.; Toti, L.; Moscone, D.; Palleschi, G. Development and Application of an Electrochemical Plate Coupled with Immunomagnetic Beads (ELIME) Array for Salmonella Enterica Detection in Meat Samples. *J. Agric. Food Chem.* **2009**, *57* (16), 7200–7204. <https://doi.org/10.1021/jf901181m>.
- (19) Xu, M.; Wang, R.; Li, Y. Rapid Detection of Escherichia Coli O157:H7 and Salmonella Typhimurium in Foods Using an Electrochemical Immunosensor Based on Screen-Printed Interdigitated Microelectrode and Immunomagnetic Separation. *Talanta* **2016**, *148*, 200–208. <https://doi.org/10.1016/j.talanta.2015.10.082>.
- (20) Tu, S.-I.; Reed, S.; Gehring, A.; He, Y.; Paoli, G. Capture of Escherichia Coli O157:H7 Using Immunomagnetic Beads of Different Size and Antibody Conjugating Chemistry. *Sensors* **2009**, *9* (2), 717–730. <https://doi.org/10.3390/s90200717>.
- (21) Carrell, C. S.; Wydallis, R. M.; Bontha, M.; Boehle, K. E.; Beveridge, J. R.; Geiss, B. J.; Henry, C. S. Rotary Manifold for Automating a Paper-Based *Salmonella* Immunoassay. *RSC Adv.* **2019**, *9* (50), 29078–29086. <https://doi.org/10.1039/C9RA07106G>.
- (22) Call, Z. D.; Dolence, A.; Boes, J.; Henry, C. S. MagnEtophoretic Slider Assay (MeSA): A Simple Platform for Point-of-Care Diagnostics. *Front. Sens.* **2023**, *3*, 1080037. <https://doi.org/10.3389/fsens.2022.1080037>.
- (23) Foddai, A.; Elliott, C. T.; Grant, I. R. Maximizing Capture Efficiency and Specificity of Magnetic Separation for *Mycobacterium Avium* Subsp. *Paratuberculosis* Cells. *Appl Environ Microbiol* **2010**, *76* (22), 7550–7558. <https://doi.org/10.1128/AEM.01432-10>.
- (24) Srisa-Art, M.; Boehle, K. E.; Geiss, B. J.; Henry, C. S. Highly Sensitive and Rapid Detection of Salmonella Typhimurium Using a Colorimetric Paper Based Analytical Device Coupled with Immunomagnetic Separation.
- (25) Cinti, S.; Volpe, G.; Piermarini, S.; Delibato, E.; Palleschi, G. Electrochemical Biosensors for Rapid Detection of Foodborne Salmonella: A Critical Overview. *Sensors* **2017**, *17* (8), 1910. <https://doi.org/10.3390/s17081910>.
- (26) Chan, K.; Schillereff, D. N.; Baas, A. C.; Chadwick, M. A.; Main, B.; Mulligan, M.; O'Shea, F. T.; Pearce, R.; Smith, T. E.; van Soesbergen, A.; Tebbs, E.; Thompson, J. Low-Cost Electronic Sensors for Environmental Research: Pitfalls and Opportunities. *Progress in Physical*

Geography: Earth and Environment **2021**, 45 (3), 305–338.

<https://doi.org/10.1177/0309133320956567>.

(27) Corradini, A.; Cecchini, M.; Trevisani, M. A Rapid Enzyme-Linked Immunomagnetic Electrochemical (ELIME) Assay for the Detection of Escherichia Coli O26 in Raw Milk. *Food Anal. Methods* **2020**, 13 (6), 1366–1370. <https://doi.org/10.1007/s12161-020-01758-8>.

(28) Guo, Q.; Han, J.-J.; Shan, S.; Liu, D.-F.; Wu, S.-S.; Xiong, Y.-H.; Lai, W.-H. DNA-Based Hybridization Chain Reaction and Biotin–Streptavidin Signal Amplification for Sensitive Detection of Escherichia Coli O157:H7 through ELISA. *Biosensors and Bioelectronics* **2016**, 86, 990–995. <https://doi.org/10.1016/j.bios.2016.07.049>.

(29) Klunder, K. J.; Nilsson, Z.; Sambur, J. B.; Henry, C. S. Patternable Solvent-Processed Thermoplastic Graphite Electrodes. *J. Am. Chem. Soc.* **2017**, 139 (36), 12623–12631. <https://doi.org/10.1021/jacs.7b06173>.

(30) Inzelt, G. Pseudo-Reference Electrodes. In *Handbook of Reference Electrodes*; Inzelt, G., Lewenstam, A., Scholz, F., Eds.; Springer Berlin Heidelberg: Berlin, Heidelberg, 2013; pp 331–332. https://doi.org/10.1007/978-3-642-36188-3_14.

(31) Lee, Z. Z.; Abraham, R.; O’Dea, M.; Harb, A.; Hunt, K.; Lee, T.; Abraham, S.; Jordan, D. Validation of Selective Agars for Detection and Quantification of Escherichia Coli Strains Resistant to Critically Important Antimicrobials. *Microbiol Spectr* **2021**, 9 (3), e00664-21. <https://doi.org/10.1128/Spectrum.00664-21>.

(32) Gao, Y.; Wu, H.; Wang, L.; Zhang, H.; Ding, Z.; Wang, Z.; Gao, L.; Zhou, E.; Han, J. Effects of Buffer Type and pH on Biophysical Properties and Stability of IgG4 Monoclonal Antibodies: Uncovering Dynamic Shifts in Stability Predictors. *International Journal of Biological Macromolecules* **2026**, 336, 149425. <https://doi.org/10.1016/j.ijbiomac.2025.149425>.

(33) Alsharif, A.; Aldawsari, L.; Koukkidis, G.; Freestone, P. The Impact of Compounds Released from Damaged Salad Leaves on the Growth and Virulence of Listeria Monocytogenes. *Microorganisms* **2025**, 13 (2), 252. <https://doi.org/10.3390/microorganisms13020252>.

(34) Rasamsetti, S.; Berrang, M.; Cox, N. A.; Shariat, N. W. Selective Pre-Enrichment Method to Lessen Time Needed to Recover Salmonella from Commercial Poultry Processing Samples. *Food Microbiology* **2021**, 99, 103818. <https://doi.org/10.1016/j.fm.2021.103818>.

(35) Grace, D. Burden of Foodborne Disease in Low-Income and Middle-Income Countries and Opportunities for Scaling Food Safety Interventions. *Food Sec.* **2023**, 15 (6), 1475–1488. <https://doi.org/10.1007/s12571-023-01391-3>.

(36) Minic, R.; Zivkovic, I. Optimization, Validation and Standardization of ELISA. In *Norovirus*; Mózsik, G., Ed.; IntechOpen, 2021. <https://doi.org/10.5772/intechopen.94338>.

CHAPTER THREE: A PORTABLE 3D-PRINTED SYSTEM FOR IN-FIELD
ELECTROCHEMICAL DETECTION OF MDMA IN UNKNOWN TABLETS*

1. Introduction

During the past decade, there has been a 28% increase in drug consumption worldwide, with a global estimate of 316 million people using drugs in 2023.¹ After the COVID-19 pandemic, an increase in the use of synthetic drugs, such as amphetamine-type stimulants (ATS), cocaine, and “ecstasy”, was especially observed. Synthetic drugs are more attractive to the drug market, considering their production doesn’t require large areas of land and has shorter production times when compared to plant-based drugs.¹

MDMA (3,4-methylenedioxymethamphetamine), also known as “ecstasy”, is a stimulant and hallucinogenic drug that is widely used in recreational settings, mainly by young adults due to its effects, such as euphoria, increased sociability,² feelings of well-being, and empathy.³ However, MDMA consumption is also associated with health risks, such as increased motor activity (muscular tension, tremors, and involuntary teeth clenching), alertness, heart rate, and blood pressure, while overdose can lead to organ failure and death.⁴

This work is submitted for publication in *Analytical Chemistry* under the title ‘Portable 3D-Printed System for in-field MDMA electrochemical detection’. It is coauthored by Elisa D. Botelli, Jason Boes, Maxwell D. Bridges, Thaisa A. Baldo, Lucas F. de Lima, Larissa M. A. Melo, Wendell K. T. Coltro, Luciano C. Arantes, William R. de Ataujo, and Charles S. Henry.

In 2023, it was estimated that MDMA had 21 million users worldwide,¹ meaning rapid and affordable detection methods are needed to facilitate the screening and seizure of this drug. Traditional MDMA detection techniques include chromatography and spectroscopy, which provide accurate results but require expensive instrumentation, extensive sample preparation, and specialized personnel.⁵⁻⁷ * In contrast, portable sensors have attracted interest across many research areas due to their simplicity, including easy fabrication, short analysis times, low reagent and sample volumes, high sensitivity, and minimal user training.⁸ Besides that, compared to traditional detection methods, the possibility of miniaturization and the low-cost of these portable devices make them ideal for in-field analysis. Many manufacturing techniques for electrochemical portable sensors have been reported in the literature, such as Laser Induced, or Laser-Scribed Graphene (LIG, or LSG),⁹⁻¹¹ screen printing,^{12,13} and pencil lead-based devices,¹⁴ among others.

A study by Alves and collaborators used a carbon screen-printed electrode to detect MDMA in aqueous media by Linear Sweep Voltammetry (LSV), which demonstrated linearity over the concentration range of 10 to 100 $\mu\text{mol L}^{-1}$ MDMA, a linear coefficient of determination of 0.99 and limits of detection (LOD) and quantification (LOQ) of 1.83 $\mu\text{mol L}^{-1}$ and 6.11 $\mu\text{mol L}^{-1}$ MDMA, respectively.¹⁵ Teófilo and collaborators reported a boron-doped diamond electrode for MDMA detection using Differential Pulse Voltammetry (DPV). The authors obtained a linear concentration range of 1.0 to 500 $\mu\text{mol L}^{-1}$ and an LOD of 0.3 $\mu\text{mol L}^{-1}$ MDMA.¹⁶ Duan and collaborators developed an electrochemical sensor based on a Larginine membrane for MDMA detection in urine samples by Square Wave Voltammetry (SWV), with a linear concentration range of 0.1 to 35 $\mu\text{mol L}^{-1}$ and an LOD of 0.33 $\mu\text{mol L}^{-1}$

MDMA.¹⁷

Recently, the use of 3D-printing for electrochemical sensor manufacturing emerged as a promising approach for large-scale production. The possibility to create a wide range of designs, manufacture electrodes with virtually any geometry, and minimize both waste generation and human intervention represents some of the main advantages of this technique.^{18–20} However, as-printed electrodes rarely provide acceptable electrochemical performance; therefore surface treatments, including chemical^{21–23} and electrochemical^{24,25} strategies have been widely described in the literature to enhance electrochemical performance by removing plastic residues from the filament on the electrode surface.

In recent years, surface treatment using laser irradiation has been studied because it is a reagent-free method and requires less time than the previously mentioned treatments.²⁶ Rocha *et al.* described a photothermal approach using a CO₂ laser to enhance the electrochemical response of carbon-black/PLA-based 3D-printed electrodes. This treatment partially removed the insulating PLA layer, exposing conductive carbon black nanoparticles, as confirmed by Field Emission-Scanning Electron Microscopy (FE-SEM), Fourier Transform Infrared Spectroscopy (FTIR), Raman Spectroscopy and X-ray Photoelectron Spectroscopy (XPS).²⁶

This work describes the development of a portable electrochemical 3D-printed system for the quantitative and qualitative detection of MDMA in forensic-seized samples. To enhance the device's electrochemical behavior, the surface was treated using a CO₂ laser under optimized lasing conditions. The affordable system comprising the 3D-printed electrochemical device, 3D-printed support for simple and rapid electrode connection, and the portable

potentiostat offers a compact and efficient alternative approach for in-field MDMA detection. Designed for ease of use, the system fits in the palm of a hand and enables non-specialized personnel to perform in-field smartphone-based electrochemical analysis, making it a valuable tool for forensic and law enforcement applications.

2. Materials and methods

Chemicals and solutions

Non-conductive filaments (Basic PLA) of 1.75 mm diameter were purchased from Bambu Labs (Shenzhen, China), and conductive PLA (CDP11705) was purchased from Protopasta (Washington, USA). Potassium ferricyanide, potassium ferrocyanide, glacial acetic acid, boric acid, caffeine, benzocaine, and hexaammineruthenium (III) chloride were purchased from Sigma-Aldrich (Missouri, USA). Sodium hydroxide, potassium chloride, and 85% orthophosphoric acid were purchased from Synth (São Paulo, Brazil). P150 and P600 sandpapers were purchased from RMC Abrasives (Changzhou, China). The MDMA standard solution was purchased from Absolute Standards Inc (Connecticut, USA). The MDMA powder standard was obtained from the United Nations Office on Drugs and Crime (UNODC) (Vienna, Austria) and was used for real sample experiments. Seized samples of MDA (3,4methylenedioxyamphetamine) and amphetamine were obtained from the Criminalistics Institute of the Scientific Police of São Paulo and were used in the sensor's selectivity study. Seized samples of MDMA were provided by the Criminalistics Institute of the Civil Police of the Federal District. The ketamine standard solution was purchased from LGC Standards (Teddington, UK). Silver/silver chloride (Ag/AgCl) conductive ink was purchased from Creative Materials Inc (Massachusetts, USA). All diluted solutions were prepared using ultrapure water (Synergy®UV Water Purification System, France) with an electrical resistivity of 18.2 MΩ cm at

25 °C. Britton-Robinson (BR) buffer solution was prepared at 0.1 mol L⁻¹, and the pH values were adjusted using 1 mol L⁻¹ NaOH solution. The MDMA standard solution was diluted in 0.1 mol L⁻¹ BR buffer (pH 3.0) prior to the analyses. A stock solution of MDMA (2 mg mL⁻¹) was prepared by dilution in HPLC-grade methanol. Ketamine stock solution (1.0 mmol L⁻¹) was prepared in a 1:1 v/v (water/absolute ethanol) solution and was diluted in 0.1 mol L⁻¹ BR buffer solution (pH 3.0) prior to the analyses.

Apparatus

The 3D-printed electrochemical sensors were designed using Bambu Studio software and printed with a Bambu Labs P1S 3D printer. Under optimized conditions, a CO₂ pulsed laser (50 W Router VS4040C, Visutec, Brazil) was used to treat the working electrode surface. The electrochemical measurements were performed using a Palmsens 5 galvanostat/potentiostat and a Sensit Wearable from PalmSens with PSTouch 5.11 software running on a Galaxy M53 smartphone. The electrochemical measurements of seized MDMA samples were performed using a Sensit Smart from PalmSens (Houten, Netherlands).

Morphological characterizations were performed by Scanning Electron Microscopy (SEM using a JEOL JSM-IT800 (HL) and a FEI Quanta FEG 250, Field Emission Gun Scanning Electron Microscope (LIMicro-IQ - Microscopy Core Facility, RRID:SCR 024633). Contact angle measurements were performed using a Krüss Drop Shape Analyzer 30 with a sessile drop method and a 12 µL droplet of deionized water. Fourier Transform Infrared Spectroscopy (FTIR) was performed using a Thermo-Nicolet iS50 using attenuated total reflectance (ATR) mode.

3D-printed device manufacturing

Electrode manufacturing was accomplished with a Bambu Labs P1S 3D printer with Automatic Material System (AMS) to allow for multi-material printing. Bambu Studio software was utilized for slicing and printer control. Both non-conductive and conductive materials were printed simultaneously using a 0.4 mm nozzle. The textured build plate was utilized to improve print adhesion. Electrodes were printed with the 'working surface' oriented downwards against the build plate to minimize material changes (the main source of filament waste) and to ensure a consistent electrode surface. The build plate was able to fit 80 electrodes for simultaneous printing, using 70.2 g of nonconductive filament and 4.75 g of conductive filament per batch. Bambu Labs 'Basic PLA' in green or white was utilized for the nonconductive sections. Conductive PLA (CDP11705) from Protopasta was utilized for the electrode sections. Nonconductive electrode sections were printed using the default PLA settings. Conductive sections were printed at a slower speed, 50 mm s^{-1} , with 100% infill to create a homogenous electrode. The slower speed and high infill were necessary for good electrical connection between filament layers. Additionally, electrodes were printed using a minimum workable thickness of 1 mm to ensure low electrical resistance while maintaining reproducibility. To facilitate rapid replacement of electrodes between analysis, electrodes were fitted with a standard 3-pin male header, allowing quick connection to a 3-pin female header attached to the electrode stand.

The 3D-printed electrodes were sanded 10 times using P150 sandpaper and 10 times using P600 sandpaper. After the sanding process, the electrical contacts were positioned on the device and Ag/AgCl conductive ink was applied to enhance the adhesion and paint the pseudoreference electrode. The device was then cured at 50°C for 30 minutes. After that, the sanded working electrodes were submitted to a laser activating procedure using a CO_2 pulsed

laser at a distance of 10 mm from the laser output to the 3D-printed device. Laser parameters, such as laser power (7.8, 8.0 and 8.5%) and laser speed (30, 35 and 40 mm s⁻¹), were previously optimized to obtain the most conductive material (8% and 35 mm s⁻¹). For those optimizations, 3D-printed electrodes were submitted to laser irradiation at different conditions and were analyzed using Cyclic Voltammetry (CV). A solution of 5 mmol L⁻¹ [Ru(NH₃)₆]³⁺ in 0.5 mol L⁻¹ potassium chloride was used as the redox probe and the anodic peak current and peak-to-peak potential separation were analyzed. All sanded/laser-treated electrodes were stored in a desiccator containing silica.

Electrochemical characterizations

The non-treated (bare; BE), sanded (SE), and sanded/laser-treated (SLTE) 3D-printed electrodes were electrochemically characterized using 5 mmol L⁻¹ [Ru(NH₃)₆]³⁺ in 0.5 mol L⁻¹ KCl as a redox probe. Electrochemical Impedance Spectroscopy (EIS) studies were performed at room temperature (~25 °C) using 10 mV as the amplitude potential, 10 frequencies per decade, and a frequency interval between 10⁵ and 0.1 Hz. CV measurements were performed using a potential range of 0.5 V to -1.0 V (vs. Ag/AgCl), with a potential step of 0.01 V and a scan rate of 50 mV s⁻¹.

The developed SLTEs were used to carry out a mass transport study via CV at scan rates ranging between 5 and 200 mV s⁻¹ and with 5 mmol L⁻¹ [Ru(NH₃)₆]³⁺ in 0.5 mol L⁻¹ KCl as a redox probe. The mass transport was also evaluated using a solution of 75 μmol L⁻¹ MDMA in 0.1 mol L⁻¹ BR buffer (pH 3.0).

The effect of pH on MDMA's electrochemical properties was carried out by DPV

within the potential range of 0.5 V to 1.75 V (vs. Ag/AgCl), 0.004 V as the potential step, 0.15 V as the potential pulse, 0.02 s as the pulse time and 20 mV s⁻¹ as the scan rate. The pH was varied from 3.0 to 11.0, while using a solution of 200 μmol L⁻¹ MDMA in 0.1 mol L⁻¹ BR buffer.

Electrochemical detection of MDMA using the sanded/laser-treated 3D-printed sensor

The calibration curve of MDMA was obtained by varying the concentration from 2.5 to 200 μmol L⁻¹ of MDMA in 0.1 mol L⁻¹ BR buffer solution (pH 3.0), using the SLTE and DPV. The stability test was performed over a period of 28 days, storing the sensors in a desiccator containing silica. The measurements were carried out by CV using the SLTE in a solution containing 5 mmol L⁻¹ [Ru(NH₃)₆]³⁺ in 0.5 mol L⁻¹ KCl solution. A reproducibility test was performed by DPV using SLTEs (n = 5) from different manufacturing batches (n = 5) and a solution of 75 μmol L⁻¹ MDMA in 0.1 mol L⁻¹ BR buffer (pH 3.0).

Real samples and selectivity study

The SLTEs were used to analyze seized MDMA samples (S1–2019, S2–2017, S3–2013, and S4–2015) that were provided by the Criminalistics Institute of the Civil Police of the Federal District as tablets of different shapes and colors. These tablets were first photographed, and then one tablet from each seizure was ground using a porcelain mortar, weighed, and subsequently diluted in 1 mL of HPLC-grade methanol. This solution was vortexed for 1 min and sonicated for 5 min. From each sample, a 1 μL aliquot was taken for subsequent dilutions in 0.1 mol L⁻¹ BR buffer solution pH 3.0 and analysis by DPV. All seized MDMA samples were previously analyzed by Liquid Chromatography Quadrupole Time-of-Flight Mass

Spectrometry (Q-TOF LC-MS) at the Criminalistics Institute of the Civil Police of Distrito Federal under routine forensic casework conditions, using parameters previously described in the literature¹⁶.

Selectivity studies were carried out by DPV to evaluate possible interfering compounds that are usually found in MDMA seized samples, such as ketamine, benzocaine, caffeine, amphetamine and MDA. All compounds were evaluated in the same concentration as MDMA ($50 \mu\text{mol L}^{-1}$ in 0.1 mol L^{-1} BR buffer solution (pH 3.0)).

3. Results and discussion

3D-printed electrodes

Once the 3D-printed device was manufactured (**Figure 1A**), chemical, electrochemical, and laser-based pretreatments were evaluated, aiming to reduce the plastic content on the 3Dprinted conductive material. For the chemical treatment, the device was dipped in a 1 mol L^{-1} NaOH solution for one hour. For the electrochemical treatment, the electrode was submitted to chronoamperometry (1.4 V applied for 200 s , followed by -1.0 V applied for 200 s) with 0.5 mol L^{-1} NaOH solution.²⁷ Optimization of the laser parameters was studied using CV of 5 mmol L^{-1} $[\text{Ru}(\text{NH}_3)_6]^{3+}$ in 0.5 mol L^{-1} KCl. The optimal parameters were selected as follows: 8% of total laser power (50 W CO_2 laser tube) and 35 mm s^{-1} laser speed, as they presented the highest anodic peak current (I_{pa}) and lowest peak-to-peak potential separation (ΔE_p) (**Figure S3**).

All other treatments were studied by CV of 5 mmol L^{-1} $[\text{Ru}(\text{NH}_3)_6]^{3+}$ in 0.5 mol L^{-1}

KCl (**Figure 1B**), and the anodic peak current and the peak-to-peak potential were analyzed (**Figure 1C**). The laser treatment was chosen as the optimal treatment technique, due to its promising results and considering that it is a fast, reagent-less technique.

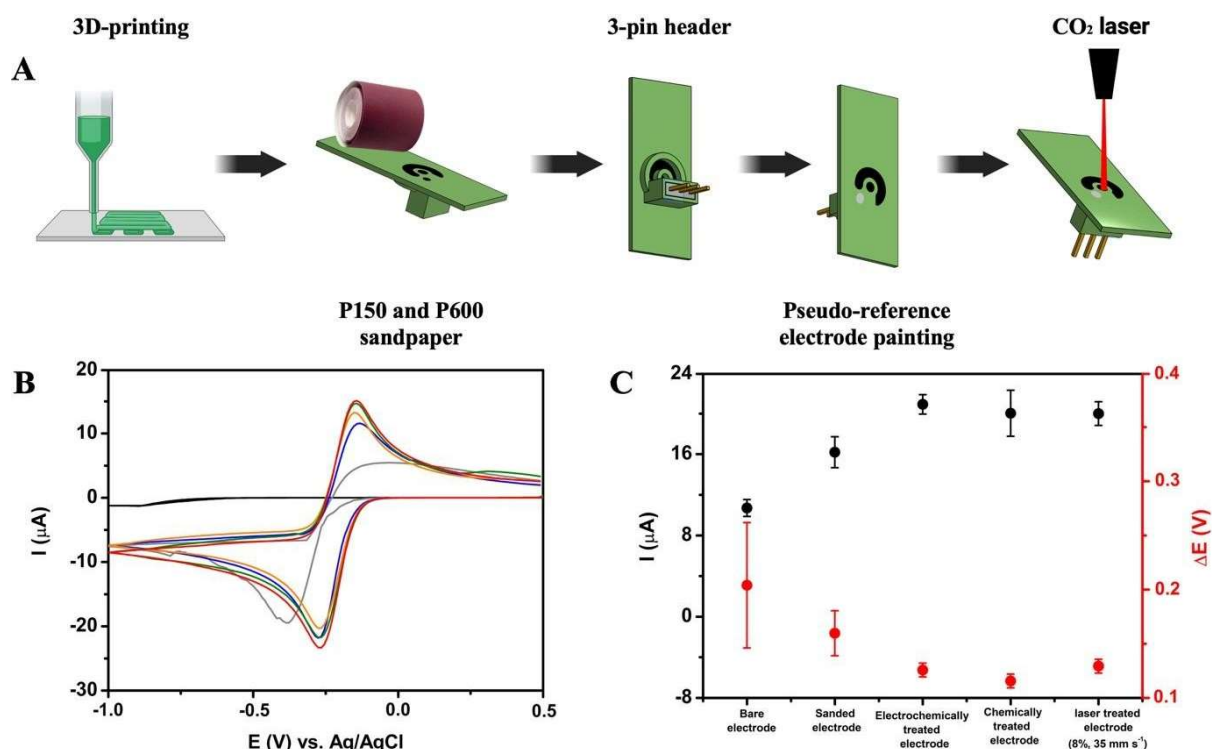


Figure 1. (A) Representation of 3D-printed electrode manufacturing process. (B) CV plots of 5 mmol L⁻¹ [Ru(NH₃)₆]³⁺ in 0.5 mol L⁻¹ KCl. Conditions: Blank solution (black line), bare 3Dprinted electrode (gray line), sanded 3D-printed electrode (blue line), sanded/chemically treated (1 mol L⁻¹ NaOH solution for 1h) 3D-printed electrode (orange line), sanded/electrochemically treated (0.5 mol L⁻¹ NaOH) 3D-printed electrode (green line), and sanded/laser-treated (8% of total laser power and 35 mm s⁻¹ laser speed) 3D-printed electrode (red line). (C) Plot of anodic peak currents and peak-to-peak potential separation (ΔE_p) values as a function of electrode pretreatment. Error bars correspond to the standard deviation of measurements using 3 different sensors.

Morphological and electrochemical characterizations

The morphology of the developed 3D-printed electrode was characterized by SEM (**Figure 2A and 2B**) at magnifications of 30x, 330x, and 1,000x for the bare and sanded

electrodes and at magnifications of 300x, 3,750x, and 4,000x for the SLTE (**Figure 2C**). As shown in **Figure 2A**, it is possible to observe the layers of filament deposited during the device manufacturing process and the rugosity of the bare electrode surface. After sanding the electrode, the surface appears rougher, likely due to partial removal of the plastic content on the surface (**Figure 2B**). The images of the SLTE can be seen in **Figure 2C**. When both treatments are combined, the electrode surface appears to be completely voided of plastic content, leading to an even rougher surface than that of a sanded electrode. This leads to a drastic increase in electrode surface area, which subsequently enhances the performance of $[\text{Ru}(\text{NH}_3)_6]^{3+}$ by providing more active sites for the redox species to undergo electron transfer around the electrode. However, these images cannot be directly compared with those of the bare and sanded electrodes, since they were obtained on different equipment and with different magnifications.

Contact angle measurements were performed on the non-treated and treated electrodes (**Figure S1A-B**), showing no significant change after the sanding process ($\text{CA}_{\text{BE}} = 71.63 \pm 0.09^\circ$, $\text{CA}_{\text{SE}} = 83.33 \pm 0.42^\circ$), which was expected given that all the filaments used in the electrode construction contain PLA in their composition. Additionally, FTIR measurements (**Figure S1C-D**) confirmed the exposure of the conductive filament after the sanding process by the appearance of the bands around 3000 and 1750 cm^{-1} , corresponding to stretching of the C=C and C=O bonds, respectively,²⁸ which are consistent with the chemical composition of the conductive filament based on PLA and carbon black.

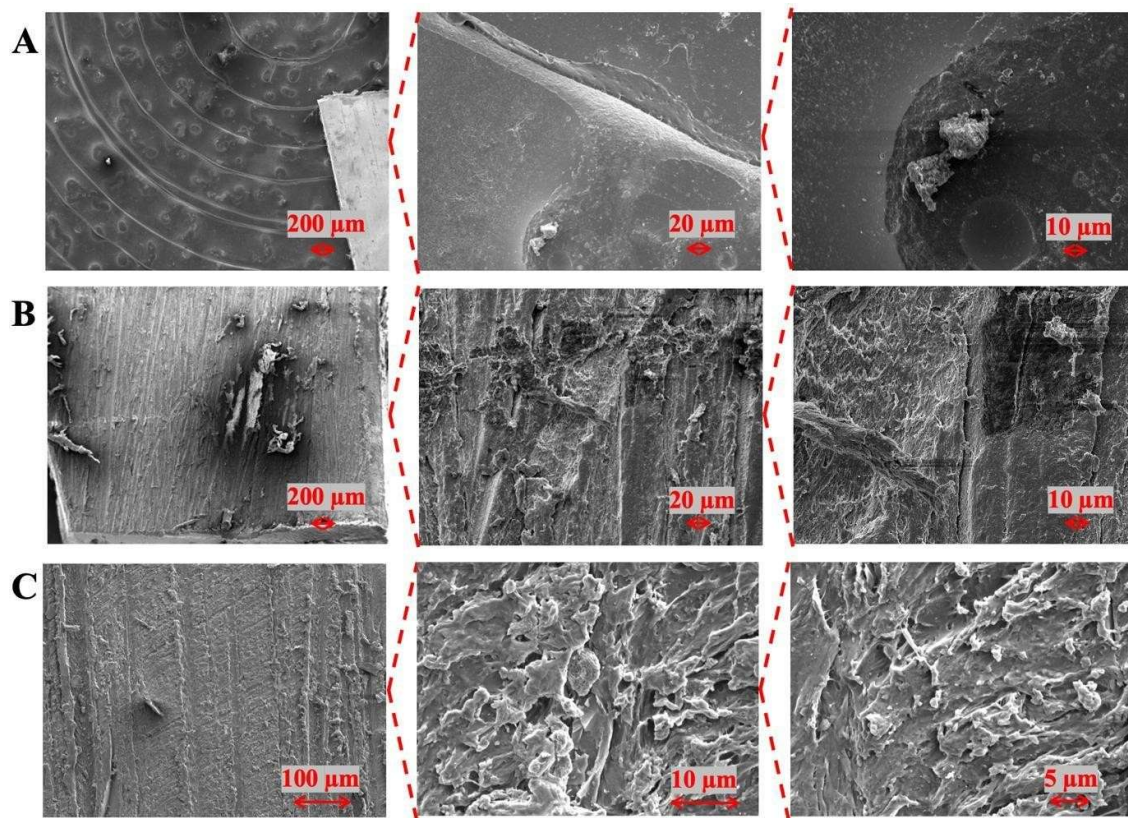


Figure 2. (A) SEM micrographs of a bare 3D-printed working electrode at magnifications of 30x, 330x, and 1,000x, respectively, from left to right. (B) SEM micrographs of a sanded 3Dprinted working electrode at magnifications of 30x, 330x, and 1,000x, respectively, from left to right. (C) SEM micrographs of a sanded/laser-treated 3D-printed working electrode at magnifications of 300x, 3,750x, and 4,000x, respectively, from left to right.

The mass transport process of $5 \text{ mmol L}^{-1} [\text{Ru}(\text{NH}_3)_6]^{3+}$ in $0.5 \text{ mol L}^{-1} \text{ KCl}$ using SE was evaluated by CV at various scan rates ranging from 5 to 200 mV s^{-1} (**Figure 3A**). Diffusioncontrolled mass transport was confirmed by the linear behavior in the anodic and cathodic peak current values as a function of the square root of the scan rate (**Figure 3B**). A scan rate study was also performed using SLTE in the presence of $5 \text{ mmol L}^{-1} [\text{Ru}(\text{NH}_3)_6]^{3+}$ in $0.5 \text{ mol L}^{-1} \text{ KCl}$ (**Figures S2A and S2B**) and in the presence of $75 \text{ μmol L}^{-1} \text{ MDMA}$ in $0.1 \text{ mol L}^{-1} \text{ BR buffer (pH 3.0)}$ (**Figures S2C and S2D**), and results from both studies indicate a diffusion-controlled mass transport in the proposed system.

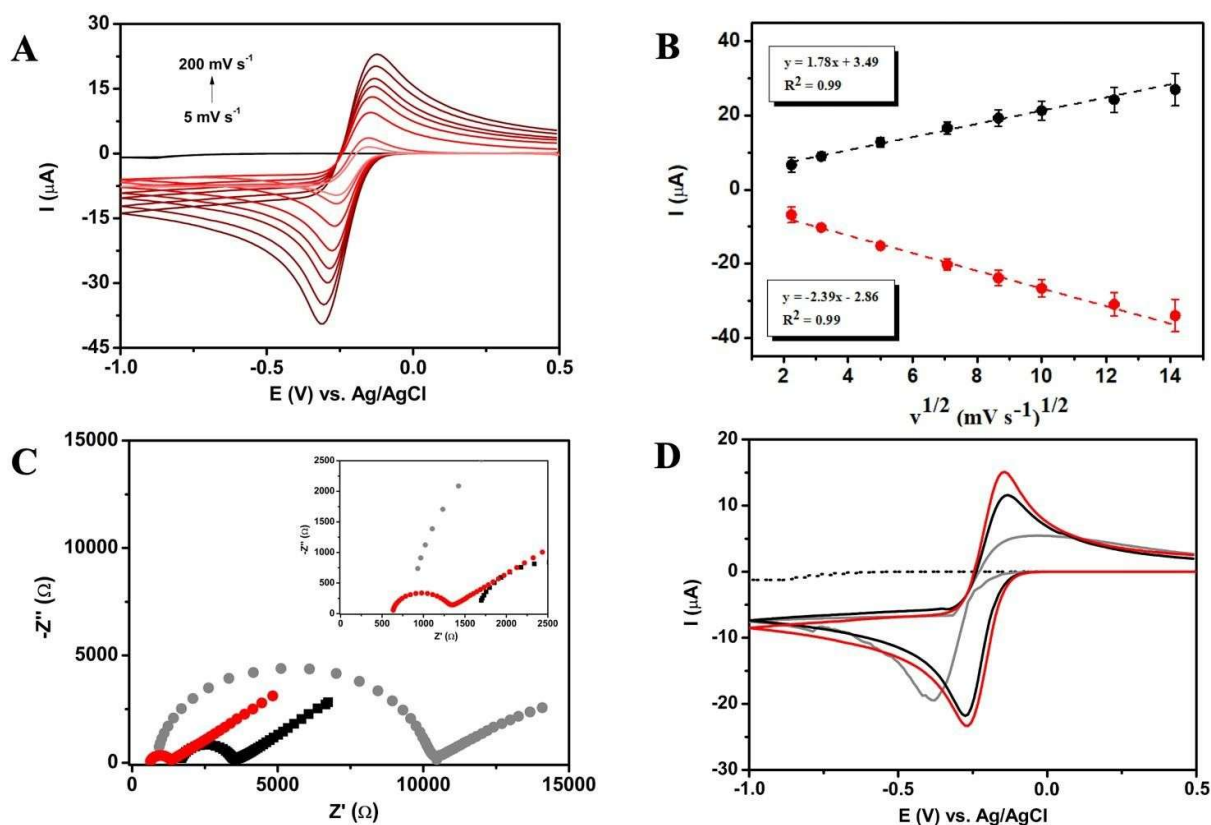


Figure 3. (A) CV plots using SEs in the presence of 5 mmol L⁻¹ [Ru(NH₃)₆]³⁺ in 0.5 mol L⁻¹ KCl at different scan rates (5, 10, 25, 50, 75, 100, 150 and 200 mV s⁻¹). (B) Plot of anodic and cathodic peak current values obtained for SEs as a function of the square root of scan rate from Figure 3A. The error bars correspond to the standard deviation of measurements from 3 different sensors. Conditions: Anodic peak current (black dots) and cathodic peak current (red dots). (C) EIS Nyquist plots using 5 mmol L⁻¹ [Ru(NH₃)₆]³⁺ in 0.5 mol L⁻¹ KCl. Conditions: BE (gray dots), SE (black squares), and SLTE (red dots). (D) CV plots of 5 mmol L⁻¹ [Ru(NH₃)₆]³⁺ in 0.5 mol L⁻¹ KCl (scan rate 50 mV s⁻¹). Conditions: Blank solution (black dots), BE (gray line), SE (black line), and SLTE (red line).

Additional electrochemical characterizations of BE, SE, and SLTE were performed by EIS and CV in the presence of 5 mmol L⁻¹ [Ru(NH₃)₆]³⁺ in 0.5 mol L⁻¹ KCl. Electrochemical behavior was significantly enhanced after sanding and laser irradiation, confirming that the treatments removed the plastic layer from the electrode surface, exposing the conductive filament material. The Nyquist plots shown in **Figure 3C** demonstrate an enhancement in the electron transfer kinetics at the SLTE when compared to the BE and SE, by the decrease in charge

□
□

transfer resistance (R_{CT}) values ($R_{CT|BE} = 10,500 \, \Omega$, $R_{CT|SE} = 3,500 \, \Omega$ and $R_{CT|SLTE} = 1,350 \, \Omega$). CV analysis of the BE, SE, and SLTE revealed that I_{pa} increases and ΔE_p decreases with each modification step, leading to the best results after both sanding and laser-treatment (**Figure 3D**). Values of I_{pa} and ΔE_p for each electrode type are outlined in **Table 1**.

Table 1. Values of I_{pa} and ΔE_p for each 3D-printed electrode modification determined from CV of $[Ru(NH_3)_6]^{3+}$

3D-printed electrode type	Anodic peak current ($I_{pa} / \mu A$)	Peak-to-peak separation ($\Delta E_p / mV$)
Bare	10.71 $\pm$ 0.83	204 $\pm$ 53
Sanded	16.19 $\pm$ 1.53	159 $\pm$ 21
Sanded/lasertreated	19.99 $\pm$ 1.17	129 $\pm$ 6

Finally, the effect of pH on the voltammetric response of MDMA was evaluated by DPV using a solution of 200 $\mu\text{mol L}^{-1}$ MDMA in 0.1 mol L^{-1} BR buffer while varying the pH from 3.0 to 11.0 (**Figure 4 A-C**). The voltammetric response of MDMA was clearly pH dependent, as indicated by significant changes in peak shape and location as the pH was adjusted. A pH of 3.0 was chosen as the optimal condition since the response is presented as a more well-defined peak when compared to that of pH values 5.0 and 7.0, which present less defined peaks in the voltammograms despite having similar current magnitudes to the response at pH 3.0. A broadening of the oxidation peaks is observed from pH 3.0 onwards, which may be

associated with splitting of electron transfer into two redox processes. The appearance of a second redox process for MDMA, mainly in less acidic conditions, has already been documented for other carbon-based electrodes.^{29,30} The more pronounced dependence in the voltammetric response at pH 9.0 and 11.0 may be attributed to the pKa value of MDMA being 9.9, with the protonated form exhibiting better electrochemical behavior as seen at lower pH values.³¹ When peak potential is plotted against pH, a slope of 36 mV/pH was obtained from the linear regression, which indicates a 2:1 electron:proton ratio in the electrooxidation of MDMA on our 3D-printed electrochemical system, similar to the behavior observed in previous studies.^{29,30}

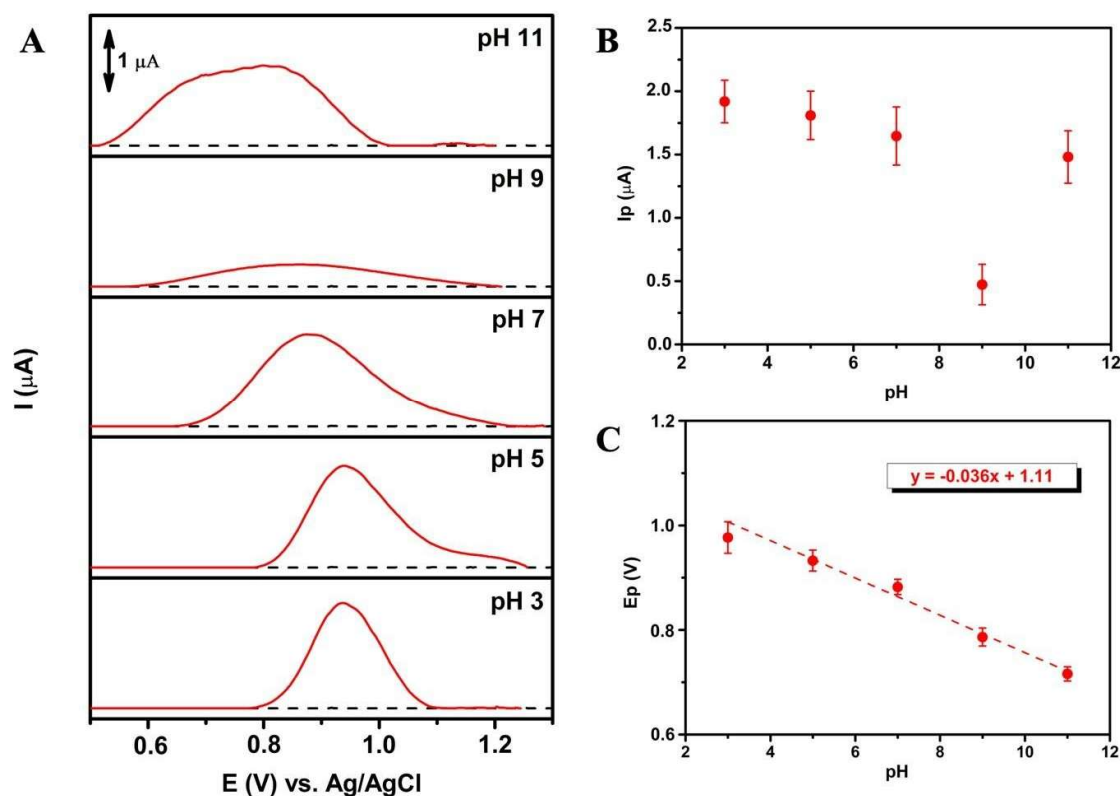


Figure 4. (A) Baseline corrected DPV plots of the SLTE in the presence of a 200 $\mu\text{mol L}^{-1}$ MDMA in 0.1 mol L^{-1} BR buffer solution at different pH values. Conditions: Blank solution (black dash), and 200 $\mu\text{mol L}^{-1}$ MDMA in 0.1 mol L^{-1} BR buffer solution (red line). (B) Plot of anodic peak current obtained from DPV data of Figure 4A as a function of pH value. (C) Plot of anodic peak potential obtained from DPV data of Figure 4A as a function of pH value.

The error bars correspond to the standard deviation of measurements across 3 different sensors.

All electroanalytical characterizations and analyses of MDMA were performed on a Sensit wearable potentiostat connected to a smartphone by Bluetooth (**Figure 5**), which facilitates the in-field applications.

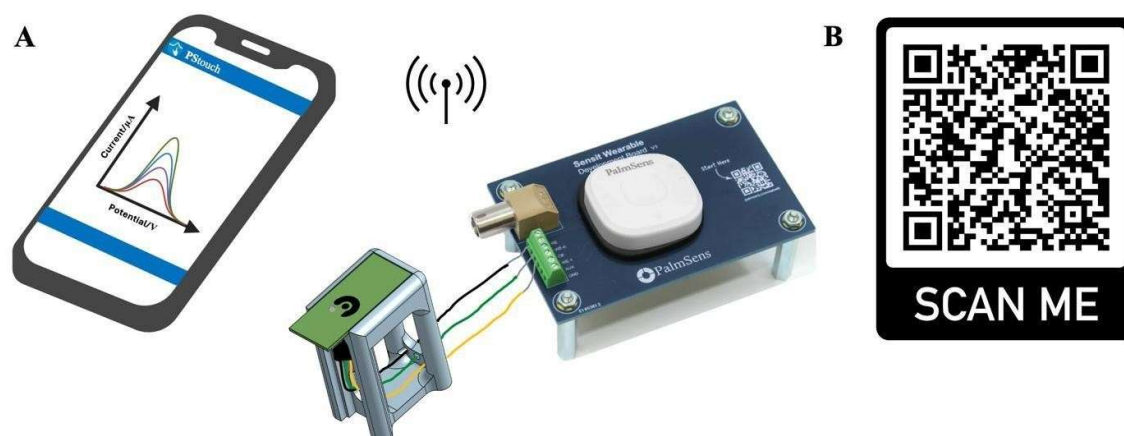


Figure 5. (A) Image of the electrochemical setup used for MDMA analysis. (B) QR code for a video showing the portable electrochemical measurement and wireless data acquisition.

Voltammetric study of MDMA

A calibration curve was built using I_{pa} values obtained from the baseline-corrected DPV plots (**Figure 6A**) in the presence of MDMA concentrations ranging from 2.5 to 200 $\mu\text{mol L}^{-1}$ (**Figure 6B**) at around 0.95 V (vs. Ag/AgCl) using the SLTE. A linear response was obtained within the concentration range of 2.5 to 100 $\mu\text{mol L}^{-1}$ MDMA in 0.1 mol L^{-1} BR buffer (pH 3.0), with a determination coefficient (R^2) of 0.99. The LOD and LOQ were calculated according to the International Union of Pure and Applied Chemistry (IUPAC) equations,³² i.e., $\text{LOD} = 3 \times (\sigma_{\text{blank}}/s)$ and $\text{LOQ} = 10 \times (\sigma_{\text{blank}}/s)$, where σ_{blank} corresponds to the standard deviation of the blank solution measurements in triplicate ($n = 3$), and s is the slope of the calibration curve. We obtained an LOD of 0.08 $\mu\text{mol L}^{-1}$ MDMA and an LOQ of 0.27 $\mu\text{mol L}^{-1}$ MDMA.

A reproducibility study, using five ($n = 5$) SLTEs from different manufacturing batches, was carried out by DPV in the presence of $75 \mu\text{mol L}^{-1}$ MDMA in 0.1 mol L^{-1} BR buffer (pH 3.0) and resulted in a relative standard deviation (RSD) of 5.5%, which is adequate for 3Dprinting electrode manufacturing, considering the surface treatments performed prior the analysis (**Figure 6C**).

A stability study over the course of 28 days was carried out by CV in the presence of 5 mmol L^{-1} $[\text{Ru}(\text{NH}_3)_6]^{3+}$ in 0.5 mol L^{-1} KCl and using SLTEs stored in a desiccator containing silica. The anodic peak current was evaluated (**Figure 6D**), and a 13% decrease was observed on the 28th day, confirming acceptable stability of the device over time in this storage condition. This behavior demonstrates the acceptable shelf life of the device, eliminating the need for additional treatments before the electrochemical measurements.

The developed 3D-printed, sanded/laser-treated sensor displayed comparable analytical performance to other electrochemical MDMA sensors reported in the literature (**Table 2**). One noteworthy aspect of our sensor is that it is highly comparable to sensors that use additional surface modifications like molecularly imprinted polymers (MIPs) and nanoparticles to enhance surface area and sensitivity. However, our sensor does not have any extra surface modifications and offers other advantages such as easy and scalable fabrication, being a completely portable system, and potential for in-field screening analysis.

Based on the results of this study, we highlight that our electrochemical device is suitable for simple, in-field MDMA detection, with measurement times around 1 min. Besides that, the estimated manufacturing cost of the system is estimated to be US\$0.18 per device, considering only the costs of commercial filaments and materials used for treatment. Considering the biodegradability of PLA, adequate manufacturing reproducibility, and the low

cost device construction, the sensor can be used as a disposable, one-sensor-per-analysis device while facilitating its use by non-electrochemist individuals. Additionally, the sensor presented good sensitivity and stability towards MDMA, once again confirming its suitability for in-field MDMA analysis.

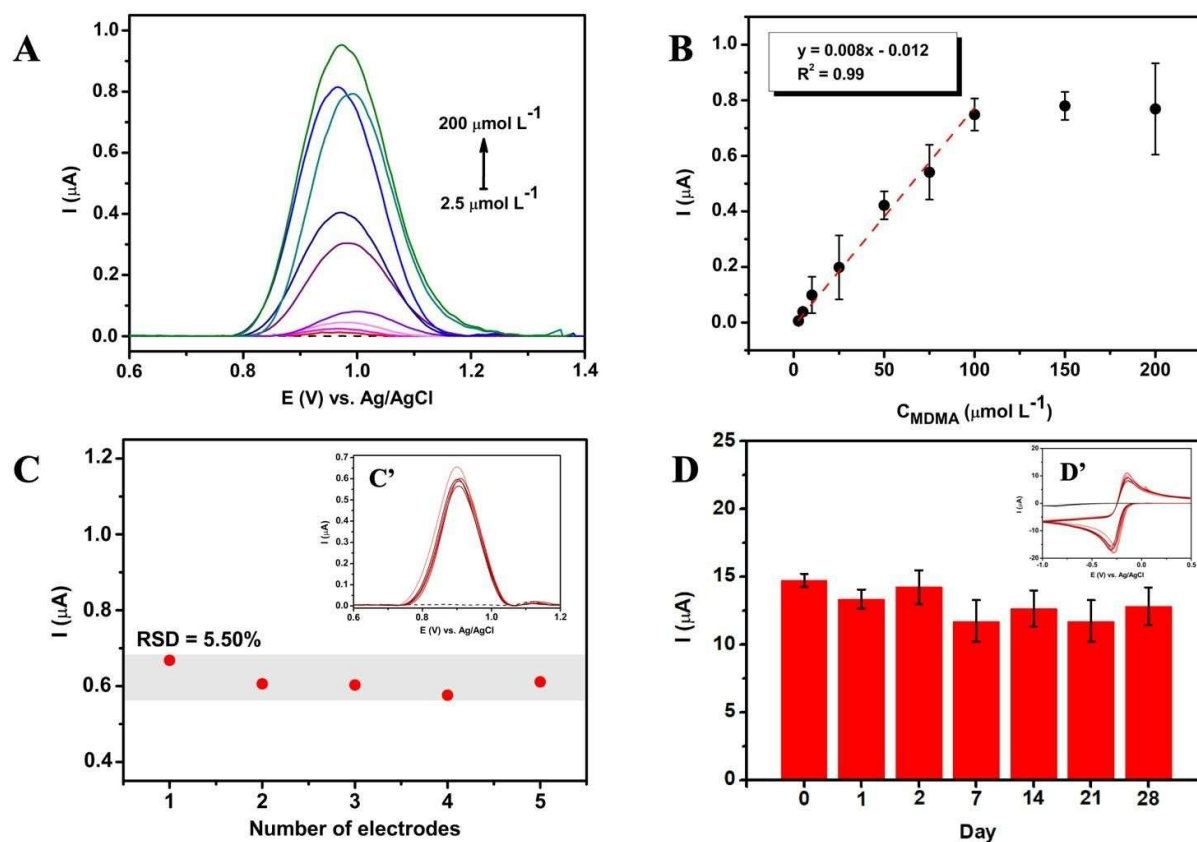


Figure 6. (A) Baseline corrected DPV plots recorded at different MDMA concentrations (from 2.5 to $200 \mu\text{mol L}^{-1}$) in 0.1 mol L^{-1} BR buffer (pH 3.0) using the SLTE. (B) Calibration curve constructed using the mean of peak current values as a function of MDMA concentration. The error bars correspond to the standard deviation of measurements using three different sensors. (C) Reproducibility test ($n = 5$) with $75 \mu\text{mol L}^{-1}$ MDMA in 0.1 mol L^{-1} BR buffer (pH 3.0). (C') Baseline corrected DPV plots recorded using five different SLTEs in the presence of $75 \mu\text{mol L}^{-1}$ MDMA in 0.1 mol L^{-1} BR buffer (pH 3.0). Conditions: Blank solution (black dash), SLTE (red lines). (D) 28-day stability study using the SLTE in the presence of 5 mmol L^{-1} $[\text{Ru}(\text{NH}_3)_6]^{3+}$ in 0.5 mol L^{-1} KCl. (D') CV plots using the SLTE in the presence of 5 mmol L^{-1} $[\text{Ru}(\text{NH}_3)_6]^{3+}$ in 0.5 mol L^{-1} KCl on different days after manufacturing (0, 1, 2, 7, 14, 21, and 28 days). Conditions: Blank solution (black lines), SLTE (from day 0, lighter red, to day 28, darker red).

Table 2. Comparison of different electrochemical sensors for MDMA detection in the literature. Abbreviations: SPE: Screen-Printed Electrode; P-L-arg/GCE: larginine membrane on Glassy Carbon Electrode; MIP@PDA/Au: Molecular Imprinted Polydopamine film on Au Electrode; CNHs-CHI@PtNPs/GCE: High Surface Area Carbon Nanohorns decorated with Pt nanoparticles on Glassy Carbon Electrode; C-SPE: Carbon Screen-Printed Electrode; 3D-printed Gr/PLA: 3D-printed Graphite/Polylactic Acid Electrode; 3D-printed CB/PLA: 3D-printed Carbon-black integrated Polylactide Electrode; SLTE: sanded/laser-treated electrode.

Sensor	Electrochemical technique	LOD ($\mu\text{mol L}^{-1}$)	Linear range ($\mu\text{mol L}^{-1}$)	Ref.
Commercial Graphite SPE (PalmSens)	SWV	4.03	25 – 1,500	³¹
P-L-arg/GCE	SWV	0.033	0.1 - 35	¹⁷
MIP@PDA/Au	DPV	0.054	-	³²
CNHs-CHI@PtNPs/GCE	DPV	0.018	2.5 – 35.4	³³
C-SPE	LSV	1.83	10 - 100	¹⁵
3D-printed Gr/PLA	DPV	0.1	1 – 175	³⁴
3D-printed CB/PLA	CSWV	1.8	5.8 - 35	³⁵
SLTE	DPV	0.08	2.5 - 100	This Work

Electrochemical detection of MDMA in real samples

We applied the SLTE for an in-field analysis of MDMA using seized tablets that were suspected of containing MDMA, the most common form of the drug's presentation. All tablet samples (diluted at least 4,000x in BR buffer solution) showed an electrochemical profile similar to the $10\ \mu\text{mol L}^{-1}$ MDMA standard in $0.1\ \text{mol L}^{-1}$ buffer (pH 3.0), with a visible oxidation process around +1.0 V (vs. Ag/AgCl), as shown in **Figure 7A-D**. This behavior is reported in the literature and may be related to the protonated form of the molecule at pH values below 8.0.³⁸

After verifying that the samples exhibited behavior similar to the analytical MDMA standard, aliquots containing $10\ \mu\text{mol L}^{-1}$ of MDMA (stock solution) were added to the samples to evaluate the recovery of the electrochemical signal, as shown by the dark lines in Figure 7AD. The MDMA-containing tablet samples did not show significant matrix effects, yielding mean recoveries between 81–135%, as shown in **Table 3**.

As demonstrated, the method shows good accuracy, with acceptable recovery values using the SLTE. Furthermore, based on the dilution steps and the calibration curve ($I_p = 0.008*[\text{MDMA}] - 0.012$), it was possible to quantify and estimate the mass of MDMA present in the samples. The amounts found, ranging from 10 to 90 mg of MDMA, are consistent with values reported in seizures, databases such as DrugsData, and reports from the European Union Drugs Agency (EUDA).^{39–43} Furthermore, as described in section 2.6, the presence of MDMA in the samples was previously confirmed by Q-TOF LC-MS by the Civil Police of Distrito Federal. Additionally, the most common adulterants found in seized MDMA samples (ketamine, benzocaine, caffeine, amphetamine, and MDA), except for MDA, did not exhibit peaks in the same potential range of MDMA, as shown in the selectivity study in **Figure 7E**,

allowing for voltammetric MDMA screening. The voltammetric differentiation between MDMA and MDA can be achieved by observing the reduction peak of the MDA oxidation product at around +0.2 V (vs. Ag/AgCl) in the reverse scan, which did not occur for MDMA.²⁴

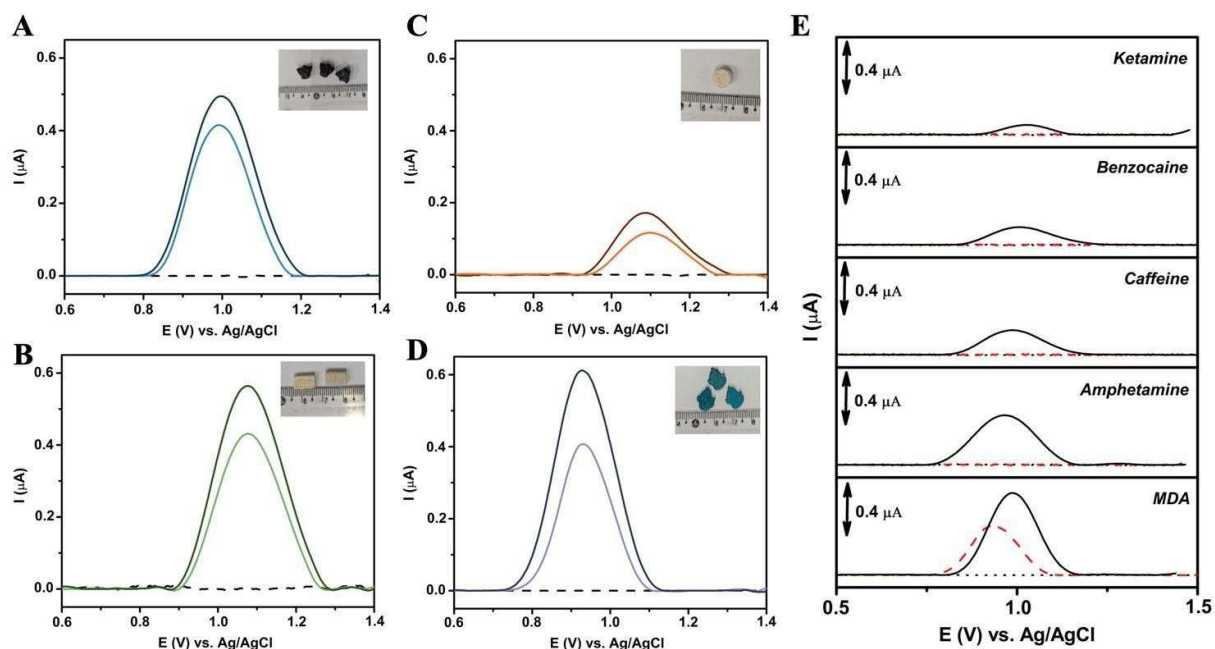


Figure 7. (A) Baseline corrected DPV plots of sample 1 (S1–2019) diluted in 0.1 mol L⁻¹ BR (pH 3.0) before (light blue line) and after (dark blue line) the addition of 10 μmol L⁻¹ MDMA standard solution. (B) Baseline corrected DPV plots of sample 2 (S2–2017) diluted in 0.1 mol L⁻¹ BR (pH 3.0) before (light green line) and after (dark green line) the addition of 10 μmol L⁻¹ MDMA standard solution. (C) Baseline corrected DPV plots of sample 3 (S3– 2013) diluted in 0.1 mol L⁻¹ BR (pH 3.0) before (light orange line) and after (dark orange line) the addition of 10 μmol L⁻¹ MDMA standard solution. (D) Baseline corrected DPV plots of sample 4 (S4– 2015) diluted in 0.1 mol L⁻¹ BR (pH 3.0) before (light purple line) and after (dark purple line) the addition of 10 μmol L⁻¹ MDMA standard solution. Conditions in A-D: Blank solution (black dashed line). (E) Baseline corrected DPV plots of possible interfering species to MDMA detection: ketamine, benzocaine, caffeine, amphetamine, and MDA at the same MDMA concentration (50 μmol L⁻¹ in 0.1 mol L⁻¹ BR buffer solution (pH 3.0)). Conditions: blank solution (black dots), interfering species (red dashed line), and the mixture of interfering species and MDMA standard solution (black line).

Table 3. Study of MDMA recovery in seized tablet samples using SLTE.

Sample	MDMA	%Recovery ± RSD	MDMA
	concentration added ($\mu\text{mol L}^{-1}$)		mass in sample (mg)
S1–2019	10	108 ± 9	47.9
S2–2017	10	128 ± 19	10.8
S3–2013	10	81 ± 8	38.2
S4–2015	10	135 ± 6	86.1

Based on the results, the SLTE developed in this study proved suitable for both qualitative and quantitative in-field detection of MDMA in tablet samples. Enabled by a scalable fabrication process, a simple setup that allows rapid analysis (approximately 1 min), and a low production cost (around US\$0.18 per device), the system supports a one-sensor-peranalysis approach that can be operated by a non-electrochemist analyst using portable and userfriendly equipment, making it highly suitable for on-site applications. In addition, considering the LOD, LOQ, and linear range achieved, the proposed voltammetric approach

presents analytical characteristics that may complement existing MDMA screening techniques, particularly with respect to specificity and performance at higher analyte concentrations.

Although the present work focused on seized tablet samples, the obtained performance parameters suggest that further studies could explore the applicability of this method to biological matrices, such as urine, blood, and saliva.⁴⁴⁻⁴⁷

4. Conclusions

This work reports the development of a sensitive, simple, and portable 3D-printed electrochemical system for in-field MDMA detection. By sanding the electrode surface with sandpaper and subsequently irradiating the surface with a CO₂ pulsed laser, the PLA content from the conductive thermoplastic filament was partially removed, exposing conductive carbon-based material, as confirmed by SEM, FTIR, and CA analyses. Electrochemical characterizations (EIS and CV) demonstrated an enhancement of the electrochemical performance after surface treatment. The reagent-free treatment method provided sensitive MDMA detection, with estimated LOD and LOQ values of 0.08 $\mu\text{mol L}^{-1}$ and 0.27 $\mu\text{mol L}^{-1}$ MDMA, respectively. The manufacturing process ensured good reproducibility (RSD = 5.50%, n = 5) and stability, with only a 13% decrease in peak current after 28 days of storage in a desiccator with silica. With minimal sample consumption (100 μL of solution for voltammetric analysis) and waste generation, a large-scale manufacturing approach, and an estimated cost of US\$0.18 per device, this system presents a promising alternative for on-site MDMA screening tests. Furthermore, the sanded/laser-treated 3D-printed electrodes demonstrated adequate performance for both qualitative and quantitative in-field analysis, supporting a one-sensorperanalysis approach that can be operated by a non-electrochemist analyst. In

summary, the developed electrochemical sensor provides a sensitive, portable, and low-cost solution for infield MDMA detection, featuring a palm-sized system that allows non-electrochemist individuals to perform analyses and obtain results directly on a smartphone.

Acknowledgments

The authors are thankful to the Civil Police of the Federal District for providing the forensic samples used in the present study. We are also thankful to the Brazilian agencies CAPES, FAPESP (Grant numbers: 2023/00246-1, 2023/12589-0 and 2022/03250-7), CNPq (Grant numbers: 406309/2025-6, 405620/2021-7, 310282/2022-5, 308136/2025-0 and 442598/2023-8), INCTBio (grant 408338/2024-5, and National Institute of Science and Technology on Psychoactive Substances (INCT-SP) (CNPq grant number: 406958/2022-0) for supporting this work. We would also like to thank the Analytical Resources Core (ARC) at Colorado State University for access to and assistance with scanning electron microscopy measurements (RRID: SCR_021758).

5. References

1. UNODC. *World Drug Report* 2025. https://www.unodc.org/documents/dataandanalysis/WDR_2025/WDR25_Special_points_of_interest.pdf (2025).
2. Costa, G. & Gołembiowska, K. Neurotoxicity of MDMA: Main effects and mechanisms. *Exp Neurol* **347**, 113894 (2022).
3. Holze, F. *et al.* Distinct acute effects of LSD, MDMA, and d-amphetamine in healthy subjects. *Neuropsychopharmacology* **45**, 462–471 (2020).
4. Department of Justice/Drug Enforcement Administration. Drug Fact Sheet: Ecstasy/MDMA. Preprint at <https://www.dea.gov/sites/default/files/202303/EcstasyMDMA%202022%20Drug%20Fact%20Sheet.pdf> (2022).
5. Concheiro, M., de Castro, A., Quintela, O., López-Rivadulla, M. & Cruz, A. Determination of MDMA, MDA, MDEA and MBDB in oral fluid using high performance liquid chromatography with native fluorescence detection. *Forensic Sci Int* **150**, 221–226 (2005).
6. De Martinis, B. S., Barnes, A. J., Scheidweiler, K. B. & Huestis, M. A. Development and validation of a disk solid phase extraction and gas chromatography–mass spectrometry method for MDMA, MDA, HMMA, HMA, MDEA, methamphetamine and amphetamine in sweat. *Journal of Chromatography B* **852**, 450–458 (2007).
7. Pirnay, S. O., Abraham, T. T. & Huestis, M. A. Sensitive Gas Chromatography-Mass Spectrometry Method for Simultaneous Measurement of MDEA, MDMA, and Metabolites HMA, MDA, and HMMA in Human Urine. *Clin Chem* **52**, 1728–1734 (2006).
8. de Araujo, W. R. *et al.* Portable analytical platforms for forensic chemistry: A review. *Anal Chim Acta* **1034**, 1–21 (2018).
9. Bottelli, E. D., de Lima, L. F., Paixão, T. R. L. C. & de Araujo, W. R. Laser-scribed graphene toward scalable fabrication of electrochemical paper-based devices for lidocaine detection in forensic and pharmaceutical samples. *Electrochim Acta* **507**, 145162 (2024).
10. Pinheiro, T. *et al.* Laser-Induced Graphene on Paper toward Efficient Fabrication of Flexible, Planar Electrodes for Electrochemical Sensing. *Adv Mater Interfaces* **8**, (2021).
11. de Lima, L. F. & de Araujo, W. R. Laser-scribed graphene on polyetherimide substrate: an electrochemical sensor platform for forensic determination of xylazine in urine and beverage samples. *Microchimica Acta* **189**, 465 (2022).
12. Baldo, T. A. *et al.* Automated enzyme-linked immunosorbent assay for point-of-care COVID-19 testing. *Electrochim Acta* **497**, 144525 (2024).
13. Hayat, A. & Marty, J. Disposable Screen Printed Electrochemical Sensors: Tools for Environmental Monitoring. *Sensors* **14**, 10432–10453 (2014).
14. Torre, R., Costa-Rama, E., Nouws, H. P. A. & Delerue-Matos, C. A do-it-yourself electrochemical cell based on pencil leads and transparency sheets: Application to the enzymatic determination of histamine. *Talanta* **266**, 124980 (2024).

15. Murilo Alves, G., Soares Castro, A., McCord, B. R. & de Oliveira, M. F. MDMA Electrochemical Determination and Behavior at Carbon Screen-printed Electrodes: Cheap Tools for Forensic Applications. *Electroanalysis* **33**, 635–642 (2021).
16. Teófilo, K. R. *et al.* Electrochemical detection of 3,4methylenedioxymethamphetamine (ecstasy) using a boron-doped diamond electrode with differential pulse voltammetry: Simple and fast screening method for application in forensic analysis. *Microchemical Journal* **157**, 105088 (2020).
17. Duan, S. *et al.* A simple polyarginine membrane electrochemical sensor for the determination of MDMA and MDA. *Anal Biochem* **688**, 115478 (2024).
18. Abdalla, A. & Patel, B. A. 3D Printed Electrochemical Sensors. *Annual Review of Analytical Chemistry* **14**, 47–63 (2021).
19. Cardoso, R. M. *et al.* Additive-manufactured (3D-printed) electrochemical sensors: A critical review. *Anal Chim Acta* **1118**, 73–91 (2020).
20. Muñoz, J. & Pumera, M. Accounts in 3D-Printed Electrochemical Sensors: Towards Monitoring of Environmental Pollutants. *ChemElectroChem* **7**, 3404–3413 (2020).
21. Gusmão, R., Browne, M. P., Sofer, Z. & Pumera, M. The capacitance and electron transfer of 3D-printed graphene electrodes are dramatically influenced by the type of solvent used for pre-treatment. *Electrochem commun* **102**, 83–88 (2019).
22. Manzanares Palenzuela, C. L., Novotný, F., Krupička, P., Sofer, Z. & Pumera, M. 3DPrinted Graphene/Polylactic Acid Electrodes Promise High Sensitivity in Electroanalysis. *Anal Chem* **90**, 5753–5757 (2018).
23. Muñoz, J. & Pumera, M. Accounts in 3D-Printed Electrochemical Sensors: Towards Monitoring of Environmental Pollutants. *ChemElectroChem* **7**, 3404–3413 (2020).
24. de Faria, L. V. *et al.* Cyclic square-wave voltammetric discrimination of the amphetamine-type stimulants MDA and MDMA in real-world forensic samples by 3Dprinted carbon electrodes. *Electrochim Acta* **429**, 141002 (2022).
25. Rocha, D. P., Squissato, A. L., da Silva, S. M., Richter, E. M. & Munoz, R. A. A. Improved electrochemical detection of metals in biological samples using 3D-printed electrode: Chemical/electrochemical treatment exposes carbon-black conductive sites. *Electrochim Acta* **335**, 135688 (2020).
26. Rocha, D. P. *et al.* Reagentless and sub-minute laser-scribing treatment to produce enhanced disposable electrochemical sensors via additive manufacture. *Chemical Engineering Journal* **425**, 130594 (2021).
27. de Faria, L. V. *et al.* Cyclic square-wave voltammetric discrimination of the amphetamine-type stimulants MDA and MDMA in real-world forensic samples by 3Dprinted carbon electrodes. *Electrochim Acta* **429**, 141002 (2022).
28. Table of Characteristic IR Absorptions. *Department of Analytical Chemistry, UCT Prague*.
29. Garrido, E.M.P.J. *et al.* Electrochemical oxidation of amphetamine-like drugs and application to electroanalysis of ecstasy in human serum. *Bioelectrochemistry* **79**, 77 – 83 (2010).
30. Teófilo, K. R. *et al.* Electrochemical detection of 3,4methylenedioxymethamphetamine (ecstasy) using a boron-doped diamond electrode with differential pulse voltammetry: Simple and fast screening method for application in forensic analysis. *Microchemical Journal* **157**, 105088 (2020).

31. Rosa Boleda, M., Huerta-Fontela, M., Ventura, F. & Galceran, M. T. Evaluation of the presence of drugs of abuse in tap waters. *Chemosphere* **84**, 1601–1607 (2011).
32. Mocak, J., Bond, A. M., Mitchell, S. & Scollary, G. A statistical overview of standard (IUPAC and ACS) and new procedures for determining the limits of detection and quantification: Application to voltammetric and stripping techniques (Technical Report). *Pure and Applied Chemistry* **69**, 297–328 (1997).
33. Van Echelpoel, R. *et al.* Validated portable device for the qualitative and quantitative electrochemical detection of MDMA ready for on-site use. *Microchemical Journal* **190**, 108693 (2023).
34. Li, C. *et al.* Polydopamine-based molecularly imprinted electrochemical sensor for the highly selective determination of ecstasy components. *Analyst* **147**, 3291–3297 (2022).
35. Zhang, R. *et al.* Highly sensitive electrochemical sensor based on Pt nanoparticles/carbon nanohorns for simultaneous determination of morphine and MDMA in biological samples. *Electrochim Acta* **370**, 137803 (2021).
36. Melo, L. M. A. *et al.* Combined colorimetric and electrochemical screening method using 3D printed devices: Towards the selective detection of MDMA in forensic samples. *Electrochim Acta* **483**, 144041 (2024).
37. de Faria, L. V. *et al.* Cyclic square-wave voltammetric discrimination of the amphetamine-type stimulants MDA and MDMA in real-world forensic samples by 3Dprinted carbon electrodes. *Electrochim Acta* **429**, 141002 (2022).
38. Chemicalize - Instant Cheminformatics Solutions.
<https://chemicalize.com/app/calculation>.
39. O'Reilly, M. J. A. *et al.* A quantitative analysis of <scp>MDMA</scp> seized at New South Wales music festivals over the 2019/2020 season: Form, purity, dose and adulterants. *Drug Alcohol Rev* **41**, 330–337 (2022).
40. Byrska, B. & Stanaszek, R. Chemical composition of Ecstasy tablets seized in Poland between 2005 and 2020. *Forensic Toxicol* **43**, 22–32 (2025).
41. Cole, J. C., Bailey, M., Sumnall, H. R., Wagstaff, G. F. & King, L. A. The content of ecstasy tablets: implications for the study of their long-term effects. *Addiction* **97**, 1531–1536 (2002).
42. *EU Drug Market: MDMA — Retail Markets*. https://www.euda.europa.eu/publications/eu-drug-markets/mdma_en#retail (2025).
43. DrugsData.org - Lab Test Results (EcstasyData).
https://www.drugsdata.org/results.php?search_field=all&s=5-MeO-MiPT.
44. Abraham, T. T. *et al.* Urinary MDMA, MDA, HMMA, and HMA Excretion Following Controlled MDMA Administration to Humans. *Journal of Analytical Toxicology*, **33** 439-446 (2009).
45. Kolbrich E. A. *et al.* Plasma Pharmacokinetics of 3,4-Methylenedioxymethamphetamine After Controlled Oral Administration to Young Adults. *Therapeutic Drug Monitoring*, **30**, 320-332 (2008).
46. Rey, E. G. *et al.* Mitigating the Hook Effect in Later Flow Sandwich Immunoassays Using Real-Time Reaction Kinetics. *Analytical Chemistry*, **89**, 5095-5100 (2017).
47. Lua, A. C. *et al.* Evaluation of immunoassays for the determination of MDMA and cannabinoids in urine samples. *Journal of Food and Drug Analysis*, **11**, (2003).

CHAPTER FOUR: A LOW-COST 3D-PRINTED PRECONCENTRATION AND FOLDABLE PAPER CARD DETECTION SYSTEM FOR HEAVY METALS ANALYSIS IN RIVER AND DRINKING WATER*

1. Introduction

Water pollution is an increasing global concern that poses significant threats to both human health and the environment. For the past decade, the World Economic Forum has consistently ranked the degradation of water resources among the top ten most critical global risks.^{1,2} Worldwide, 80% of the wastewater remains untreated, carrying a mixture of home wastes and harmful industrial pollutants.³ These contaminants disrupt the delicate ecosystem balances and cause widespread environmental damage.⁴ Exposure to these environmental toxins contributes to a significant portion (almost 1/4th) of the global disease burden with 50% of global child death attributed to poor water quality.^{6, 8}

Among different classes of water contaminants, heavy metals have garnered significant attention across various sectors due to their detrimental effects on human health and ecological balance.⁴ While heavy metals can naturally occur in the environment, anthropogenic activities are the predominant source of water contamination.^{4,9} The inherent properties of heavy metals, including their water solubility, resistance to biological degradation, and high bioavailability, contribute to their persistent presence in the environment.⁵

* This work is published in ACS Sensors, titled 'Fill, Fold, Photo: Preconcentration and Multiplex Detection of Trace Level Heavy Metals in Water'. The work was coauthored by Prakash Aryal, Jason Boes, Eric Brack, Todd Alexander, and Charles S. Henry. ACS Sensors 2024, 9(10):5479-5488. doi: 10.1021/acssensors.4c01708

Heavy metal toxicity can result in various health issues, including gastrointestinal and renal disorders, neurological disorders, dermatological issues, vascular harm, impaired immune system function, congenital disabilities, and malignancy.¹⁰⁻¹² Despite existing regulations, many households and public water systems across the United States continue to have elevated levels of heavy metals present in their water supplies.¹³⁻¹⁶ Numerous studies have shown that drinking water in the United States can contain toxic metals such as Pb(II), Cu(II), and As(III&V), posing significant health risks.¹⁷⁻²⁰ The situation is even more dire in underdeveloped countries, where regulatory frameworks and resources for water quality management are further limited. The lack of accessible, affordable, and user-friendly water testing systems in these resource-limited settings further compounds this

problem.^{16, 21-23} Traditional techniques for quantifying heavy metals include Inductive Coupled Plasma Mass Spectrometry (ICP-MS) and Optical Emission Spectrometry (ICP-OES), as well as Atomic Absorption Spectrometry (AAS).²⁴⁻²⁶ These techniques offer notable advantages in sensitivity, specificity, and precision, especially when measuring low concentrations. Molecular spectroscopy methods, such as UV/Vis molecular absorption and fluorescence spectroscopy, provide indirect approaches for detecting heavy metals.²⁷ Other techniques include electrochemical and chromatographic methods, which have proven valuable in the realm of heavy metal detection.²⁸⁻³³ Despite the wide range of techniques available for heavy metal analysis, these methods come with limitations, including time-consuming processes, the need for costly equipment, and skilled operators. As a result, the practical considerations of conducting

point-of-need testing in resource-limited and economically disadvantaged areas become challenging, if not impossible.

The emergence of paper-based analytical devices (PADs) has played a significant role in bridging the gap between contaminant testing and end-users. These platforms enable point-of-use analysis with their affordability, diverse designs, and compact size, making them highly portable.³⁴⁻³⁷ Common chromogenic agents such as Dimethylglyoxime, Bathocuproine, Bathophenanthroline, Phenanthroline, Diphenyl Carbazone, Rhodizonic acid, Dithizone, 4(2pyridylazo) resorcinol (par), Ferrozine, Xylenol Orange, Chrome azurol S, natural dyes etc. are employed for the colorimetric detection of different heavy metals in water.⁴⁶⁻⁵⁰

While various paper-based analytical systems have utilized these reagents to detect heavy metals in water, most face challenges in achieving regulated detection limits.³⁶ Developing a sensor capable of detecting metals at levels compliant with relevant legislative guidelines, typically in parts per billion (ppb), is necessary to execute a system suitable for monitoring water contamination. Therefore, developing a simple, cost-effective, and user-friendly technique for heavy metal monitoring is crucial, particularly for resource-limited locations. The technique should prioritize ease of use and widespread accessibility while ensuring it adheres to established legislative guidelines for accuracy and reliability.

A preconcentration technique can be used to detect the analytes at very low concentrations.^{51, 52} In a preconcentration system, the capture and concentration of target analyte onto a preconcentration medium results in accumulating these analytes in a confined area. Concentrating the analytes increases the overall available mass of the target elements, leading to an increased color signal from the targeted analyte. Some methods have been

proposed for preconcentration systems for colorimetric heavy metals detection. Satarpai et al. devised a cost-effective and straightforward method utilizing a PAD modified with Zirconium Silicate for the on-site sampling, preconcentration, and analysis of Pb(II) in water samples.⁵³ Ninwong et al. developed a sensitive distance-based method for quantifying mercury ions using fluorescent distance-based paper device coupled with an evaporating preconcentration system.⁵⁴ Suarez et al. developed a preconcentration system that uses a chitosan and superabsorbent pads to preconcentrate and detect Co(II) in water.⁵⁵ It is crucial to highlight that non-substrate-modified paper-based multiplexed systems for heavy metal detection often lack the sensitivity to attain environmentally relevant Limits of Detection (LoD). In contrast, preconcentration systems achieving the necessary LoD are focused on single heavy metal detection or require extensive capture substrate modification. Therefore, the challenge lies in developing a method that overcomes the sensitivity limitations while also allowing for multiplexed detection, ensuring a more comprehensive and practical solution for environmental monitoring.

To overcome the aforementioned shortcomings, we developed a 3D-printed preconcentration system combined with colorimetric paper-based detection, enabling the simultaneous detection of seven different metal ions at low ppb levels in water. The proposed system can process up to 250 mL of water to concentrate heavy metals on Ahlstrom Blotting Grade 319 (AB319) paper. Out of the seven metals analyzed, the system achieved a LoD well below the legislative guideline values for six metal ions with minimal paper substrate modification. Our smartphone-enabled system allows for on-site quantification of the color signals. Various masking strategies were implemented to attain a higher level of selectivity for

the targeted metals with minimal interference. The sensor demonstrates stability suitable for batch manufacturing and timely delivery to end-users. Real water samples were tested to validate the sensor's effectiveness, and the results were benchmarked against industry-standard inductively coupled plasma mass spectrometry (ICP-MS) data. Spike recovery analysis was also used to further confirm the sensor's accuracy.

2. Materials and Methods

Materials and Chemicals

All metal standards, reagents, and masking agents were obtained from Sigma-Aldrich (St. Louis, MO, USA), while hydroxylamine hydrochloride and buffer components were sourced from Fisher Scientific (Waltham, MA, USA). Double-sided adhesive (DSA, 468MP) and transparency film (9984) were purchased from 3M (St. Paul, MN, USA), and Whatman paper was obtained from Fisher Scientific (Waltham, MA, USA). Additionally, PVC foil (0.7 mm) was purchased from Deecraft company in Wang Thonglang-Bangkok, Thailand. The resin for stereolithography was purchased from Formlabs.

Instrumentation

The adhesive tapes, PVC sheets, paper circles, glass fiber (GF) circles, and transparency films were cut using an Epilog Engraver WinX64 Zing laser cutter. The preconcentration tank was printed using a Formlabs Form 3 3D Printer. Method validation was performed using Perkin Elmer NexION 350D ICP-MS.

Device Design and Fabrication

The reagent card was fabricated by bonding a white PVC sheet and DSA. The dimensions of the reagent card are 10 cm X 5 cm. Using the laser cutter, eight 4 mm (diameter) circular

holes are cut on the left side of PVC and DSA. Between these holes, eight 6 mm AB 319 grade paper circles were inserted within the layers. On the right side of the DSA, eight 6 mm circular holes were laser cut. Eight 8 mm circular glass fibers were placed between the layers in this region, as illustrated in Figure 1A. To ensure controlled water flow and filtration exclusively through the detection circles, the left side of the reagent card is attached with a 6 mm wide inner DSA lining. This design element acts as a barrier, preventing lateral water leakage and guaranteeing that the water sample flows vertically through the intended detection areas. The reagent card features a laser-cut perforated fold line running precisely through its center. This fold line allows the left and right sides of the card to be folded together, enabling the delivery of the colorimetric substrates for analysis. The preconcentration tank measures 4.2 cm × 4.2 cm × 14.2 cm. The tank is connected to the card station using a hinge. Please see supplementary information Figure S1 for detailed schematic on 3D printed preconcentration tank. To operate, the top DSA lining is first peeled off, and the preconcentration zone inserted into the card station, followed by closing the tank. Next, the tank was filled with the sample, and vertical filtration was allowed until the water reached the 150 mL mark. Once the water reached the 150 mL mark, the excess water was drained, the card was removed, the back DSA layer was peeled off, and the card was folded along the perforation line to get the colorimetric signal (Figure 1B).

Colorimetric Metal Detection Reagents

Metal standards, including Nickel(II) sulfate hexahydrate, Copper(II) sulfate pentahydrate, Iron(III) chloride hexahydrate, Potassium Dichromate (VI), Mercury(II) Chloride, Lead(II) nitrate, and Manganese(II) Sulfate, were prepared at a concentration of 1000 ppm in 18.2 MΩ cm1

Millipore water. Additionally, 15 solutions containing other non-targeted metallic and nonmetallic ions were prepared to analyze potential interferences.

Assay Card Preparation

To prepare the assay card, reagent and pretreatment solutions were added to the paper preconcentration pads & the glass fiber reagent pads. All the respective concentrations for each assay are outlined in Table S1 and Assays S1, supplementary information. After each reagent addition, the card was left to dry completely at room temperature. Post-fabrication, cards were stored as indicated in packaging protocols, below.

Image Analysis

After the optimized reaction time, the MATLAB Phone app captured images under controlled lighting. The code automates analysis: it identifies the circular region of interest (ROI), creates a mask to isolate it, calculates its average gray value, and normalizes it using a background ROI. The normalization step in the code accounts for variations in lighting conditions during

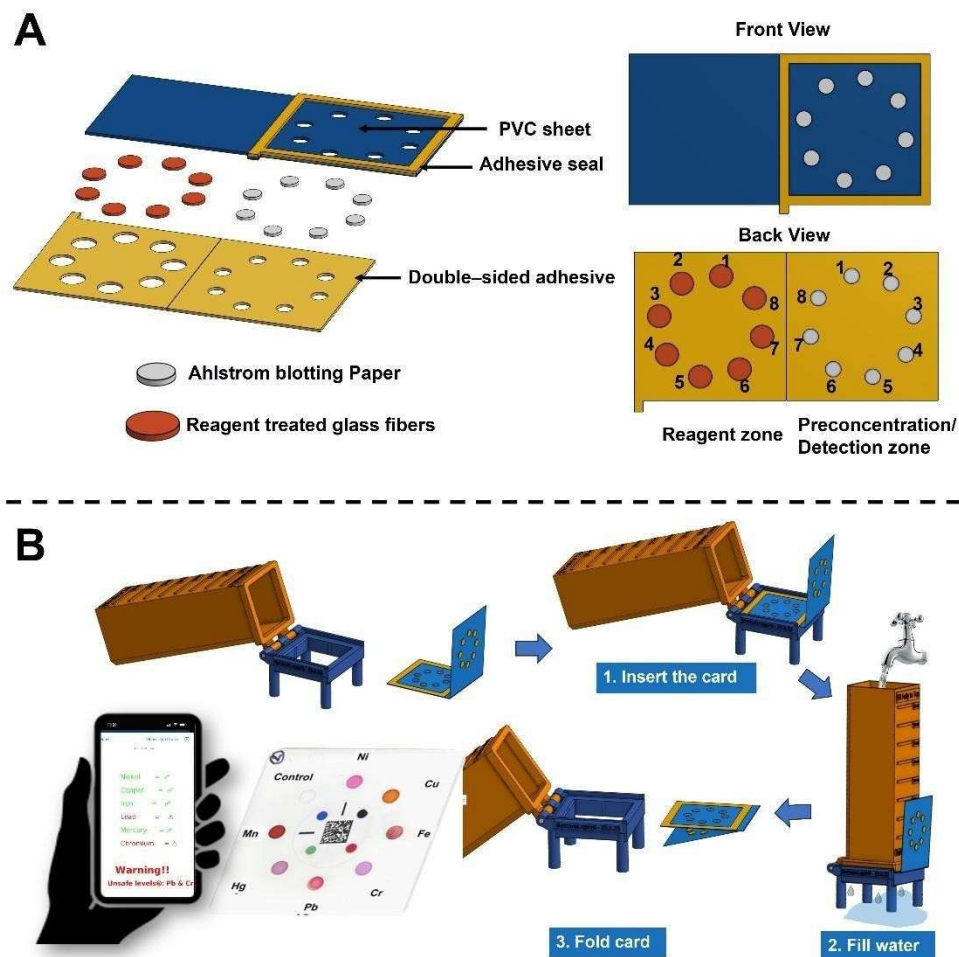


Figure 1. Device schematic and running. (A) Lateral view schematic of reagent card (left), front and back view of the reagent card (right). The detection paper (AB 319) and glass fibers were attached to the left and right side of the reagent card respectively by laser cutting holes into the PVC sheet and Double-Sided Adhesive (DSA). (B) Device running. The reagent card is inserted into the preconcentration tank using adhesive lining. Subsequently, water is added to the tank until it reaches full capacity. The tank is then allowed to filter until it reaches the 150 mL mark. Following this, the adhesive on the backside is peeled off, and the card is folded to observe the color signal.

image capture, enhancing the method's applicability in field settings. Additionally, it analyzes grayscale intensity in specific color channels for optimal sensitivity to different analytes. This process runs in parallel for all ROIs. Ni(II), Fe(III), Cr(VI), Pb(II), Hg(II), and Mn(II) were calibrated in the green channel, and Cu(II) in the blue channel (see supplementary information, Figure S2).

Since the sensor is primarily designed for drinking water systems, the samples used in this study are colorless water samples. However, if the water already exhibits some color, the 8th circle can be utilized as the control zone. As shown in equation 1, this approach will address both lighting inconsistencies and variations in analyte color.

$$Relative\ Intensity = \frac{I(reaction\ zone) - I(control\ zone)}{I(reference\ zone)} \quad (Eq1) \quad 39$$

3. Results and Discussion

Paper Substrate Optimization

To maximize ionic retention, it is crucial to use an optimal paper substrate that effectively captures the metal ions and exhibits a sensitive color intensity when the paper is folded. We examined various paper types (Whatman 1, 3, and 4, Ahlstrom Blotting grade 319), revealing that the highest color intensity was observed with AB 319 grade paper (see supplementary information, Figure S3). Although we also tested other Whatman papers (Grade 5 and Grade 6) with smaller pore sizes, the filtration time was excessively long. As a result, the AB 319 grade paper emerged as the best compromise between color intensity and filtration time.

We hypothesize that the metal ion trapping ability of Ahlstrom AB 319 paper can be attributed to its unique combination of thickness, structure, surface area, tortuosity, and composition. The increased thickness of AB 319 (483 μm) compared to W1 (180 μm) and W4 (210 μm) provides a more extended diffusion pathway, increasing the likelihood of metal ions colliding with the paper fibers, promoting adsorption. Additionally, the denser structure and greater thickness offer a larger surface area for adsorption and introduce more tortuous paths that metal ions must navigate. This increased tortuosity hinders the rapid movement of metal ions, thereby

enhancing their interactions with the fibers and facilitating their retention. Furthermore, the proprietary nature of blotting paper manufacturing suggests the possible presence of undisclosed functional groups that may exhibit a specific affinity for metal ions, forming complexes and contributing significantly to the observed improvement in metal ion trapping during filtration.

Sample Volume Optimization

The sample volume was optimized to process the maximum volume while maintaining a consistent flow rate. The preconcentration tank is designed to hold 250 mL of water. Filtration is facilitated solely by the force of gravity, eliminating the need for an external pump. We observed that the first 150 mL of water flows within a duration of 11.77 ± 1.23 minutes ($n=5$). However, filtering the remaining 100 mL would take nearly an hour due to the decrease in water pressure as the liquid height decreases. Consequently, we halted the process after filtering 150 mL of sample. The excess water is discarded, and the reagent card is removed from the preconcentration tank and folded. In instances where the treated preconcentration pads exhibited hydrophobic properties (in the case of Cu-Bc and Hg-Dtz assay), the reagents were mixed with PEG-400 to make these regions hydrophilic. This modification was implemented to guarantee that the flow rate/filtration rate remained consistent across all detection pads (see supplementary information, Figure S4).

Signal Homogeneity

Because most reagents were immobilized on the glass fibers, establishing a tight seal between the detection pads and fibers after folding was crucial. In this configuration, the thicker paper fibers and active evaporation upstream resulted in convective mass transport, causing the

color compounds to migrate up toward the surface of the detection pad. Glass fibers were chosen over paper for reagent immobilization due to their higher surface energy, which promotes expanded interaction sites. Their higher surface energy facilitates improved wetting, adhesion, and allows for the easy release of reagents into solution.

Reagent Optimization

The concentration of reagents for each assay was optimized to ensure that the analyte consistently functions as the limiting reactant while allowing for the optimal utilization of reagents. Reagent optimization figures can be found in Figures S5 through S11 in supplementary information. The selected optimum concentrations for the reagents are as follows: 60 mM for NiDMG, 8.5 mg/mL for Cu-Bc, 1.25 mg/mL for Fe-Bph, 15 mg/mL for Cr-DPC, 0.625 mg/mL for Pb-Rh, 12 μ g/mL for Hg-Dtz, and 5 mM for Mn-PAR assay.

Reaction Time

Multiplexed detection involving seven metal ions necessitates consideration of varying reaction kinetics for each complexation chemistry. While most reactions reached peak intensity within 3 minutes after folding, Mn-PAR and Cr-DPC complexations required an 8-minute reaction period. To ensure uniform performance across all assays and facilitate better imaging, 8 minutes post-folding was chosen as the optimal reaction time. Figure S12 in supplementary information demonstrates this optimization, where signal intensities stabilize after 8 minutes for each assay.

Metal Ion Retention

To assess the system's ability to capture a specific amount of metal at various concentrations consistently, tests were conducted at low, medium, and high concentrations for each metal under neutral pH (with $n = 5$ devices for each concentration). Significant differences

in color signals were found among the tested gradients. Moreover, the RSD for all examined points remained below 10%, as shown in supplementary information, Figure S13. This indicates a reliable and consistent metal retention across the concentrations. The analyte retention rate was assessed by comparing the signal obtained from the sensor with the signal obtained from a direct application of the same amount of mass onto the paper substrate (spot test). The analyte retention rate was calculated to be $25.8 \pm 3.2 \%$.

To study the effect of pH on metal retention, Ni(II) ion solutions were processed through the sensor at various pH levels. After filtration, pH was adjusted directly on the paper to optimal detection pH. No significant effect on metal retention was observed between pH 1 and 7. However, at pH 9, the metal retention decreased by approximately 5% (see Supplementary Information, Figure S14). While most natural waters containing metals are either acidic or neutral, the presence of non-dissolved metal species at higher pH levels may occasionally affect metal retention. Our study focused exclusively on ionic species due to the detection chemistries employed. In realworld applications, where we anticipate the system's use after acid digestion, the presence of nonionic species should not be a concern.

Detection Pad Calibration

Ni(II) was detected by Chugaev's method, which involves reacting dimethylglyoxime (DMG) with nickel (Ni^{2+}) ions under basic conditions, resulting in the formation of a pink-colored nickel dimethylglyoxime (NiDMG_2) complex.⁵⁶ The pH was adjusted using an ammonium hydroxide/ammonium chloride buffer at the glass fiber. The calibration for Ni concentration is illustrated in Figure 2A, with a calibration curve established for the range of 20–400 ppb. Beyond 400 ppb, the color intensity exhibited non-linearity due to surface saturation. The surface

saturation is common in colorimetric assays.⁵⁷⁻⁵⁹ The estimated LoD and LoQ were calculated to be 15 ppb and 50 ppb, respectively. These values were calculated using the equations $(3s)/b$ and $10s/b$, where "s" is the standard deviation of the blank ($n = 8$ pads per device), and "b" is the slope of the calibration curve.⁶⁰ Importantly, the LoD is well below the guideline limit for Ni(II) in water.⁶¹ Copper was detected using a Cu–bathocuproine assay known for its selectivity and

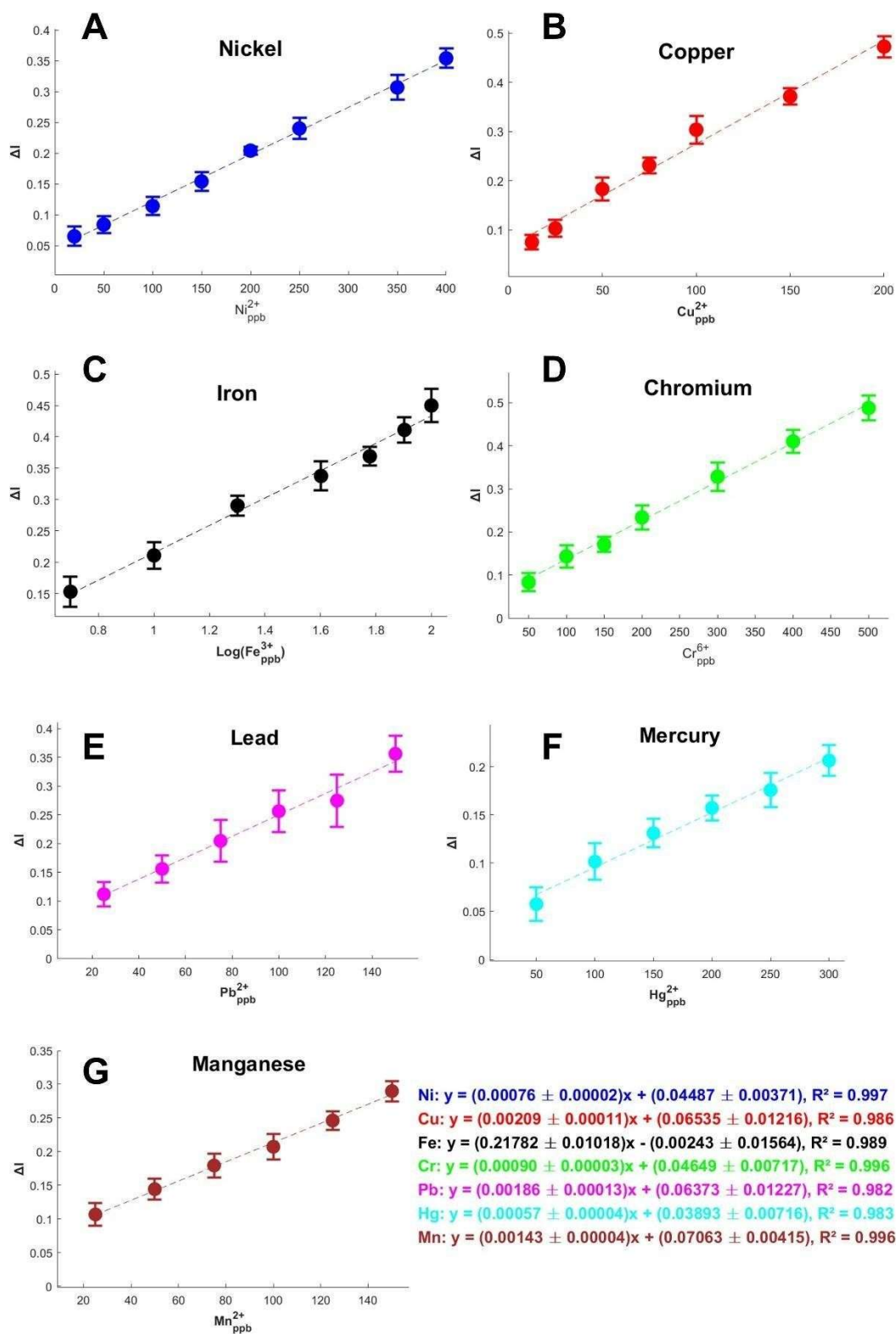


Figure 2. Calibration for Ni-DMG (A), Cu-Bc (B), Fe-Bph (C), Cr-DPC (D), Pb-Rh (E), Hg-Dtz (F), and Mn-PAR (G) assays on the developed sensor. The error bars represent the standard deviation among eight replicate pads on a single device.

sensitivity.⁶² In this method, Cu^+ reacts with Bathocuproine under acidic conditions, forming an orange-colored complex. Bathocuproine exhibits selectivity for Cu detection as it does not produce any color with Fe(II) , unlike other cuproine compounds.^{62, 63} The reduction of Cu(II) to Cu(I) is done by using hydroxylamine as a reducing agent. The presence of Cl^- in the NaCl /acetic acid buffer helps stabilize the cationic Cu-Bc complex.⁶⁴ The hydrophobic nature of the Cu-Bc complex hindered its movement upstream for color formation. Therefore, the reagent was treated directly within the detection pads. However, this adjustment led to an extended filtering time exceeding 30 minutes. To enhance hydrophobicity and maintain an efficient flow rate, an optimized solution containing 0.12 g/mL PEG-400 was mixed with the reagent. Consequently, the flow and filtering rates matched those achieved with other detection pads. The quantitative calibration curve for Cu is depicted in Figure 2B, showcasing a linear calibration range from 12.5 to 200 ppb. Estimated LoD and LoQ were calculated to be 7 ppb and 24 ppb, respectively. The LoD falls significantly below the guideline value for Cu(II) in water.⁶¹

Fe(III) detection was performed using bathophenanthroline, a compound that reacts with Fe^{2+} under acidic conditions, forming a red-colored complex. Hydroxylamine served as the chosen reducing agent to convert Fe(III) to Fe(II) in an acetate buffer. A quantitative calibration curve for Fe(III) is shown in Figure 2C. A log-linear range was established specifically for 5 to 80 ppb. The estimated LoD and LoQ were calculated to be 2 ppb and 9 ppb, respectively. These values significantly fall below the established guideline limits for Fe(III) in water.⁶¹

Chromium was detected through the complexation reaction of Cr(VI) with diphenylcarbazide (DPC), forming a purple-colored complex on paper.⁶⁵ A phthalic anhydride, polydiallyldimethylammonium chloride (PDDA), and DPC mixture was prepared in acetone. PDDA and phthalic anhydride were added to stabilize the reaction product between Cr and 1,5-DPC and prevent the edge effect, while phthalic anhydride stabilized 1,5-DPC on the glass fibers.^{65, 66} Figure 2D illustrates the calibration of the Cr-DPC assay on the proposed sensor. A calibration curve was established within the linear range of 50 to 500 ppb. The estimated LoD and LoQ were calculated to be 20 ppb and 67 ppb, respectively. These values are well below the legislative guideline for Cr(VI) in water, which is set at 100 ppb.⁶¹

Lead metal detection was achieved utilizing sodium rhodizonate as a complexation agent, where the chemical reaction induces a color transition from colorless to red in acidic conditions.^{53, 67} A 10 mM tartrate buffer solution was used to enhance the stability of the complexation reagent.⁶⁸ Figure 2E includes a linear curve established within 25 to 150 ppb range for the Lead-Rh assay. The estimated LoD and LoQ were calculated to be 13 ppb and 43 ppb, respectively. Importantly, the LoD falls below the EPA action limit for lead in drinking water.⁶⁹

Dithizone (DTZ) and Hg(II) readily form a stable complex with a high formation constant ($\log K_f$, $\text{Hg}-(\text{DTZ})_2 = 40.3$),⁷⁰ evident in the dramatic color shift from yellow to reddish-purple.⁷¹ ⁷² Similar to challenges encountered in the Cu-Bc assay with glass fibers, treating the reagents directly on paper resulted in color formation in glass fibers rather than the paper itself. Like the Cu-Bc assay, all the reagents were added in the detection zone itself for this assay to overcome this issue. Similarly, the paper's hydrophilicity was fine-tuned by mixing 0.08 g/mL PEG-400 with

the reagent, improving both flow rate and filtering efficiency. Figure 2F illustrates the calibration results for this assay. A linear calibration range was established within the range of 50 ppb to 300 ppb. Estimated LoD and LoQ were calculated to be 26 ppb and 89 ppb, respectively.

While the LoD in this assay is lower than that of many multiplexed platforms, it still exceeds the legislative guidelines, which are established at 2 ppb.⁶¹ Ongoing and future studies include exploring additional strategies for paper modification as part of efforts to enhance the sensor's sensitivity and bring it within the desired cutoff range.

4-(2-Pyridylazo) resorcinol (PAR) is a commonly employed analytical reagent for heavy metal analysis.^{46, 49} PAR exhibits the ability to form red-colored complexes with various metal ions. This versatility makes PAR a highly used colorimetric reagent, particularly for Mn(II), Ni(II), Zn(II), and Pb(II) detection under basic conditions.⁷³ In prior work, we have successfully developed a strategy for masking interference against the Mn-PAR assay, resulting in a high degree of selectivity.⁴⁹ In this colorimetric reaction, the observed color change progresses from yellow to red. Borate buffer was used to stabilize the pH, and Polydiallyldimethylammonium Chloride (PDADMAC) was added to the reagent to ensure the stability of the Mn-PAR complex. Figure 2G presents the calibration results for the Mn-PAR assay. A linear calibration range was defined from 25 ppb to 150 ppb. The estimated LoD and LoQ were calculated to be 15 ppb and 49 ppb, respectively. Importantly, these values fall well below the legislative limit for Mn(II) in water.⁶¹

Table 1 presents a comparative analysis of our Fill, Fold, Photo system with previously documented μ PADs in the literature, focusing on their capability for the multiplexed colorimetric determination of metal ions. Our system stands out in two key aspects: first, it can detect up to

seven metal ions simultaneously without substrate modification, surpassing the capabilities of previously reported designs. Second, our limits of detection fall within the lower ppb range, adhering to legislative requirements.

Interference Study

We implemented multiple masking approaches to achieve selectivity across various assays on the device. This involved strategically applying masking reagents directly on the detection pads themselves or adding them to the glass fibers. The masking process was optimized to establish the highest possible tolerance limit without adversely impacting the vertical flow time and crossreactivity among the reagents. To determine this tolerance limit, we conducted testing at analyte: interferent ratios of 1:1, 1:5, and 1:10. In this context, the tolerance ratio is defined as the specific ratio between targeted and non-targeted ions that results in a change exceeding 5% in the control signal, which originates from the targeted or non-targeted ion. All masking strategies are comprehensively explained in masking protocol S1 in Supplementary Information. In summary, most tested ions exhibited no interference at a 1:1 ratio, and we achieved over 80% selectivity even at a higher mixing ratio of 1:10, as shown in Figure 3. While increasing the concentration of masking reagents on the detection pad could further broaden the tolerance limit, this strategy was not pursued due to the detrimental effects on flow rate/filtration time.

Stability and Packaging Protocols

The stability of the developed sensor was evaluated by storing them under various conditions: room temperature (23°C), fridge (5°C), and freezer (-20°C). Across all these conditions, consistent signals were obtained for Ni(II), Cu(II), Fe(III), Cr(VI), and Mn(II) throughout the 28-day study period. However, for Pb(II) and Hg(II), better stability was observed under fridge

and freezer conditions, as depicted in supplementary information, Figure S16A. In order to improve the stability of reagents for Pb(II) and Hg(II) at room temperature, the sensors were stored in nitrogen-purged bags with impulse heat sealing. This packaging protocol resulted in retaining over 90% of the signal for up to 5 days at room temperature as shown in supplementary

Table 1. Comparison of analytical performance of the developed system with other multiplexed colorimetric paper-based systems.

Ref	Ion	Reagent	Linear range (ppm)	LoD (ppm)
[75]	Ni(II)	Dimethylglyoxime	15-60	4.8
	Cr(VI)	PAR	0.5-10	0.18
	Cu(II)	Bathocuproine	5.0-80	1.6
[76]	Fe(III)	Bathophenanthroline	20-1300	20
	Ni(II)	Dimethylglyoxime	100-1100	100
	Cu(II)	Bathocuproine	100-1300	100
[48]	Fe(III)	1,10-Phenanthroline	0.3-18 0.4-	0.2 0.3
	Ni(II)	Dimethylglyoxime	23	0.1
	Cr(VI)	1.5-DPC	0.2-17	
	Cu(II)	Bathocuproine	0.05-24	0.03
	Al(III)	Chromeazurol S	0.1-10	0.08
	Zn(II)	Dithizone	0.05-24	0.04
[46]	Cu(II)	Bathocuproine	0.32-63.55	0.32 0.59
	Co(II)	PAR	0.59-4.71	5.87
	Ni(II)	Dimethylglyoxime	5.87-352.16	
	Hg(II)	Dithizone	0.20-12.04	0.2
	Mn(II)	PAR	0.11-0.55	0.11
[77]	Fe(III)	1,10-Phenanthroline	30-300	15
	Ni(II)	Dimethylglyoxime	30-300	15
	Cr(VI)	1,5-DPC	7.6-120	2.4
	Cu(II)	Bathocuproine	60-300	15
This Work	Ni(II)	Dimethylglyoxime	0.02-0.4	0.015 0.007
	Cu(II)	Bathocuproine	0.0125-0.2	0.002 0.020
	Fe(III)	Bathophenanthroline	0.005-0.1	0.013
	Cr(VI)	Diphenylcarbazide	0.05-0.5	
	Pb(II)	Rhodizoinic Acid	0.025-0.15	
	Hg(II)	Dithizone	0.05-0.3	0.026
	Mn(II)	PAR	0.025-0.15	0.015

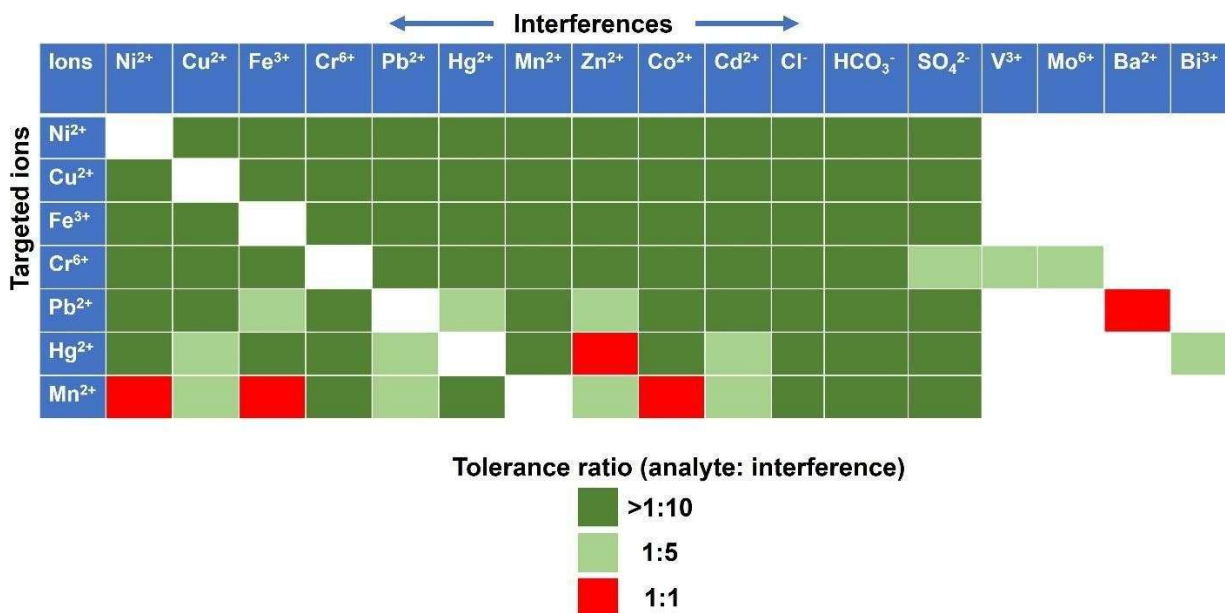


Figure 3. Interference study on the developed sensor. The targeted ions are down the rows, and interfering ions are across the columns. Dark green boxes indicate the interference at >1:10 (targeted: non-targeted), light green at 1:5, and red boxes at 1:1.

information, Figure S16B. This indicates that with our packaging protocols, the sensors can be shipped to end users, refrigerated upon arrival, and subsequently utilized for monitoring purposes. *Method Validation*

Device reproducibility was validated by spiking drinking water samples with varying concentrations of each metal ion. Figure 4 shows the representative images of the sensor for three different levels of spike recovery analysis. Table 2 shows the results of spike recovery analysis for all metal ions. All RSD values remained below 15%, indicating consistent device performance across varying concentrations. These results suggest the sensor's potential for reliable and precise heavy metal detection in real drinking water.

Moreover, to evaluate the sensor's applicability for real water samples, we conducted tests using various samples: drinking water, laboratory water samples, and water samples from a river in Colorado. Details regarding the locations and sample collection protocols are provided in Sampling protocols S1. Additionally, the sensor's performance was validated utilizing results

obtained from ICP-MS analysis following EPA method 200.8 guidelines.⁷⁴ The evaluation of the proposed sensor for metal ion detection in various water sources yielded promising results. Notably, Fe(III) levels in drinking water remained within regulatory limits, with a recovery of 77%. In both Lab Water 1 and 2, the sensor successfully detected Cu(II) and Fe(III), with recoveries ranging from 81% to 94% and relative standard deviations (RSD) between 6.4% and 10.8%, demonstrating good accuracy and precision.

Furthermore, the sensor detected Ni(II), Cu(II), and Fe(III) in river water in Colorado, achieving recoveries between 80% and 92% and RSDs below 15%. Most of the detected levels were below the guideline limits. The on-card color response for each water sample is shown in the supplementary information, Figure S17. The developed sensor demonstrated a performance



Figure 4. Representative images for spike recovery analysis showing on-card responses for lower to higher spiking concentrations (left to right).

comparable to the established industry standard (ICP-MS) as shown in Table 3. The on-card responses for the real samples and full results are included in the supplementary information, Figure S17 and Table S2 respectively. These results highlight its potential for widespread adoption in environmental monitoring applications, offering valuable insights for future implementation.

Hg	NT	ND	100	75.75 ± 11.09	76	14.6
			200	154.89 ± 14.65	77	9.5
			300	231.47 ± 30.86	77	13.3

Metal	ICP-MS (ppb)	Sensor (ppb)	Spiked	Recovered	Rec	RSD
	(n=3)	(n=3)	(ppb)	(ppb)	%	%
Ni (II)	0.11	ND	75	70.97 ± 5.21	95	7.3
			150	139.85 ± 10.62	93	7.6
			300	281.68 ± 18.96	94	6.7
Cu	6.00	ND	30	26.27 ± 2.68	88	10.2
			60	56.16 ± 8.20	94	14.6
			100		91	13.9
Fe	13.20 ± 0.37	10.16 ± 1.84	10	91.25 ± 12.71	85	13.1
			20	8.46 ± 1.11	89	14.7
			50	17.89 ± 2.63	90	13.0
			50	44.98 ± 5.83		
Cr	ND	ND	75	63.01 ± 7.9	84	12.5
			150	130.21 ± 15.83	87	12.2
			300	264.18 ± 36.05	88	13.6
Pb (II)	0.28	ND	50	42.18 ± 6.02	84	14.3
			100	87.94 ± 11.94	88	13.6
			150	127.46 ± 11.78	85	9.2
Mn(II)			50	57.55 ± 8.11	115	14.1
			100	118.11 ± 17.14	118	14.5
			150	178.23 ± 19.33	119	10.8

Table 2. Spike recovery analysis for the developed sensor in real drinking water samples (ND = not detected, NT = not tested).

Table 3. Summary of metal ion detection results in various water sources using the developed sensor.

Sample Type	Metal Ion	ICP-MS (ppb) (n=3)	Sensor (ppb) (n=3)	Rec (%)	RSD (%)
Drinking water	Fe(III)	13.20 ± 0.37	10.16 ± 1.84	77	18.1
Lab water 1	Cu(II)	627.67 ± 8.18	540.11 ± 34.67	86	6.4
	Fe(III)	15.86 ± 0.45	12.83 ± 1.39	81	10.8

Lab water 2	Cu(II)	690.00 ± 5.72	645.37 ± 41.10	94	6.4
	Fe(III)	7.71 ± 0.10	5.32 ± 0.97	69	18.3
River water	Ni(II)	50.77 ± 2.01	40.39 ± 5.85	80	14.5
	Cu(II)	689.00 ± 24.59	621.13 ± 43.14	90	7.0
	Fe(III)	721.33 ± 20.81	662.72 ± 71.15	92	10.7

The sensor has certain limitations. In natural water, heavy metal ions can exist in various forms, including precipitates, colloidal particles, and those bound to organic matter. However, the system's colorimetric chemistry is designed to detect only dissolved ion species. Therefore, metal digestion is necessary to ensure that all forms of metal ions are detected. Our ongoing work includes the development of a portable and user-friendly acid digestion system. Additionally, the system's metal retention capacity requires a careful balance between filtration rate and tank height.

Lastly, although the system is more user-friendly compared to existing multiplexed systems, the reliance on a smartphone for on-site quantification may be challenging in resource-limited settings.

However, the color change is sufficiently pronounced to allow for semi-quantitative analysis.

4. Conclusions

In this study, we developed a novel preconcentration system coupled with colorimetric detection to simultaneously detect seven heavy metal ions at low ppb levels in water. The system achieved guideline-relevant LoDs of 15 ppb (Ni(II)), 7 ppb (Cu(II)), 2 ppb (Fe(III)), 20 ppb (Cr(VI)), 13 ppb (Pb(II)), 26 ppb (Hg(II)), and 15 ppb (Mn(II)). The simple preconcentration method allows for ease of use and eliminates the need for complex pre-treatment steps. Furthermore, the Fill, Fold, Photo detection method offers a portable, user-friendly quantification platform.

Implementing various masking strategies ensured high selectivity for the targeted metals, minimizing interference from other potentially present elements. The system maintained stable signal performance with the proper packaging and storage protocols for over four weeks, highlighting its long-term usability and suitability for mass production and deployment. Different real-world water samples were analyzed, demonstrating good recovery compared to the standard ICP-MS method. Spike recovery analysis further confirmed the sensor's accuracy and precision, achieving less than 15% RSD in all samples. This work presents a significant advancement in the field of low-cost on-site environmental monitoring. We envision this technology evolving into a convenient and portable solution for in-field monitoring of various water contaminants, including pesticides, PFAS, fertilizers, and beyond.

Additional Content

The supplementary information contains details of reagents, masking agents, and hydrophilicity treatments, as outlined in Table S1 and Assays S1. The schematic of the preconcentration tank is depicted in Figure S1. Further optimization efforts included refining imaging channels (Figure S2), substrate properties (Figure S3), and polyethylene glycol (PEG) conditions for specific metal-ion assays (Figures S4–S11). Additionally, reaction time optimization (Figure S12), filtration consistency testing (Figure S13), and pH effects on filtration (Figure S14) are also included. Sampling protocols are detailed in Sampling Protocols S1, with real sample images demonstrating on-card response (Figure S17) and calibration color gradients (Figure S15). The stability study is shown in Figures S16A & S16B, and the reaction schematic is illustrated in Figure S18. This study was funded through a contract (W911QY2120003) awarded by the US Army DEVCOM Center.

5. References

1. The Global Risks Report 2024. World Economic Forum 2024.
2. Forum, W. E. the global risk report 2024; 2024.
3. Weerasekara, P., The United Nations world water development report 2017 wastewater. Future of Food: Journal on Food, Agriculture and Society 2017, 5 (2), 80-81.
4. Nachana'a Timothy, E. T. W., Environmental pollution by heavy metal: an overview. Chemistry 2019, 3 (2), 72-82.
5. UNICEF, WHO. 1 in 3 people globally do not have access to safe drinking water. Geneva: WHO News Release 2019.
6. WHO, World Health Organization Health Risk Assessment Toolkit. International Programme for Chemical Safety: Chemical Hazards, 2010.
7. Organization, W. H., Drinking-Water: Fact Sheet, 2017. Accessed online at <https://www.who.int/en/news-room/fact-sheets/detail/drinking-water> (<https://www.who.int/newsroom/fact-sheets/detail/drinking-water>).
8. Lin, L.; Yang, H.; Xu, X., Effects of water pollution on human health and disease heterogeneity: a review. Frontiers in environmental science 2022, 10, 880246.
9. Tchounwou, P. B.; Yedjou, C. G.; Patlolla, A. K.; Sutton, D. J., Heavy metal toxicity and the environment. Exp Suppl 2012, 101, 133-64.
10. Balali-Mood, M.; Naseri, K.; Tahergorabi, Z.; Khazdair, M. R.; Sadeghi, M., Toxic Mechanisms of Five Heavy Metals: Mercury, Lead, Chromium, Cadmium, and Arsenic. Frontiers in Pharmacology 2021, 12 (April), 1-19.
11. Costa, M., Review of arsenic toxicity, speciation and polyadenylation of canonical histones. Toxicol. Appl. Pharmacol. 2019, 375 (May), 1-4.
12. Coogan, T. P.; Motz, J.; Snyder, C. A.; Squibb, K. S.; Costa, M., Differential DNAprotein crosslinking in lymphocytes and liver following chronic drinking water exposure of rats to potassium chromate. Toxicol. Appl. Pharmacol. 1991, 109 (1), 60-72.
13. Pan, W.; Giammar, D. E., Point-of-Use Filters for Lead Removal from Tap Water: Opportunities and Challenges. Environmental Science & Technology 2022, 56 (8), 47184720.
14. Martinez-Morata, I.; Bostick, B. C.; Conroy-Ben, O.; Duncan, D. T.; Jones, M. R.; Spaur, M.; Patterson, K. P.; Prins, S. J.; Navas-Acien, A.; Nigra, A. E., Nationwide geospatial analysis of county racial and ethnic composition and public drinking water arsenic and uranium. Nature communications 2022, 13 (1), 7461.
15. Gurdian, L. K., We sampled tap water across the US – and found arsenic, lead and toxic chemicals. 2021.
16. Chowdhury, S.; Mazumder, M. J.; Al-Attas, O.; Husain, T., Heavy metals in drinking water: occurrences, implications, and future needs in developing countries. Sci. Total Environ. 2016, 569, 476-488.
17. Development, U. S. D. o. H. a. U., WELCOME TO NATIONAL LEAD POISONING PREVENTION WEEK. 2022.

18. Foster, S. A.; Pennino, M. J.; Compton, J. E.; Leibowitz, S. G.; Kile, M. L., Arsenic drinking water violations decreased across the United States following revision of the maximum contaminant level. *Environmental science & technology* 2019, 53 (19), 1147811485.
19. Schiff, K.; Brown, J.; Diehl, D.; Greenstein, D., Extent and magnitude of copper contamination in marinas of the San Diego region, California, USA. *Marine Pollution Bulletin* 2007, 54 (3), 322-328.
20. Carter, J. A.; Erhardt, R. J.; Jones, B. T.; Donati, G. L., Survey of lead in drinking water from schools and child care centers operating as public water suppliers in North Carolina, USA: implications for future legislation. *Environmental Science & Technology* 2020, 54 (22), 14152-14160.
21. Fasinu, P. S.; Orisakwe, O. E., Heavy metal pollution in sub-Saharan Africa and possible implications in cancer epidemiology. *Asian Pacific Journal of Cancer Prevention* 2013, 14 (6), 3393-3402.
22. Olade, M., Heavy Metal Pollution and the Need for Monitoring: Illustrated for Developing Countries in West Africa. 1987.
23. Fernandez-Luqueno, F.; Lopez-Valdez, F.; Gamero-Melo, P.; Luna-Suarez, S.; AguileraGonzalez, E. N.; Martínez, A. I.; García-Guillermo, M.; Hernandez-Martinez, G.; Herrera-Mendoza, R.; Álvarez-Garza, M. A., Heavy metal pollution in drinking water a global risk for human health: A review. *African Journal of Environmental Science and Technology* 2013, 7 (7), 567-584.
24. Sarojam, P., APP_Metals in Wastewater. *Perkin Elmer Instruments* 2010, 11-11.
25. Hamida, S.; Ouabdesslam, L.; Ladjel, A. F.; Escudero, M.; Anzano, J., Determination of Cadmium, Copper, Lead, and Zinc in Pilchard Sardines from the Bay of Boumerdés by Atomic Absorption Spectrometry. *Anal. Lett.* 2018, 51 (16), 2499-2506.
26. N Endah, S. R.; Surantaatmadja, S. I., The Determination of Heavy Metals Level : Lead in Cosmetic Soap Preparation by Atomic Absorption Spectrophotometer (AAS). *Journal of Physics: Conference Series* 2019, 1179 (1).
27. Lo, M.; Diaw, A. K. D.; Gningue-Sall, D.; Oturan, M. A.; Chehimi, M. M.; Aaron, J. J., A novel fluorescent sensor based on electrosynthesized benzene sulfonic acid-doped polypyrrole for determination of Pb(II) and Cu(II). *Luminescence* 2019, 34 (5), 489-499.
28. Bansod, B.; Kumar, T.; Thakur, R.; Rana, S.; Singh, I., A review on various electrochemical techniques for heavy metal ions detection with different sensing platforms. *Biosens. Bioelectron.* 2017, 94, 443-455.
29. Ruecha, N.; Rodthongkum, N.; Cate, D. M.; Volckens, J.; Chailapakul, O.; Henry, C. S., Sensitive electrochemical sensor using a graphene–polyaniline nanocomposite for simultaneous detection of Zn (II), Cd (II), and Pb (II). *Anal. Chim. Acta.* 2015, 874, 4048.
30. Lace, A.; Cleary, J., A review of microfluidic detection strategies for heavy metals in water. *Chemosensors* 2021, 9 (4), 60.
31. Silva-Neto, H. A.; Cardoso, T. M. G.; McMahon, C. J.; Sgobbi, L. F.; Henry, C. S.; Coltro, W. K. T., Plug-and-play assembly of paper-based colorimetric and electrochemical devices for multiplexed detection of metals. *Analyst* 2021, 146 (11), 3463-3473.
32. Araujo-Barbosa, U.; Peña-Vazquez, E.; Barciela-Alonso, M. C.; Costa Ferreira, S. L.; Pinto dos Santos, A. M.; Bermejo-Barrera, P., Simultaneous determination and speciation analysis of arsenic and chromium in iron supplements used for iron-deficiency anemia treatment by HPLC-ICP-MS. *Talanta* 2017, 170 (April), 523-529.

33. Wang, L.; Hu, Q.; Guangyu, Y.; Yin, J.; Yuan, Z., Determination of lead, cadmium, and mercury by on-line enrichment followed by RP-HPLC. *J. Anal. Chem.* 2003, 58 (11), 10541059.
34. Li, X.; Ballerini, D. R.; Shen, W., A perspective on paper-based microfluidics: Current status and future trends. *Biomicrofluidics* 2012, 6 (1), 011301.
35. Jokerst, J. C.; Emory, J. M.; Henry, C. S., Advances in microfluidics for environmental analysis. *Analyst* 2012, 137 (1), 24-34.
36. Aryal, P.; Hefner, C.; Martinez, B.; Henry, C. S., Microfluidics in environmental analysis: advancements, challenges, and future prospects for rapid and efficient monitoring. *Lab Chip* 2024.
37. Kung, C.-T.; Hou, C.-Y.; Wang, Y.-N.; Fu, L.-M., Microfluidic paper-based analytical devices for environmental analysis of soil, air, ecology and river water. *Sensors and Actuators B: Chemical* 2019, 301, 126855.
38. Morbioli, G. G.; Mazzu-Nascimento, T.; Stockton, A. M.; Carrilho, E., Technical aspects and challenges of colorimetric detection with microfluidic paper-based analytical devices (μ PADs)-A review. *Anal. Chim. Acta.* 2017, 970, 1-22.
39. Aryal, P.; Brack, E.; Alexander, T.; Henry, C. S., Capillary Flow-Driven Microfluidics Combined with a Paper Device for Fast User-Friendly Detection of Heavy Metals in Water. *Anal. Chem.* 2023.
40. Nery, E. W.; Kubota, L. T., Sensing approaches on paper-based devices: a review. *Analytical and bioanalytical chemistry* 2013, 405, 7573-7595.
41. Mentele, M. M.; Cunningham, J.; Koehler, K.; Volckens, J.; Henry, C. S., Microfluidic paper-based analytical device for particulate metals. *Anal. Chem.* 2012, 84 (10), 44744480.
42. Yan-Qi, L.; Liang, F., Progress in paper-based colorimetric sensor array. *Chin. J. Anal. Chem.* 2020, 48 (11), 1448-1457.
43. Brooks, J. C.; Mace, C. R., Scalable methods for device patterning as an outstanding challenge in translating paper-based microfluidics from the academic benchtop to the point-of-care. *J. Anal. Test.* 2019, 3 (1), 50-60.
44. Mahato, K.; Purohit, B.; Kumar, A.; Srivastava, A.; Chandra, P., Next-generation Immunosensing technologies based on nano-bio-engineered paper matrices. In *Immunodiagnostic technologies from laboratory to point-of-care testing*, Springer: 2021; pp 93-110.
45. Filippidou, M.-K.; Chatzandroulis, S., Microfluidic Devices for Heavy Metal Ions Detection: A Review. *Micromachines* 2023, 14 (8), 1520.
46. Kamnoet, P.; Aeungmaitrepirom, W.; Menger, R. F.; Henry, C. S., Highly selective simultaneous determination of Cu(ii), Co(ii), Ni(ii), Hg(ii), and Mn(ii) in water samples using microfluidic paper-based analytical devices. *Analyst* 2021, 146 (7), 2229-2239.
47. Aryal, P.; Indrianingsih, A. W.; Henry, C. S., Smartphone-Enabled Green Anthocyanin Sensor for Fe (III) Sensing on Paper Using Capillary-Driven Microfluidics. *Green Analytical Chemistry* 2024, 100091.
48. Li, F.; Hu, Y.; Li, Z.; Liu, J.; Guo, L.; He, J., Three-dimensional microfluidic paperbased device for multiplexed colorimetric detection of six metal ions combined with use of a smartphone. *Analytical and Bioanalytical Chemistry* 2019, 411 (24), 6497-6508.

49. Meredith, N. A.; Volckens, J.; Henry, C. S., based microfluidics for experimental design: screening masking agents for simultaneous determination of Mn (II) and Co (II). *Anal. Methods* 2017, 9 (3), 534-540.
50. Aryal, P.; Indrianingsih, A. W.; Henry, C. S., Smartphone-enabled green anthocyanin sensor for Fe (III) sensing on paper using capillary-driven microfluidics. *Green Anal. Chem.* 2024, 8, 100091.
51. Lin, C.-C.; Hsu, J.-L.; Lee, G.-B., Sample preconcentration in microfluidic devices. *Microfluidics and Nanofluidics* 2011, 10, 481-511.
52. Bruzzoniti, M. C.; Sarzanini, C.; Mentasti, E., Preconcentration of contaminants in water analysis. *J. Chromatogr. A* 2000, 902 (1), 289-309.
53. Satarpai, T.; Shiowatana, J.; Siripinyanond, A., based analytical device for sampling, onsite preconcentration and detection of ppb lead in water. *Talanta* 2016, 154, 504-510.
54. Ninwong, B.; Sangkaew, P.; Hapa, P.; Ratnarathorn, N.; Menger, R. F.; Henry, C. S.; Dungchai, W., Sensitive distance-based paper-based quantification of mercury ions using carbon nanodots and heating-based preconcentration. *RSC advances* 2020, 10 (17), 98849893.
55. Suarez, W. T.; Franco, M. O.; Capitán-Vallvey, L. F.; Erenas, M. M., Chitosan-modified cotton thread for the preconcentration and colorimetric trace determination of Co (II). *Microchem. J.* 2020, 158, 105137.
56. Tschugaeff, L., Ueber ein neues, empfindliches Reagens auf Nickel. *Berichte der deutschen chemischen Gesellschaft* 1905, 38 (3), 2520-2522.
57. Prakobdi, C.; Baldo, T. A.; Aryal, P.; Link, J.; Saetear, P.; Henry, C. S., Non-invasive iron deficiency diagnosis: a saliva-based approach using capillary flow microfluidics. *Anal. Methods* 2024, 16 (16), 2489-2495.
58. Soda, Y.; Bakker, E., Quantification of colorimetric data for paper-based analytical devices. *ACS sensors* 2019, 4 (12), 3093-3101.
59. Tan, W.; Zhang, L.; Jarujamrus, P.; Doery, J. C.; Shen, W., Improvement strategies on colorimetric performance and practical applications of Paper-based analytical devices. *Microchem. J.* 2022, 180, 107562.
60. Anderson, D. J., Determination of the lower limit of detection. *Clin. Chem.* 1989, 35 (10), 2152-2153.
61. WHO, Guidelines for drinking-water quality: 4th Edition. 2022.
62. Frederick Smith, G.; Wilkins, D. H., New Colorimetric Reagent Specific for Copper: Determination of Copper in Iron. *Anal. Chem.* 1953, 25 (3), 510-511.
63. Soares, S. A. R.; Costa, S. S. L.; Araujo, R. G. O.; Teixeira, L. S. G.; Dantas, A. F., Comparison of Spectrophotometric Methods for the Determination of Copper in Sugar Cane Spirit. *J. AOAC Int.* 2019, 101 (3), 876-882.
64. Hulthe, P., A bathocuproine reagent-paper for the rapid semi-quantitative determination of copper in the 1 to 70 p.p.m. range. *The Analyst* 1970, 95 (1129), 351-355.
65. Ege, J.; Silverman, L., Stable colorimetric reagent for chromium. *Anal. Chem.* 1947, 19 (9), 693-694.
66. Rattananarat, P.; Dungchai, W.; Cate, D. M.; Siangproh, W.; Volckens, J.; Chailapakul, O.; Henry, C. S., A microfluidic paper-based analytical device for rapid quantification of particulate chromium. *Anal. Chim. Acta.* 2013, 800, 50-55.

67. Bartsch, M. R.; Wainwright, K. P.; Kobus, H., An update on the use of the sodium rhodizonate test for the detection of lead originating from firearm discharges. *Journal of forensic sciences* 1996, 41 (6), 1046-1051.
68. Wisang, Y. F.; Sulistyarti, H.; Andayani, U.; Sabarudin, A. In *Microfluidic Paper-based Analytical Devices (μPADs) For Analysis Lead Using Naked Eye and Colorimetric Detections*, IOP Conference Series: Materials Science and Engineering, IOP Publishing: 2019; p 032033.
69. Estes-Smargiassi, S.; Cornwell, D.; Slabaugh, R.; Via, S., Understanding the Proposed Revisions to the Lead and Copper Rule. *Journal: American Water Works Association* 2020, 112 (3).
70. Budesinsky, B.; Sagat, M., Stability constants of some metal dithizonates. *Talanta* 1973, 20 (2), 228-232.
71. Shahat, A.; Ali, E.; El Shahat, M., Colorimetric determination of some toxic metal ions in post-mortem biological samples. *Sensors and Actuators B: Chemical* 2015, 221, 10271034.
72. Paradkar, R. P.; Williams, R. R., Micellar colorimetric determination of dithizone metal chelates. *Anal. Chem.* 1994, 66 (17), 2752-2756.
73. Doyle, C. M.; Naser, D.; Bauman, H. A.; Rumfeldt, J. A.; Meiering, E. M., Spectrophotometric method for simultaneous measurement of zinc and copper in metalloproteins using 4-(2-pyridylazo) resorcinol. *Anal. Biochem.* 2019, 579, 44-56.
74. Long, S.; Martin, T. Method 200.8 determination of trace elements in waters and wastes by inductively coupled plasma: Mass spectrometry. Revision 4.3; Environmental Protection Agency, Cincinnati, OH (United States ...: 1990.
75. Sun, X.; Li, B.; Qi, A.; Tian, C.; Han, J.; Shi, Y.; Lin, B.; Chen, L., Improved assessment of accuracy and performance using a rotational paper-based device for multiplexed detection of heavy metals. *Talanta* 2018, 178, 426-431.
76. Cate, D. M.; Noblitt, S. D.; Volckens, J.; Henry, C. S., Multiplexed paper analytical device for quantification of metals using distance-based detection. *Lab Chip* 2015, 15 (13), 28082818.
77. Rattanarat, P.; Dungchai, W.; Cate, D.; Volckens, J.; Chailapakul, O.; Henry, C. S., Multilayer paper-based device for colorimetric and electrochemical quantification of metals. *Anal. Chem.* 2014, 86 (7), 3555-3562.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

In 2026 and beyond, the challenges of water and food safety continue to remain relevant, especially globally in low and lower middle-income countries. As the world population continues to expand, climate change progresses, and infrastructure ages, water safety will continue to become increasingly critical. LMIC infrastructure investment will likely continue the current path; expanding access and sanitation services, while often failing to account for continual monitoring needs. To combat this, point-of-need water testing devices are poised for commercialization, widespread usage, and integration into citizen science campaigns and daily life.

Chapter Two demonstrates the ability of a commonly used preconcentration and cleaning tool to simplify assay procedure and provide a foundation for sensitive and specific bacterial detection. Focusing on ease-of-use allowed us to translate it to an external lab and achieve comparable detection results without the need for assay-specific training beyond typical protocol documentation. Moving forward in this work, microfluidic flow control could be implemented to automate the capture, label, rinse, and measure process. Additionally, data reduction utilizing logistic fit and serial dilutions can further increase the working range and linearity of the bacterial assay. Ideally, integrated processing on a disposable device can be used to run a multi-dilution assay and process the resulting data, based on factory or user calibration. Multiplexing with specific bacteria/enzyme pairs could be implemented for multiplex detection or self-referencing systems.

Chapter Three shows the effectiveness of electrochemical assays in a point of need setting to solve specific detection challenges. In this case, by developing 3D-printed quickswappable electrodes, we enabled quantitative detection of MDMA in samples that would not otherwise have been tested due to restrictions on available instrumentation. The 3D printed material allowed us to achieve improved electrochemical results while decreasing the user skill required for operating the test. It also and enabled single-use disposal of electrodes, which can be beneficial when working with unknown seized drug samples. Further work includes development of protocols for widespread testing with seized samples, usage in harm reduction contexts for drug analysis, and further device engineering towards usability.

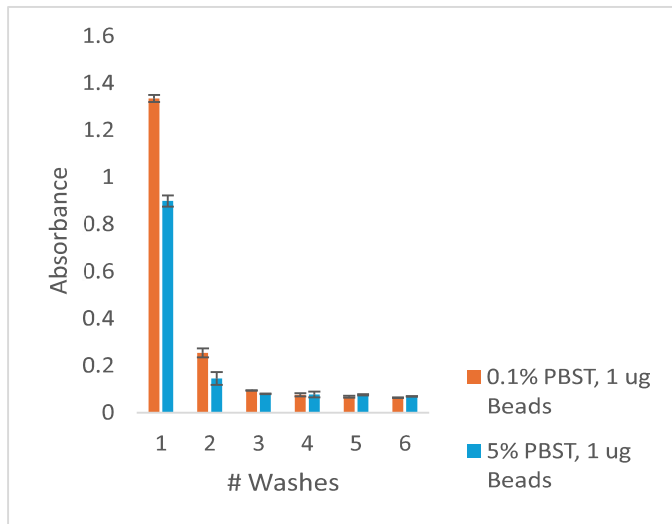
Chapter Four demonstrates that with effective engineering design, point of need sensors can improve on existing limits of detection by orders of magnitude. The combination of a tunable sensing and preconcentration platform is incredibly powerful towards the continued quantification needs at low concentrations. Going forward, the next step for this work is widespread testing and experimental design development. Releasing an app for sampling instructions, data collection, automatic geolocation, heavy metal result analysis, and shared cloud data collection and analysis would allow independent citizen science groups to take large-scale measurements across a watershed, river, or site of interest, and would offer increased ease of use compared to current techniques. Additionally, use of a centralized application and barcoded detection cards can allow sensor-specific calibration based on age, application, sensitivity, or other internal calibration tools.

In this dissertation, I hope to have highlighted the fact that many of the traditional problems with point-of-need testing devices such as sample preparation sensitivity, high limits of detection, and difficulty in practical usage have been mitigated as development into widely applicable point of need testing continues. As we have seen over the last six years, the commercial and scientific advancements in point-of-need sensing developed over the Covid-19 pandemic are paying off; over-the-counter lateral flow assays are now widely available for flu and Covid-19 biomarkers in saliva, as well as for nitrates and white blood cells in urine for UTI diagnostics, enabling easier and more extensive home health monitoring than ever before.

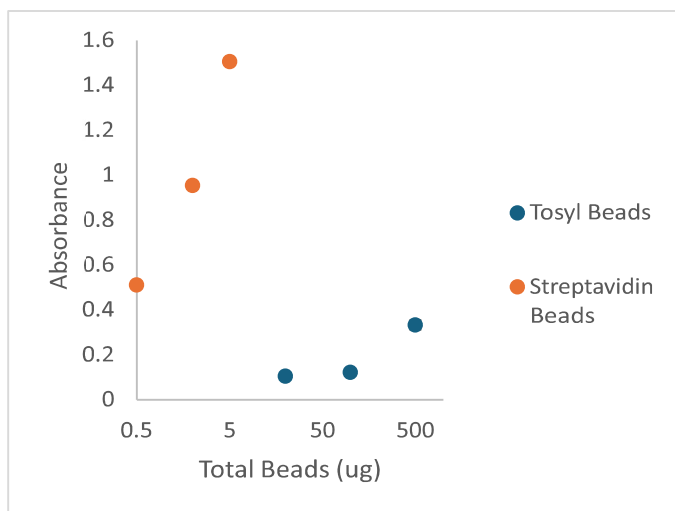
In the future, I hope to see this work expanded in widescale testing efforts. Towards this, we have developed cloud-based sensor meta-analysis and calibration software for Matlab, capable of running on an end-user device, for use in manufacturing, QA/QC, and field testing utilizing the heavy metal detection cards discussed in Chapter Four. To further our ability to produce, activate, and utilize alternative and low-cost electrode materials such as 3D-printed or carbon composites, materials studies should be undertaken to identify key electrode features that make them ideal for certain systems, as well as failure modes to ensure more consistent manufacturing. Finally, with the integration of passive microfluidic flow control, the bead ELISA could be a powerful tool for further research. To further this goal, specific antigens should be identified for 'strong' connections, allowing magnetic capture and wash. Study into mechanism and strength of bacterial retention on beads, as well as prevalence of capture-compatible antigens, could lead to wide applicability in bacterial sensing.

By utilizing the resources and the work we currently have at our disposal, we can take those lessons learned from medical device development and translate them towards environmental monitoring and analysis. We have the technology for sensitive and specific assays for bacterial and heavy metals detection and monitoring at the point of need, and my hope is that my work as a Ph.D. student has demonstrated the immense power of the combination of simply designed analytical systems, point-of-need testing, and electroanalytical measurement for both new and existing problems in the environmental and health monitoring spaces. I believe that the effective integration of disposable point of need devices, large-scale citizen science, and effective testing plans has the potential to help solve some of the most complicated global health challenges.

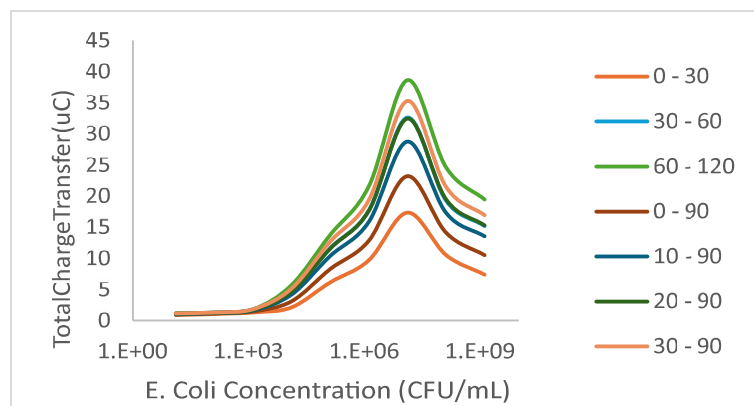
APPENDIX A – CHAPTER TWO SUPPLEMENTARY



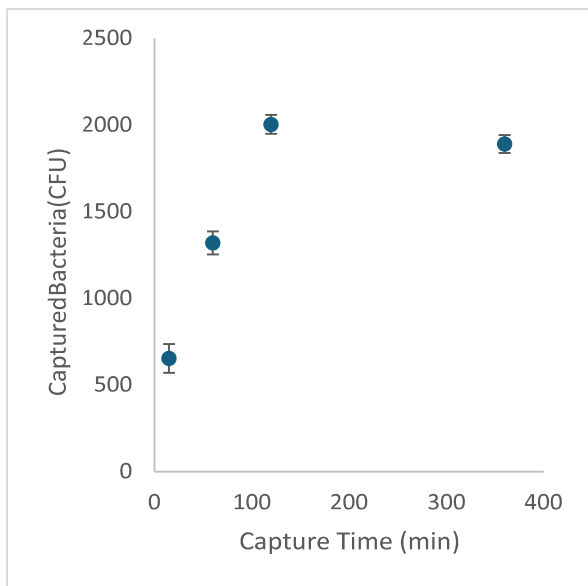
SI Figure 1: Microwell TMB Analysis of HRP in Supernatant Following PBST Washes



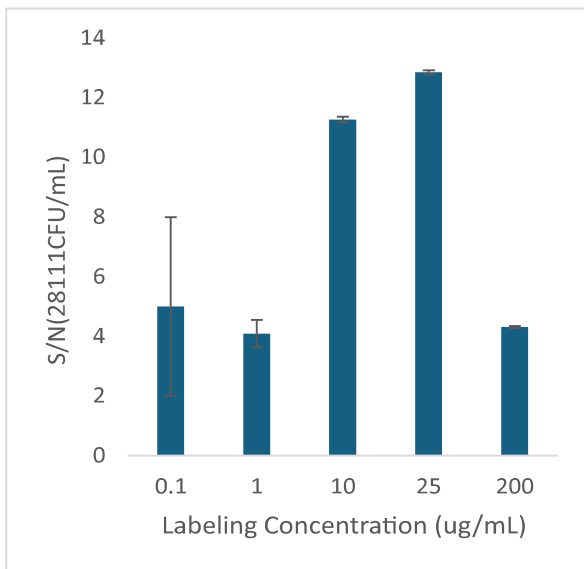
SI Figure 2: Microwell TMB Analysis of HRP Bound to MMBs following PBST washes



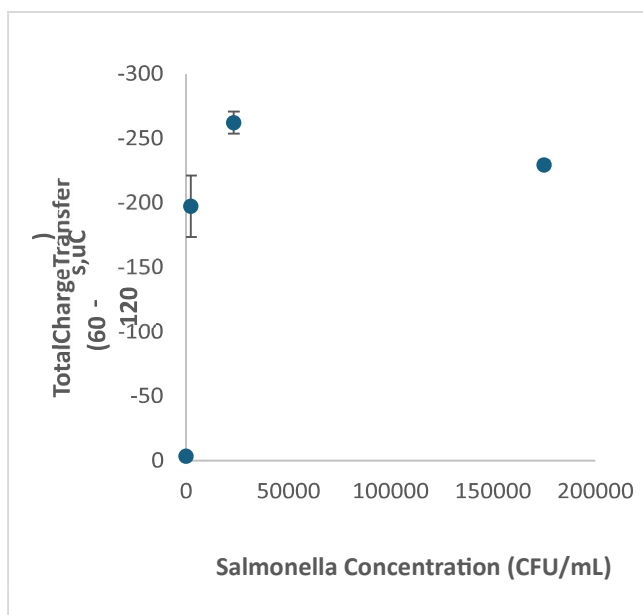
SI Figure 3: Analysis of total charge transfer over different time periods



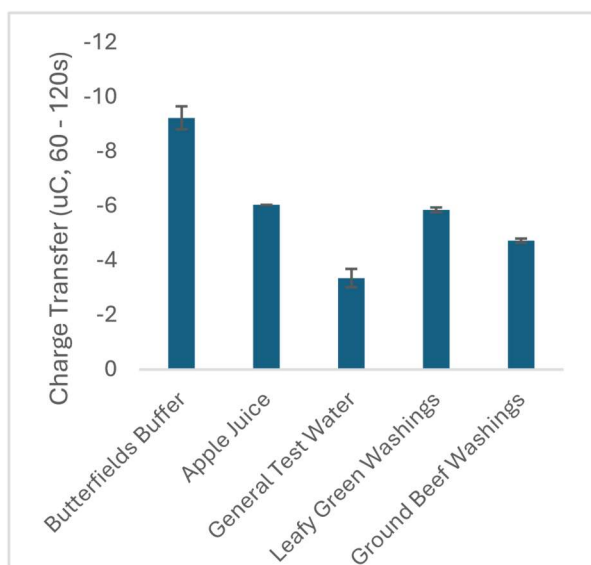
SI Figure 4: Capture time optimization results in a 10^3 CFU/mL e. Coli solution



SI Figure 5: Secondary Antibody Concentration Optimization Results in a 2.8×10^4 CFU/mL e. Coli solution



SI Figure 6: Nonlinear response of assay to *Salmonella enterica* in PBS buffer



SI Figure 7: E. Coli assay blanks collected for each complex matrix

Bead Preparation Protocol V3 – 3/2/2024 SUPPLIES

Name	Source	Batch	Conc	Prepped?
Tosylactivated Magnetic Particles (2.8 um)	Dynabeads M-280 Tosylactivated		30 mg/mL	
Anti-Bacteria Ab	Abcam ab137967		4 mg/mL	
PBS Buffer	Lab-Made (below)		See Protocol	
PBST 0.1%	Lab-Made (below)		1 mg/mL	
0.5% BSA in PBS	Lab-Made (below)		5 mg/mL	
0.1% BSA in PBS	Lab-Made (below)		1 mg/mL	
Borate Buffer (0.1M, pH 9.5)	Lab-Made (below)		0.1M	
Ammonium Sulfate Borate Buffer (3M, in borate buffer)	Lab-Made (below)		3M, 0.1M	

EQUIPMENT

Name	Notes	Prepped?
Micropipettes	1000 uL, 100 uL, 5 uL	
Vortexer	Any, Setting 5 (med)	
Rotator	Any, Med-Slow, approximately 1 inversion / second	
Magnetic Stand	Thermo 12321D	

Timer		

PREPARATION

Phosphate Buffered Saline (PBS) – Prepare as per instructions on tablets. Typically, 1 tablet makes 500 mL PBS at pH 7.4. If pH needs to be adjusted, do so with 1M NaOH or HCl. Autoclave and store refrigerated up to 2 months.

0.1% PBST – 1 mg/mL Tween-20 in PBS – Dissolve 20 mg Tween-20 in 20 mL PBS. Store refrigerated up to 1 month.

0.5% BSA in PBS – 5 mg/mL BSA in PBS – Dissolve 100 mg BSA in 20 mL PBS. Store refrigerated up to 2 weeks.

0.1% BSA in PBS – 1 mg/mL BSA in PBS – Dissolve 20 mg BSA in 20 mL PBS OR Dilute 4 mL “0.5% BSA in PBS” in 16 mL PBS. Store refrigerated up to 2 weeks.

Borate Buffer (0.1M, pH 9.5) – Dissolve 3.09g H_3BO_3 in 500 mL distilled water. Adjust pH to 9.5 with 5M NaOH.

Ammonium Sulfate Borate Buffer (3M, 0.1M, pH 9.5) – Dissolve 39.64g $(\text{NH}_4)_2\text{SO}_4$ in 80 mL Borate Buffer (above). Adjust pH to 9.5 with NaOH or HCl. Adjust volume to 100mL with borate buffer.

PROTOCOL

1. Resuspend beads by vortexing bulk solution 30s. If beads are still seen on bottom of vial, vortex a further 30s.
2. Transfer 335 uL beads to a 2mL microcentrifuge tube.
3. Add 1 mL Borate buffer. Vortex 10s.
4. Place tube in magnet for 1 minute. Pipette away and discard supernatant.
5. Add 200ug antibody (50 uL)* to tube, bring **total volume to 150 uL** with borate buffer (add 100 uL). Vortex 5s.
* With Ab 137967, Ab is at 4 mg/mL = 4000 ug/mL, 200 ug = 50 uL. If Ab is at less than 2 mg/mL, see 5 mg bead recommendations in manufacturer instructions.
6. Add 100 uL borate-sulfate buffer to tube. Vortex 5s.
7. Incubate at room temperature for 24-36 hours with mixing (end over end on rotator)
8. Place tube on magnet for 2 minutes, remove and discard supernatant.
9. Resuspend in 1 mL 0.5% BSA in PBS, incubate for 3 hours at room temperature with mixing (end over end on rotator).
10. Place tube on magnet for 2 minutes, remove and discard supernatant.
11. Resuspend in 1 mL 0.1% BSA in PBS, vortex 10s.
12. Place tube on magnet for 2 minutes, remove and discard supernatant.
13. Repeat steps 11 & 12 once.
14. Resuspend in 480 uL 0.1% BSA in PBS to a final bead concentration of 20 mg/mL.

NOTES:

SINGLEPLEX ASSAY PROTOCOL V4.1 – 4/8/2024 Supersedes all

previous.

SUPPLIES

Name	Source	Batch	Conc	Prepped ?
Anti-Bacteria Magnetic Particles	Lab-Made (below)		2 mg/mL	
PBST 0.1%	Lab-Made (below)		1 mg/mL	
Tween-20	Sigma P9416		Bulk liquid	
Anti-Bacteria Ab, HRP Conjugated	Lab-Made		1 mg/mL	
PBS Buffer pH 7.4	Lab-Made		See Protocol	
TMB Solution	Sigma T0440		Bulk liquid	

EQUIPMENT

Name	Notes	Prepped?
Micropipettes	1000 uL, 200 uL	
Rotator	Any, medium-slow, ~1 inversion/second	
Timer	Any	
Magnet		
Vortexer		
Microcentrifuge Tubes		
Flow Channels		
Electrodes		
Electrode Holder		

Optional: LB Agar Plates		
--------------------------	--	--

PREPARATION

PBS Buffer – Prepare as per instructions on tablets. Typically, 1 tablet makes 500 mL PBS at pH 7.4. If pH needs to be adjusted, do so with 1M NaOH or HCl. Autoclave and store refrigerated up to 2 months.

0.1% PBST – Dissolve 100 mg Tween-20 in 100 mL PBS buffer.

2Ab Solution – Dilute pre-conjugated secondary antibody to 25 ug/mL in PBS buffer. (From 1 mg/mL, dilute 25 uL to 1 mL in PBS).

Beads – Complete protocol BEAD PREP V3, dilute to 2 mg/mL.

PROTOCOL Capture

1. Prepare 1 mL of each sample in 2 mL microcentrifuge tubes.
 - 1a. If desired, plate 100uL of sample in triplicate to determine bacterial concentration, dilution may be appropriate, plate-counting is most reliable at 200-2000 CFU/mL.
2. Pipette 50 uL of prepared beads (2.0 mg/mL, 100 ug total) into each tube.
3. Incubate 1 hour at room temperature, with constant rotation, tubes turning end-over-end.
4. Add tube to magnet. Let separate for 1 minute.
5. Remove supernatant via pipetting. Discard.
6. Resuspend in 1 mL PBST. Vortex 30s to rinse.
7. Add tube to magnet. Let separate for 1 minute.
8. Remove supernatant via pipetting. Discard.
9. Repeat steps 6-8, for a total of two washes.

Labeling

10. Resuspend in 50 uL 2Ab solution at 25 ug/mL. Vortex 10s to mix. Incubate 20 minutes at room temperature with constant rotation, tubes rotating on their axis (not end-over-end).

11. Add tube to magnet. Let separate for 1 minute.
12. Remove supernatant via pipetting. Discard.
13. Resuspend in 1 mL PBST. Vortex 30s to wash.
14. Transfer the entire solution to a clean tube.
15. Add tube to magnet. Let separate for 1 minute.
16. Remove supernatant via pipetting. Discard.

Washing

17. Resuspend in 1 mL PBST. Vortex 30s to wash.
18. Add tube to magnet. Let separate for 1 minute.
19. Remove supernatant via pipetting. Discard.
20. Repeat steps 17 - 19 twice for a total of three washes.
21. Resuspend in 1 mL PBST. Vortex 10s to mix.

Electrochemical Measurement

1. Add 10 μ L of bead mixture to channel inlet.
2. Add 30 μ L TMB to channel inlet. Allow to incubate 15 minutes.
3. Attach potentiostat to electrodes, measure via chronoamperometry at -0.2 V.
4. Signal is average of current from 50 – 60 seconds.

NOTES:

Bring all solutions to RT before use.



Pre-Assay Preparation



Assay Preparation Needs

Component Needed	Volume Needed
Sample	1 mL
Capture Beads at Working Concentration	50 <u>uL</u> / sample
0.1% PBST	7 mL / sample
Secondary Ab at Working Concentration	50 <u>uL</u> / sample
Microfluidic Channel	1 / sample*
TMB Solution	50 <u>uL</u> / sample*

*: Assuming 1 sample = 1 measurement. The assay procedure will result in ~ 1mL bead-bacteria-label solution, enough for 100 replicate measurements.

Suggested Workflow

1. Determine # of samples to test.
2. Reference 'Assay Preparation Needs' table to determine needs.
3. Compare to current prepared stocks.
4. If necessary, continue to Bead Preparation Protocol or Conjugated Antibody Protocol as needed.
5. Reference respective tables for preparation needs.

Bead Preparation** Needs

Reagent Needed	Volume Needed
<u>Dynabead M-280 Tosylactivated Beads</u> (30 mg/mL)	335 <u>uL</u>
Capture Antibody	See Protocol**
Borate Buffer	1.1 mL
Ammonium Sulfate Borate Buffer	100 <u>uL</u>
0.5% BSA in PBS	1 mL
0.1% BSA in PBS	2.5 mL
Makes 480 <u>uL</u> Concentrated Capture Beads (20 mg/mL); 4.8 mL Capture Beads at Working Concentration (2 mg/mL); Enough for 96 samples.	
Store at 4C up to 2 months.	

Conjugated Antibody Preparation** Needs

Reagent Needed	Volume Needed
Secondary Ab	@ 4 mg/mL: 50 <u>uL</u> @ 2 mg/mL: 100 <u>uL</u> @ 1 mg/mL: 200 <u>uL</u>
Neutravidin-HRP	133 <u>uL</u>
PBS Buffer	1500 <u>uL</u>
Makes 1.5 mL Concentrated Conjugated Antibody Solution (200 ug/mL); 12 mL Conjugated Antibody Solution at Working Concentration (25 ug/mL); Enough for 240 samples.	
Store at 4C up to 2 months.	

**See Bead Preparation Protocol and Secondary Ab Conjugation Protocol.

Equipment Needs

Electrode Holder
2 mL Microcentrifuge Tubes
IMS Magnet Stand
Micropipette & Micropipette Tips
<u>Vortexer</u>
Electrodes
(Optional) LB Agar Plates
<u>Potentiostat & Controlling Laptop</u>
Rotator
Timer
<u>Whirlpak Bags</u>



Consumables & Equipment Purchasing List



Consumables		
Item	Source	Qty
PBS Tablets (100)	Medicago 09-8912-100 (Fisher 11340439)	1x 100
Boric Acid	Thermo 327132500	1x 250 g
Ammonium Sulfate	Sigma a5132	1x 500 g
Sodium Hydroxide 5M	Sigma S8263-150ML	1x 150 mL
Hydrochloric Acid 1M	Sigma H9892	1x 100 mL
Tween-20	Sigma P1379-100ML	1x 100 mL
BSA Fraction V	Equitech Bio BAC61 OR VWR 103219-148	1x 50g
HRP-Neutravidin	Fisher 31030	1x 0.5 mL
E. Coli Secondary Ab-Biotin	Abcam ab20640	1x 250 uL
Dynabeads M-280 Tosylactivated	Fisher 14203	1x 2 mL
E. Coli Capture Antibody	Abcam ab137967	1x 500 uL
Whirlpak Bags for Sample Prep	Whirlpak B01385	1x 250
TMB Substrate Solution	Sigma T0440	1x 100 mL
Listeria Capture Antibody	Abcam ab35132	1x 1mL
Listeria Secondary Antibody	Abcam ab20766	1x 250 uL
Salmonella Capture Antibody	Abcam ab8274	1x 250 uL
Salmonella Secondary Antibody	Fisher PA1-73022	1x 1 mL

Equipment		
Item	Source	Qty
Micropipette & Micropipette Tips	Any	
2 mL Microcentrifuge Tubes	Any (Sterile, amber, non-coated)	
IMS Magnet Stand	Fisher 12321D	1
Vortexer	Any	
Potentiostat & Controlling Laptop	Palmsens	
Rotator	Any	
Timer	Any	
Assorted bottles for solution storage	Any	



Buffer & Solution Preparation



General Buffer Preparation Procedure

1. In a 1 L autoclavable bottle prepare buffer by dissolving solid and liquid constituents in a total volume of 500 mL using distilled water.
2. Check pH of buffer, adjust to necessary value with HCl or NaOH as necessary.
3. Aliquot if desired and autoclave buffer if needed. All buffers used in assay and prep can be stored at 4C or room-temp long term. PBS may be stored at RT only if sterile.

Buffer Preparation

Buffer Name	Needed Reagents	Final Concentrations	Final pH	Notes
Phosphate Buffered Saline (PBS)	1 PBS tablet, 500 mL distilled water	0.14 M NaCl, 0.0027 M KCl, 0.010 M Phosphate	7.4	Autoclave. Store at 4C. If unopened after autoclaving, shelf life > 6 months.
Borate Buffer	3.09g Boric Acid (H_3BO_3) 500 mL distilled water	0.1 M Borate	9.5	No need to autoclave. Store in plastic at 4C. Shelf life > 6 months.
Ammonium Sulfate Borate Buffer	39.64g Ammonium Sulfate ($(NH_4)_2SO_4$) 100 mL Borate Buffer (Above)	0.1 M Borate, 3 M Ammonium Sulfate	9.5	No need to autoclave. Store in plastic at 4C. Shelf life > 6 months.

Use 'General Buffer Preparation Procedure'. Use 3M NaOH to adjust pH of Borate buffer. Use 1M NaOH or HCl to adjust pH of PBS. Borate Buffer & Ammonium Sulfate Borate Buffer are utilized for capture bead coupling only.

Solution Preparation

Solution	Needed Reagents	Final Concentrations	Notes
0.1% PBST	100 mL PBS Buffer (Above) 100 mg Tween-20	0.1% W/V Tween-20, PBS Buffer	Do not autoclave. Store at 4C up to 1 month.
0.5% BSA in PBS	10 mL PBS Buffer (Above) 50 mg BSA Fraction V	0.5% W/V BSA, PBS Buffer	Do not autoclave. Store at 4C up to 2 weeks.
0.1% BSA in PBS	2 mL 0.5% BSA in PBS (Above) 8 mL PBS Buffer (Above)	0.1% W/V BSA, PBS Buffer	Do not autoclave. Store at 4C up to 2 weeks.
Concentrated Secondary Ab Solution*	200 ug Secondary Ab 133 ug HRP-Neutravidin 1500 μ L PBS Buffer (Above)	200 ug/mL Conjugated Secondary Ab, PBS Buffer	Do not autoclave. Store at 4C up to 1 month.
Working Secondary Ab Solution	100 μ L Concentrated Secondary Ab Solution 700 μ L PBS Buffer	25 ug/mL Conjugated Secondary Ab, PBS Buffer	Do not autoclave. Store at 4C up to 1 month.
Concentrated Capture Bead Solution*	See Bead Prep	20 mg/mL Capture Beads, 0.1% BSA, PBS Buffer	Do not autoclave. Store at 4C up to 2 months.
Working Capture Bead Solution	50 μ L Concentrated Capture Bead Solution 450 μ L PBS Buffer (Above)	2 mg/mL Capture Beads, 0.01% BSA, PBS Buffer	Do not autoclave. Store at 4C up to 2 months.

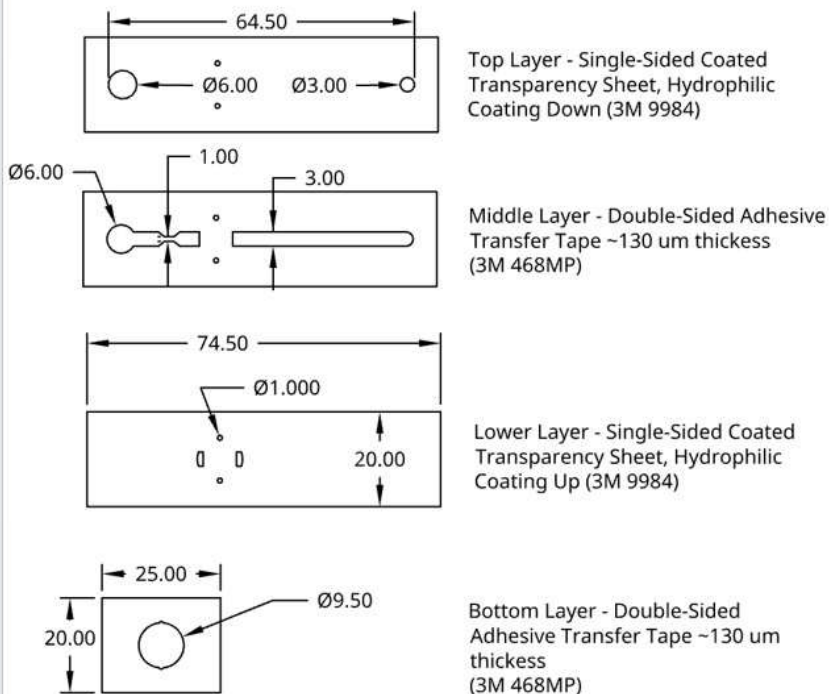
*See Bead Preparation Protocol and Secondary Ab Conjugation Protocol.
For all other solutions, mix needed reagents until dissolved, and store at 4C.



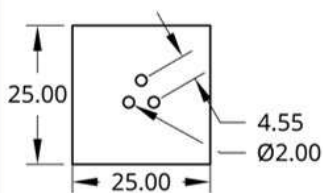
Electrode & Channel Version 12.1 Technical Specifications



Flow Channel

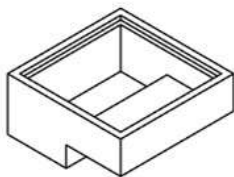


Thermoplastic Electrode



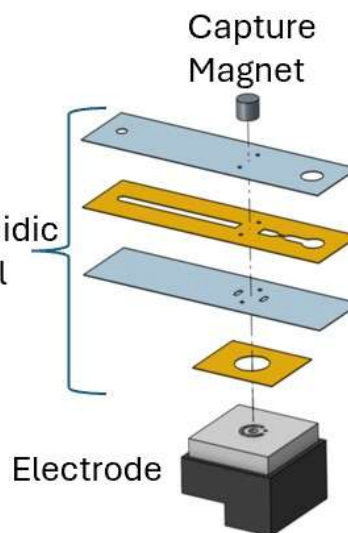
Mold Material: Laser-patterned polymethyl methacrylate (PMMA)

Electrode Material: Polystyrene-based graphite thermoplastic electrode



Electrode Casing: 3D Printed Resin (Formlabs)
Potentiostat Connection: 20G Copper Wire
Electrode-Wire Connection: Conductive Silver Paint (SPI 05001-AB)

Microfluidic Channel



APPENDIX B – CHAPTER THREE SUPPLEMENTARY

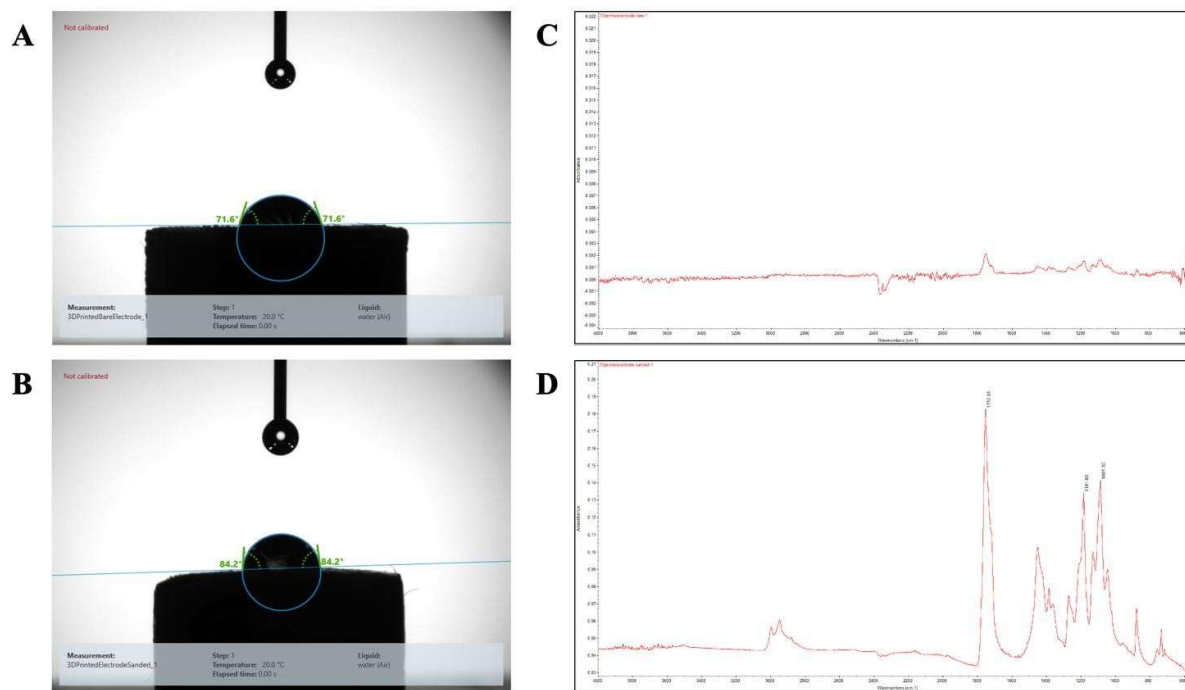


Figure S1. (A) CA image of a bare electrode (BE). **(B)** CA image of a sanded electrode (SE). **(C)** Fourier-transform infrared spectroscopy (FTIR) spectrum of a BE. **(D)** FTIR spectrum of a SE.

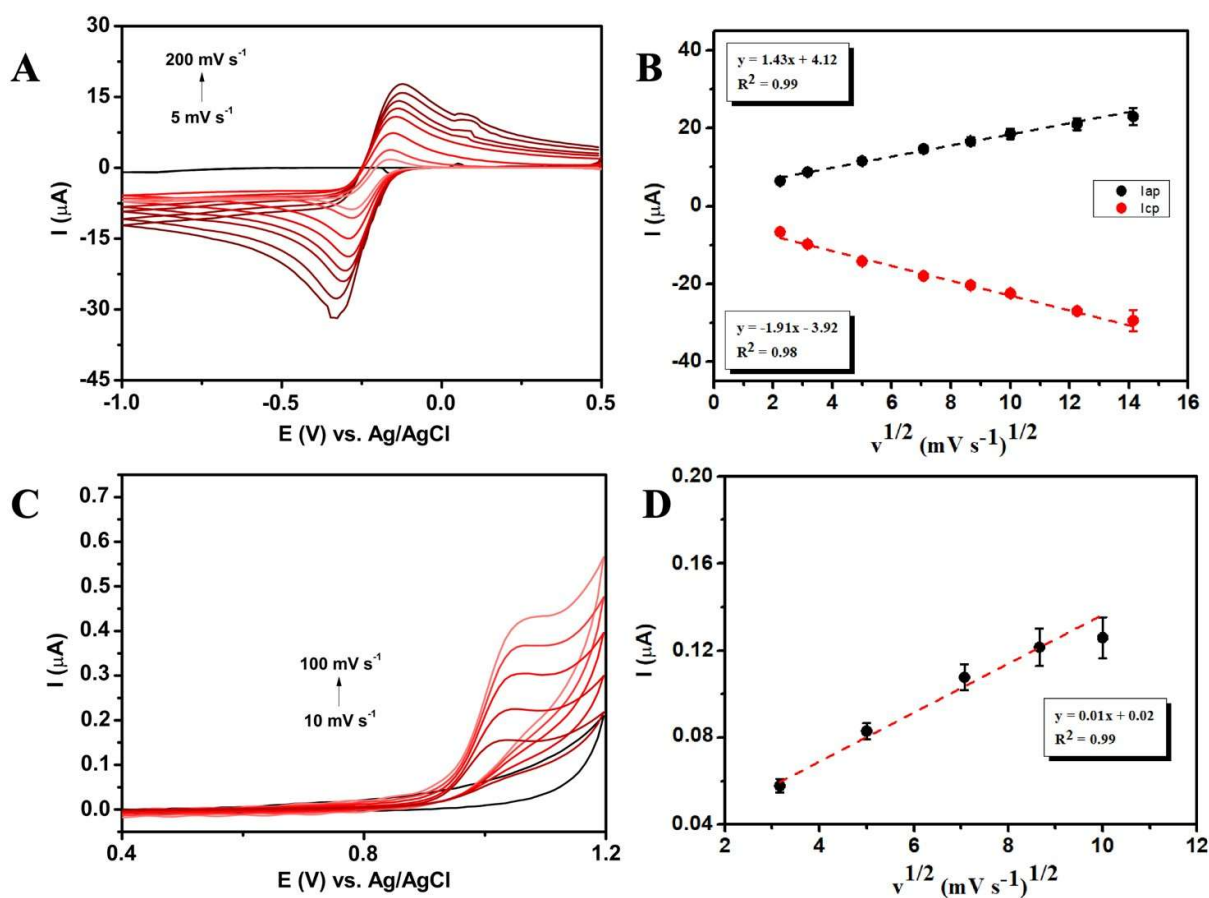


Figure S2. (A) CV plots of 5 mmol L⁻¹ hexaamineruthenium (III) in 0.5 mol L⁻¹ KCl at different scan rates (5, 10, 25, 50, 75, 100, 150 and 200 mV s⁻¹) on sanded/laser treated electrode (SLTE). (B) Plot of anodic and cathodic peak current values obtained for the SLTE as a function of the square root of scan rate from Figure S2A. (C) CV plots of 75 μmol L⁻¹ MDMA in 0.1 mol L⁻¹ BR buffer (pH 3.0) at different scan rates (5, 10, 25, 50, 75, 100, 150 and 200 mV s⁻¹) on the SLTE. (D) Plot of anodic peak current values obtained for the SLTE as a function of the square root of scan rate from Figure S2C. Error bars correspond to the standard deviation of measurements using 3 different sensors.

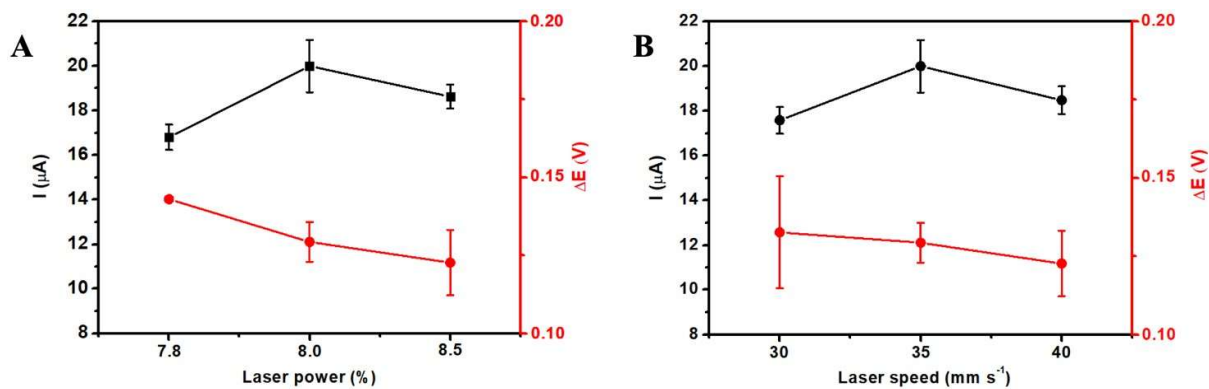


Figure S3. (A) Plot of anodic peak currents and peak-to-peak potential (ΔE_p) values as a function of laser power. **(B)** Plot of anodic peak currents and ΔE_p values as a function of laser speed. Error bars correspond to the standard deviation of measurements using 3 different sensors.

APPENDIX C – CHAPTER FOUR SUPPLEMENTARY

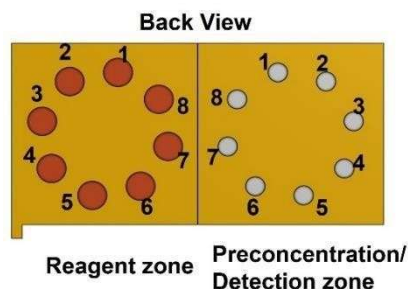


Table S1. List of reagents (red), masking agents, and hydrophilicity treatment in each zone.

Assay	DZ	Preconcentration/Detection Zone (DZ) paper Reagents	GF	Reagent Zone /Glass Fiber (GF) Reagents	Hydrophilicity Treatment
Ni-DMG Assay	1	5 µL 6.0 M ammonium acetate 5 µL 0.5 M NaF	1	7.5 µL NH ₄ OH/NH ₄ Cl buffer (pH 9) Two 10 µL aliquots of 60 mM dimethylglyoxime in methanol	NA
Cu-Bc Assay	2	5 µL hydroxylamine (0.2 g/mL, Acetate/NaCl buffer, pH 4.5) 10 µL solution of 8.5 mg/mL Bathocuproine, 0.12 g/mL PEG 400	2	0.12 g/mL reagent NA	PEG added to the mixture
Fe-Bph Assay	3	5 µL hydroxylamine (0.2 g/mL, acetate buffer, pH 4.5)	3	20 µL solution of 1.25 mg/mL bathophenanthroline in isopropanol	NA
Cr-DPC Assay	4	5 µL 0.1 M phosphoric acid	4	20 µL solution of 15 mg/mL Diphenyl Carbazide, 0.04 g/mL phthalic anhydride, 1 mM ceric(IV) ammonium nitrate, 5% w/v PDCA in acetone	NA
Pb-Rz Assay	5	NA	5	20 µL Rhodizate (0.625 mg/mL in Tartrate buffer, pH 3)	NA

Hg-Dtz 6 Assay	5 µL Ethylenediamine (3 M) 5 µL trans-1,2diaminocyclohexaneN,N,N',N'-tetraacetic acid (DCTA) (0.1 M) 10 µL diluted stock solution of dithizone (12 µg/mL in dichloromethane) + PEG 400 (0.08 g/mL)	6 NA	0.08 g/mL PEG added to the reagent mixture
-------------------	--	-----------------	---

Mn-PAR 7 Assay	Combination of dimercaptosuccinic acid and triethylenetetramine in pH 9.3 borate buffer	7
-------------------	---	---

Assays S1

20 µL solution of 2.5 mM 4-(2-pyridylazo) resorcinol mixed with 5% w/w PDADMAC in borate buffer (pH 9.3, 0.1 M)	NA
---	----

Ni-Dimethylglyoxime (Ni-DMG) Assay. 5 µL of 6.0 M ammonium acetate followed by 5 µL of 0.5 M NaF was added to the detection zone 1. Subsequently, 7.5 µL of NH₄OH/NH₄Cl buffer (pH 9) was added to the glass fiber 1, followed by the addition of two 10 µL aliquots of 60 mM dimethylglyoxime prepared in methanol. After each reagent addition, the detection zone and reagent zone were left to dry at room temperature. Ultimately, 250mL of the standard solution was added to the preconcentration tank.

Cu-Bathocuproine (Cu-Bc) Assay. 5 µL of hydroxylamine, prepared in Acetate/NaCl buffer at a concentration of 0.2 g/mL (pH 4.5), was added to detection zone 2 and dried. A solution containing 8.5 mg/mL Bathocuproine was prepared in dichloromethane. A 10 µL of this solution was added to detection zone 2. After each reagent addition, the detection zone was dried completely. Ultimately, 250mL of the standard solution was added to the preconcentration tank. In this assay, all the reagents were added to the detection zone itself.

Iron-Bathophenanthroline (Fe-Bph) Assay. In detection zone 3, 5 µL of 0.2 g/mL of hydroxylamine, prepared in a 6 M acetate buffer with a pH of 4.5, was initially added. Then, 20 µL of 1.25 mg/mL of bathophenanthroline, prepared in isopropanol, was added to glass fiber 3. After each reagent

addition, the detection zone and reagent zone were dried completely. Ultimately, 250mL of the standard solution was added to the preconcentration tank.

Chromium-Diphenylcarbazide (Cr-DPC) Assay. 5 μ L of 0.1 M phosphoric acid was added to detection zone 4. A solution was prepared by mixing 15 mg/mL of Diphenyl Carbazide (DPC), 0.04 g/mL of phthalic anhydride, along with 1 mM ceric(IV) ammonium nitrate and polydiallyldimethylammonium chloride (PDDA) (5% w/v) in acetone. 20 μ L of this solution was added to glass fiber 4. After each reagent addition, the detection zone and reagent zone were dried completely. Ultimately, 250mL of the standard solution was added to the preconcentration tank.

Lead- Rhodizate (Pb-Rh) Assay. 20 μ L of Rhodizate at a concentration of 0.625 mg/mL in Tartrate buffer (pH 3) was added to the glass fibers 5 and exposed to drying. Following this, 250mL of the standard solution was added to the preconcentration tank.

Mercury- Dithizone (Hg-Dtz) Assay. In the detection zone 6, 5 μ L of Ethylenediamine (3 M) was added, followed by the addition of 5 μ L of *trans*-1,2-diaminocyclohexane-N,N,N', N'-tetraacetic acid (DCTA) (0.1 M). Subsequently, a stock solution of dithizone at a concentration of 12 μ g/mL in dichloromethane was prepared. This stock solution was then diluted 20 times in 5 mM NaOH, mixed with 0.08 g/mL of PEG-400 and 10 μ L of the final diluted solution was added to detection zone 6. After each reagent addition, the detection zone was dried completely. Ultimately, 250mL of the standard solution was added to the preconcentration tank. Similar to copper, all the reagents were added in the detection zone itself for this assay.

Manganese- 4-(2-pyridylazo) resorcinol (Mn-Par) Assay. In detection zone 7, a combination of dimercaptosuccinic acid and triethylenetetramine in pH 9.3 borate buffer was added and dried. Subsequently, a solution containing 2.5 mM of 4-(2-pyridylazo) resorcinol (PAR), mixed with 5% w/w PDADMAC, was prepared in borate buffer (pH 9.3, 0.1 M). A 20 μ L aliquot of this solution was applied to the glass fiber 7. After each reagent addition, the detection and reagent zones were completely dried. Ultimately, 250mL of the standard solution was added to the preconcentration tank.

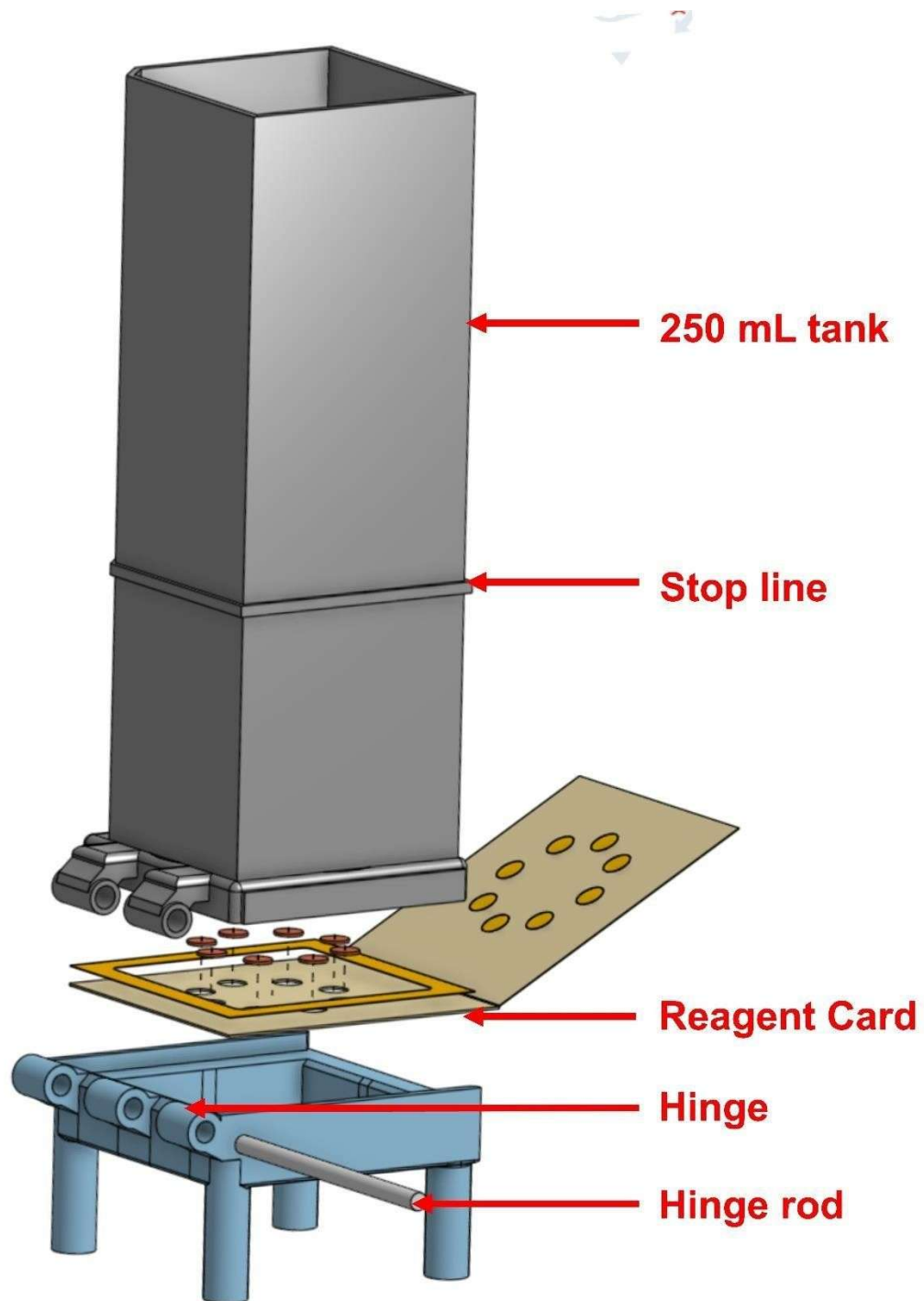


Figure S1. Schematic of 3D printed preconcentration tank

Video S1. Device running video

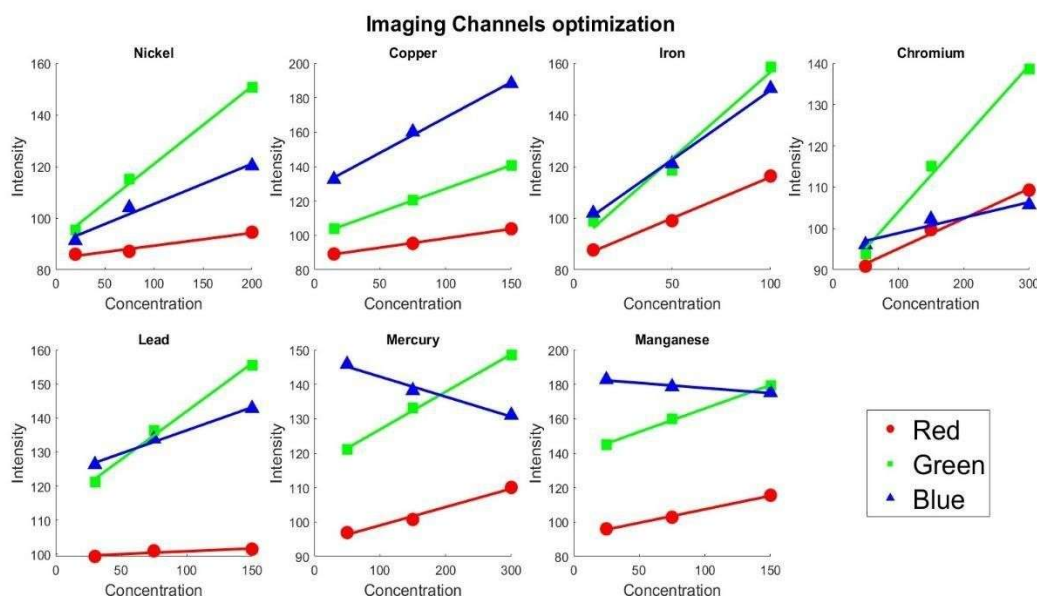


Figure S2. Imaging Channels optimization. The image channels were optimized for green, red, and blue channels. ImageJ established A linear plot for each channel using three points (low, medium, and high concentrations). Subsequently, the plot with the steepest slope was chosen for calibration. All metals were calibrated under the green channel except for copper, which was calibrated under the blue channel.

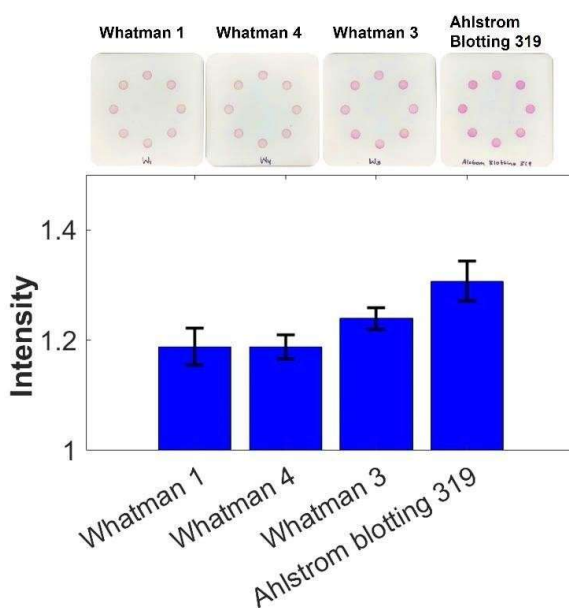


Figure S3. Substrate optimization. The increased thickness of Ahlstrom AB 319 (Thicker Paper (483 μ m for AB 319 vs. 180 μ m for W1 and 210 μ m for W4) provides an extended diffusion pathway, enhancing the chances of metal ions colliding with the paper fibers. Moreover, AB 319's higher thickness and denser structure likely provide a greater surface area for adsorption. The thicker nature of Ahlstrom AB 319 introduces more tortuous paths for metal ions to navigate. This tortuosity, coupled with potential functional groups, hinders the swift movement of metal ions, increasing the likelihood of interactions with the paper fibers and subsequent retention. In addition, considering the proprietary nature of AB 319, it's reasonable to speculate that the paper may contain undisclosed functional groups that could have an affinity for metal

ions, forming complexes that contribute significantly to the enhanced metal ion trapping observed during filtration.

In summary, while the specific chemical composition of Ahlstrom AB 319 remains proprietary, its superior metal ion trapping capabilities can be attributed to a combination of increased thickness, enhanced surface area for adsorption, and tortuosity. The presence of functional groups may play a crucial role in complexing with metal ions, further enhancing the paper's effectiveness in retaining these ions during the experimental filtration process.

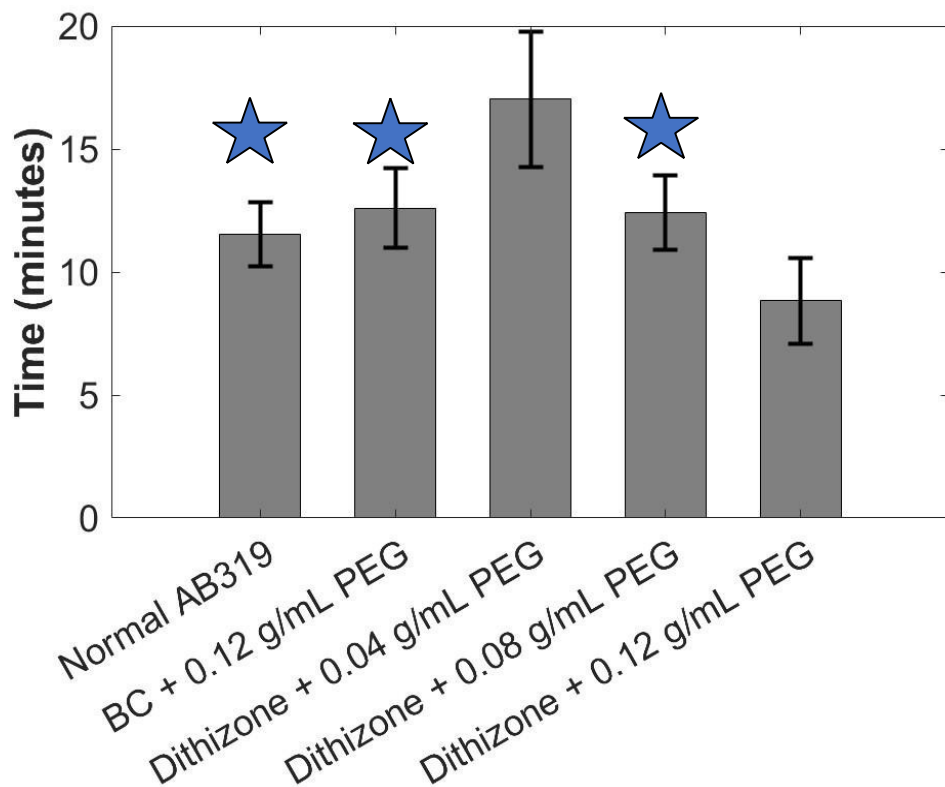


Figure S4. Substrate modification with PEG for Cu-Bc and Hg-Dtz assay to increase the hydrophobicity of the detection zone. The optimization for PEG was to ensure that we had the same filtration time for the reagent-treated paper as compared to those not treated in the case of the other five metal ions. Therefore, the reagents for Cu(II), and Hg(II) were mixed with optimized PEG concentrations.

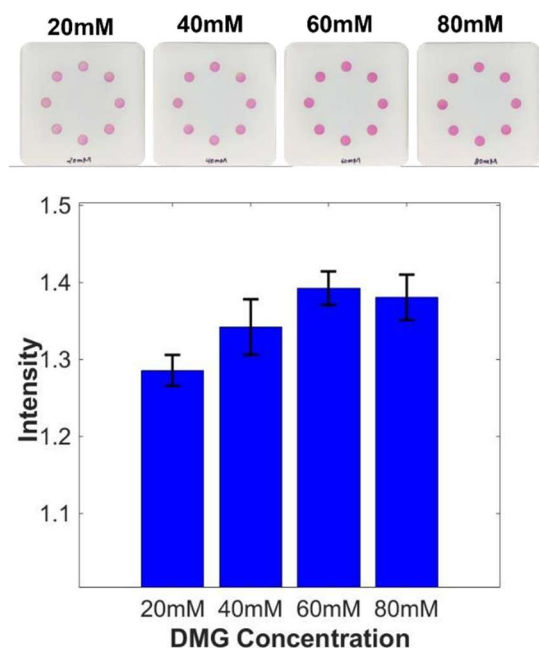


Figure S5. Dimethylglyoxime (DMG) concentration optimization. The best concentration for the optimal color response was determined to be 60mM. This concentration gave the highest average signal with lower standard deviation. Although the average signal difference was not statistically significant compared to neighboring concentration in the T-test ($p > 0.05$), this concentration was chosen for better consistency.

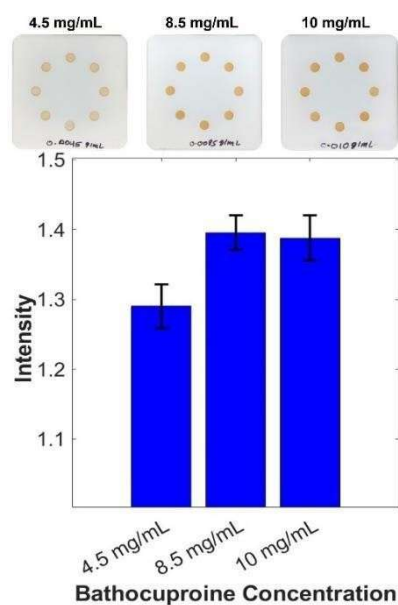


Figure S6. Bathocuproine concentration optimization. The best concentration for the optimal color response was determined to be 8.5 mg/ μ L. This concentration gave the highest average signal with lower standard deviation. Although the average signal difference was not statistically significant compared to 10mg/mL in the T-test ($p > 0.05$), this concentration was chosen for better consistency.

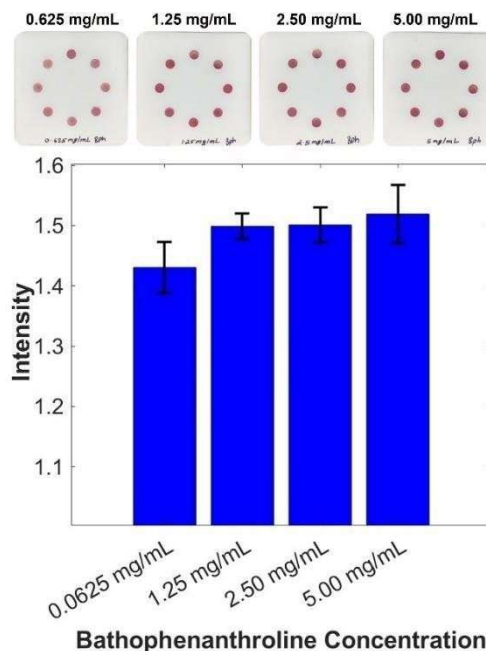


Figure S7. Bathophenanthroline concentration optimization. The best concentration for the optimal color response was determined to be 1.25mg/mL. Although 1.25, 2.5, and 5 mg/mL produced statistically similar signals ($p>0.05$), 1.25 mg/mL was chosen because it had least standard deviation.

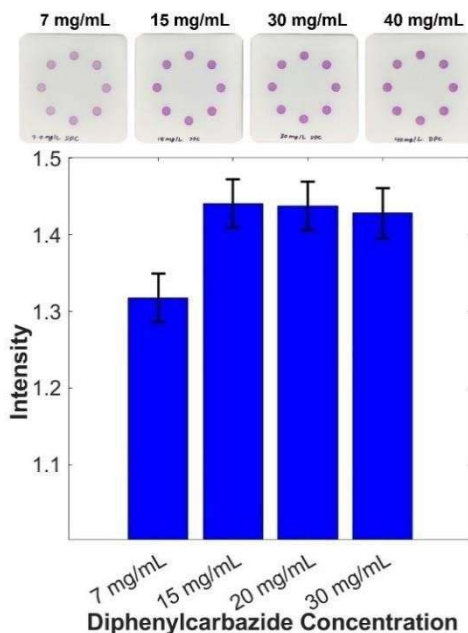


Figure S8. Diphenylcarbazide concentration optimization. The best concentration for the optimal color response was determined to be 15 mg/mL. Although 15 mg/mL, 20 mg/mL, and 30 mg/mL produced statistically similar signals ($p>0.05$), 15 mg/mL was chosen to minimize reagent use. This concentration was sufficient even at higher analyte concentrations.

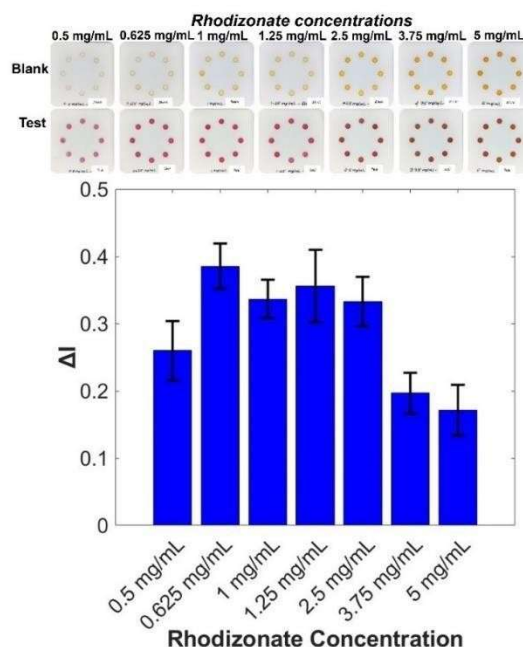


Figure S9. Rhodizonate concentration optimization. The best concentration for the optimal color response was determined to be 0.625 mg/mL. This concentration provided the highest average differentiation between the test and blank, with a lower standard deviation than the next concentration (1.25 mg/mL), which was statistically similar to 0.625 mg/mL ($p>0.05$). Additionally, this concentration was enough even at higher analyte concentration to attain the maximum signal.

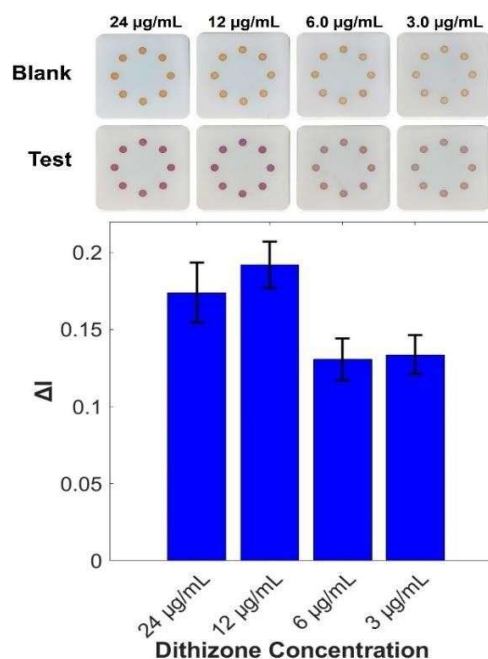


Figure S10. Dithizone reagent optimization. The optimal color response was achieved at 12 μ g/mL. While this concentration was statistically similar ($p>0.05$) to 24 μ g/mL, 12 μ g/mL had a lower standard deviation. We selected this as the minimal reagent concentration that was not reagent-limited, even at higher analyte concentrations and had better consistency.

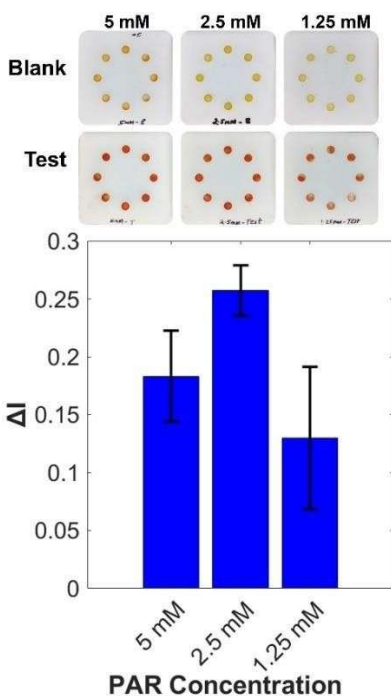


Figure S11. PAR concentration optimization. The best concentration for the optimal color response was determined to be 5 mM. This concentration clearly provided the best signal differentiation between the test and blank, with a statistically significant difference ($p < 0.05$) from its neighboring concentrations.

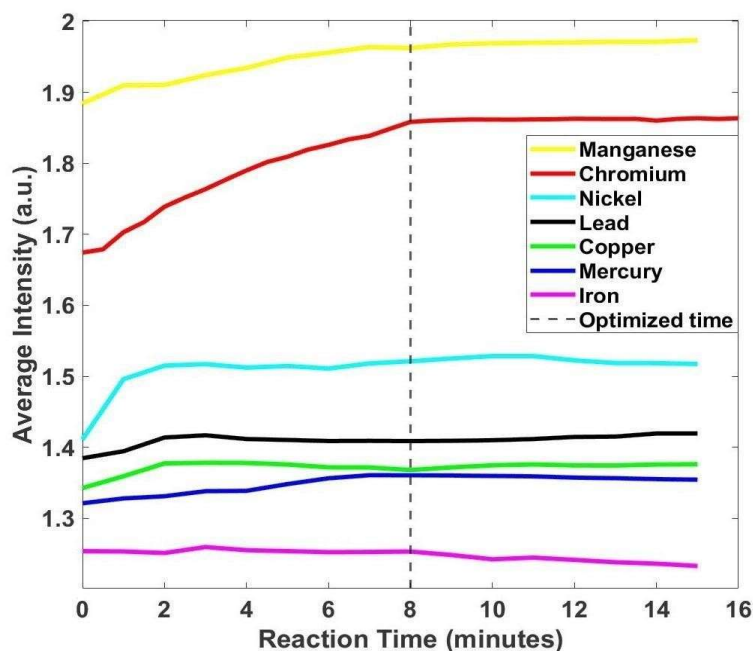


Figure S12. Reaction time optimization for developed sensor ($n=8$ for each reaction). The optimized time was determined to be 8 minutes after folding the chip.

Masking Protocols S1.

Building on our previous findings, we identified Fe, Cu, and Co as potential culprits interfering with the formation of the Ni-DMG complex in detection zone 1.¹ We modified the detection zone 1 by adding

sodium fluoride and ammonium acetate to tackle this challenge.¹ In the Cubathocuproine assay, increasing the reducing agent to 0.2 g/mL effectively mitigated interferences from Zn(II), Co(II), and Cd(II).² The same strategy was applied for Cu(II) interference in Febathophenanthroline assay by reducing it to Cu(I). In the Cr-DPC assay, significant interference from Fe(III) was addressed by adding a phosphoric acid at the detection zone 4.³ With the implementation of these masking strategies, tolerance ratios for Ni(II), Cu(II), Fe(III), and Cr(VI) were extended beyond 1:10 (analyte: interference).

Only Barium showed a significant interference at a 1:1 ratio for the Pb-Rh assay. Zn(II), Hg(II), and Fe(III) showed interference at a tolerance ratio of 1:5. In the Hg-dithizone assay, Ethylenediamine (3 M) was employed to mask Cu(II), Ni(II), Zn(II), Cd(II), and Pb(II) in detection zone 6.⁴ Additionally, DCTA (0.1 M) was added to mask Cu(II), Co(II), Ni(II), and Pb(II) at zone 6.⁵ Only Zn(II) exhibited significant interference at a 1:1 ratio among the non-targeted ions. The remaining non-targeted ions demonstrated higher tolerance limits in the Hg-Dtz assay. In the MnPAR assay, we employed masking strategies from our previous study by Meredith et al. to achieve higher selectivity for manganese detection.⁶ A combination of dimercaptosuccinic acid and triethylenetetramine in pH 10 borate buffer was added at detection zone 7 to enhance the assay's selectivity. Significant interferences were observed from Fe(III), Co(II), and Ni(II) (<±10% change) at a 1:1 ratio.

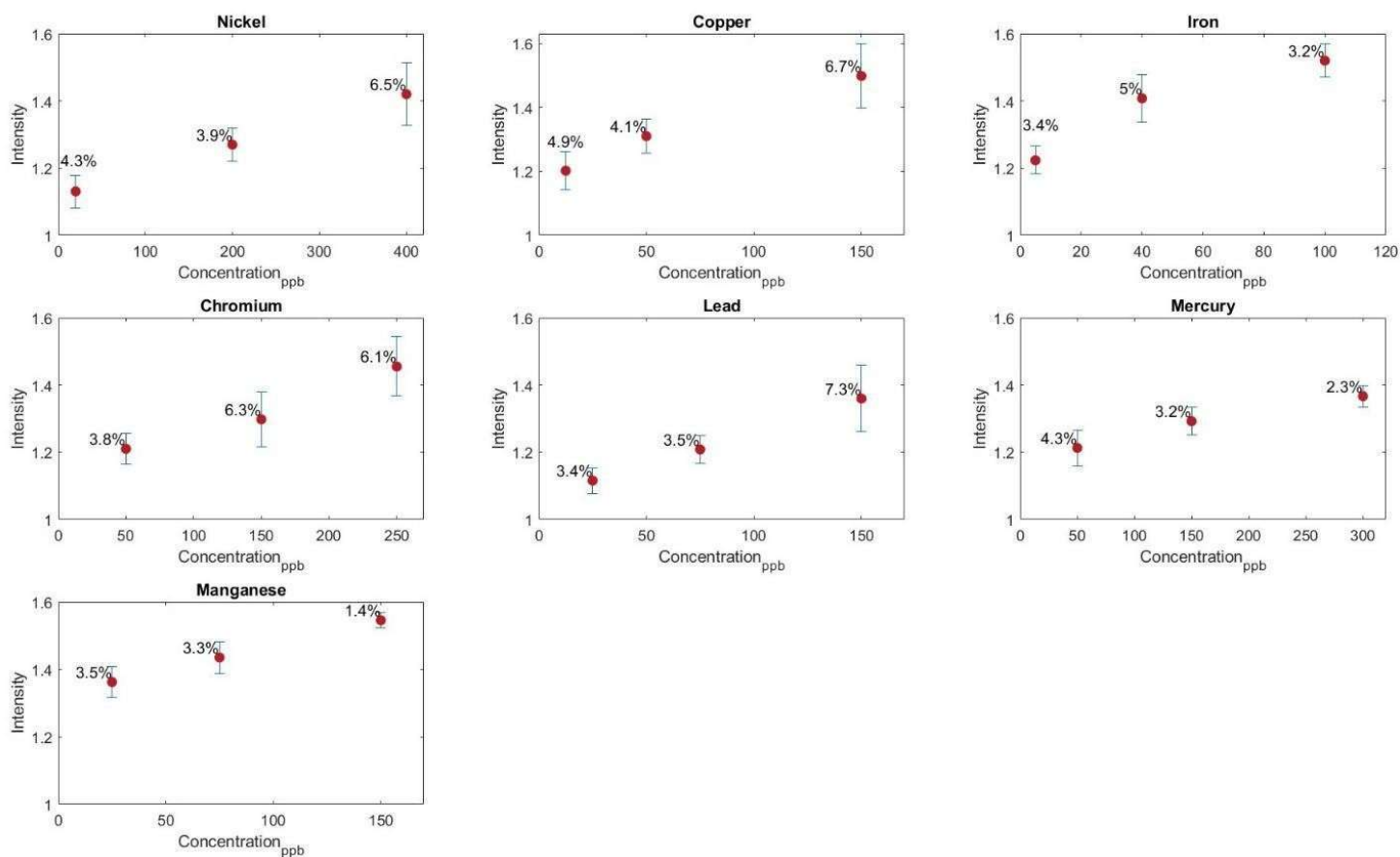


Figure. S13. Filtration consistency test (n=5 devices for each concentration). Tests were conducted at low, medium, and high concentrations for each metal. The RSD for all examined points remained below 10%.

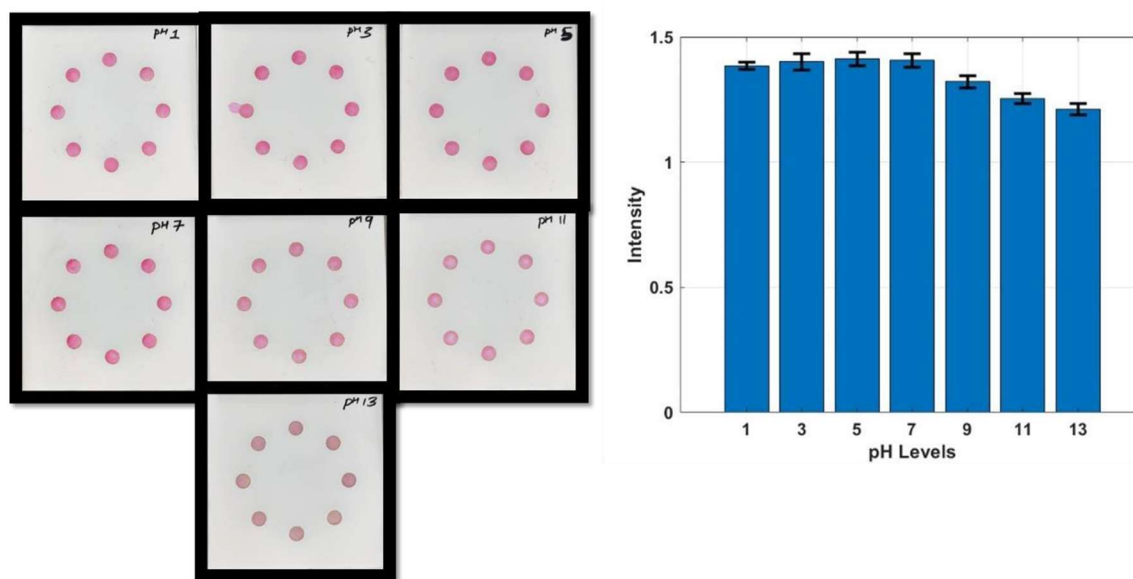


Figure S14. Effect of pH on Metal Retention

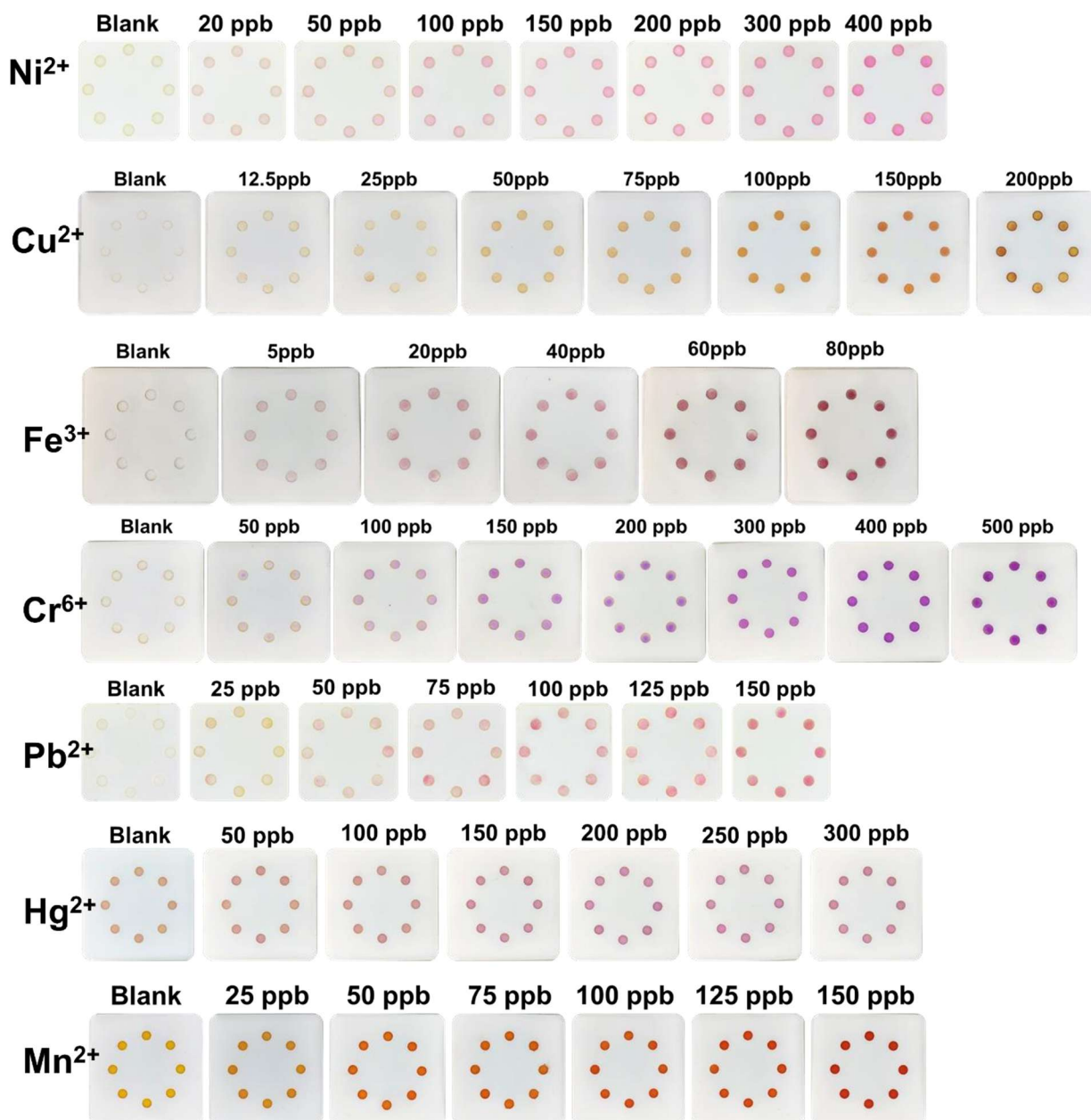


Figure S15. Color gradients for each assay for the calibration on the developed system. The linear fit and R^2 values were calculated as $y=(0.00076\pm0.00002)x+(0.04487\pm0.00371)$ with an R^2 of 0.997, $y=(0.00209\pm0.00011)x+(0.06535\pm0.01216)$ with an R^2 of 0.986, $y=(0.21782\pm0.01018)x-(0.00243\pm0.01564)$ with an R^2 of 0.989, $y=(0.00090\pm0.00003)x+(0.04649\pm0.00717)$ with an R^2 of 0.996, $y=(0.00186\pm0.00013)x+(0.06373\pm0.01227)$ with an R^2 of 0.982, $y=(0.00057\pm0.00004)x+(0.03893\pm0.00716)$ with an R^2 of 0.983, and $y=(0.00143\pm0.00004)x+(0.07063\pm0.00415)$ with an R^2 of 0.996 for Ni-DMG, Cu-Bc, Fe-Bph, Cr-DPC, Pb-Rh, Hg-Dtz, and Mn-PAR assays, respectively.

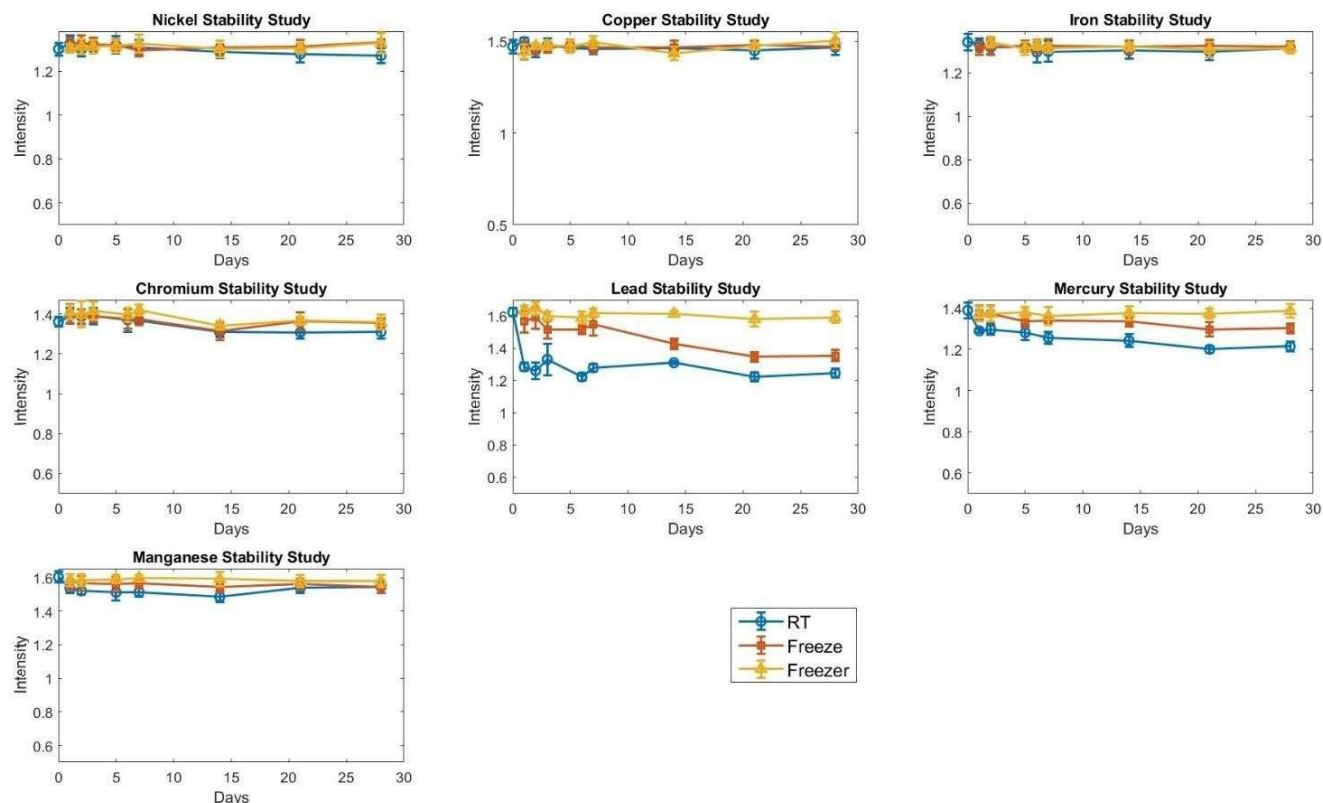


Figure S16A. Stability study for all the assays under three different conditions for four weeks. To evaluate the stability of the developed sensor, it underwent storage in three distinct conditions: room temperature (23°C), fridge (5°C), and freezer (-20 °C). Over four weeks, the color signal on the chip was systematically tested for Ni(II), Cu(II), Fe(III), Cr(VI), and Mn(II). The color signals remained stable across all storage conditions throughout the four-week testing period. However, it was observed that the freeze and freezer conditions proved to be more conducive for the Pb(II) and Hg(II) assay.

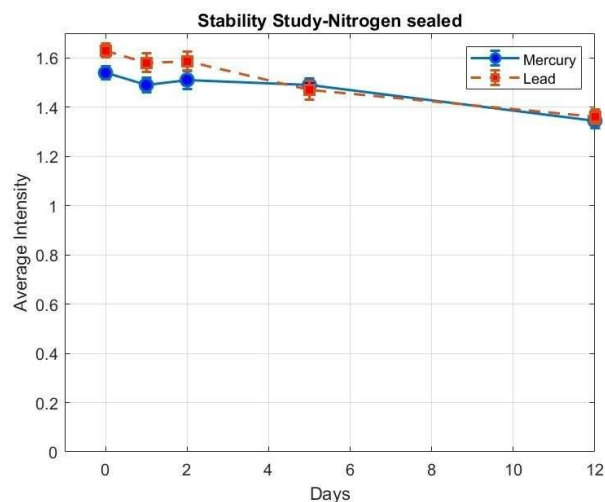


Figure S16B. In order to improve the stability of Pb(II) and Hg(II) at room temperature, the sensors were stored in nitrogen-purged bags. This packaging protocol resulted in retaining over 90% of the signal for up to 5 days.

Sample protocols S1. Drinking water samples were collected in triplicate using 50 mL Falcon tubes and spiked with 1% HNO₃. In the case of water from river, the samples were filtered and spiked in triplicate. The tubes were fully submerged, and the lids were closed inside the water. We ensured that water samples were collected from areas where the water was clear. The samples were refrigerated overnight and sent for ICP-MS analysis the following day. Metal testing was conducted using the sensor on the same day.

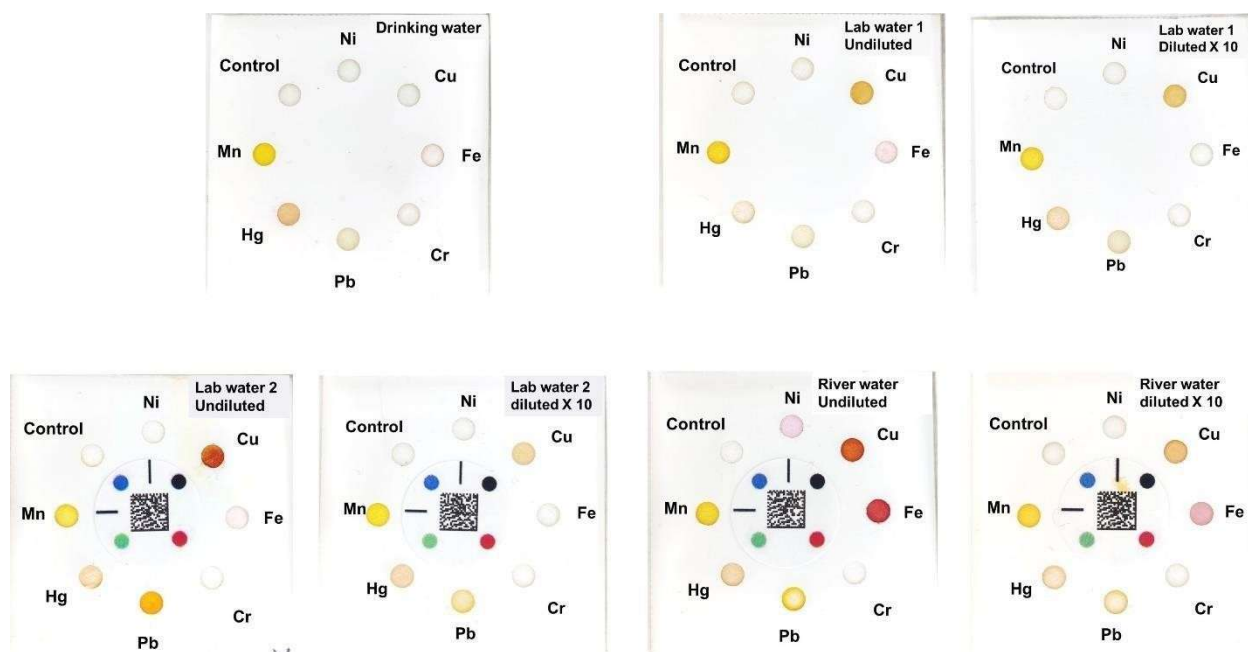


Figure S17. Representative images for Real water samples analysis on chip response. The test was done immediately for the samples that provided the signal within the signal's dynamic range. However, in case the color was already saturated, the quantification was done after 10-fold dilution.

Table S2. Summary of metal ion detection results in various water sources using the developed sensor. The results are compared with the industry-standard ICP-MS method (ND = not detected).

Sample Type	Metals	ICP-MS (ppb) (n=3)	Sensor (ppb) (n=3)	% Rec	% RSD
Drinking water	Ni(II)	0.11 ± 0.02	ND	x x	x x
	Cu(II)	6.00 ± 0.04	ND	77 x	18.1 x
	Fe(III)	13.20 ± 0.37 ND	10.16 ± 1.84 ND		
	Cr(VI)				
	Pb(II)	0.28 ± 0.03	ND	x	x
	Mn(II)	3.91 ± 0.02	ND	x	x
Lab water 1	Ni(II)	15.15 ± 0.14	<LoD	x	x
	Cu(II)	627.67 ± 8.18	540.11 ± 34.67	86	6.4
	Fe(III)	15.86 ± 0.45	12.83 ± 1.39 ND	81 x	10.8 x
	Cr(VI)	0.08 ± 0.01			
	Pb(II)	0.41 ± 0.01	ND	x	x
	Mn(II)	4.78 ± 0.02	ND	x	x
Lab water 2	Ni(II)	7.78 ± 0.38	ND	x	x
	Cu(II)	690.00 ± 5.72	645.37 ± 41.10	94	6.4
	Fe(III)	7.71 ± 0.10	5.32 ± 0.97	69	18.3
	Cr(VI)	0.07 ± 0.01	ND	x	x
	Pb(II)	0.19 ± 0.01	ND	x	x
	Mn(II)	4.56 ± 0.03	ND	x	x
River water	Ni(II)	50.77 ± 2.01	40.39 ± 5.85	80	14.5 7
	Cu(II)	689.00 ± 24.59	621.13 ± 43.14	90	10.7 x
	Fe(III)	721.33 ± 20.81	662.72 ± 71.15	92 x	
	Cr(VI)	0.34 ± 0.01	ND		
	Pb(II)	3.05 ± 0.04	ND	x	x
	Mn(II)	4.13 ± 0.00	ND	x	x

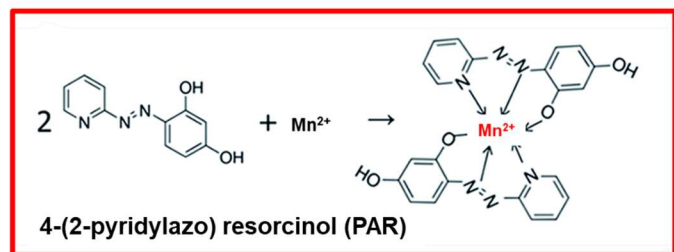
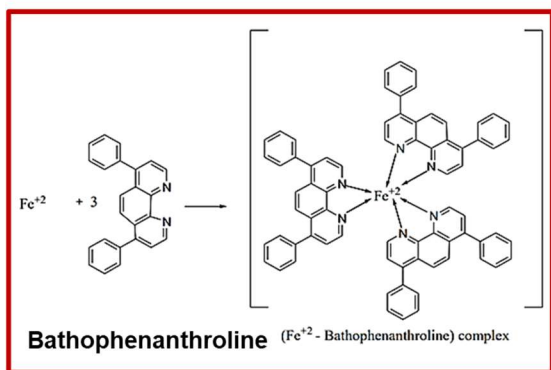
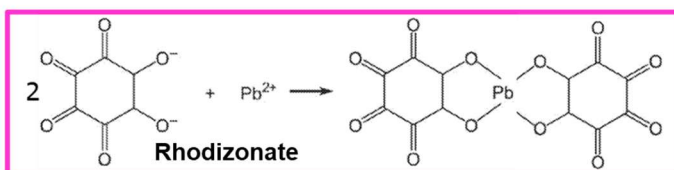
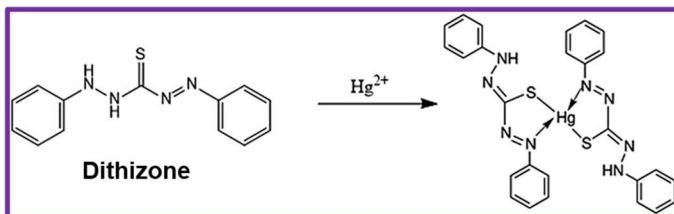
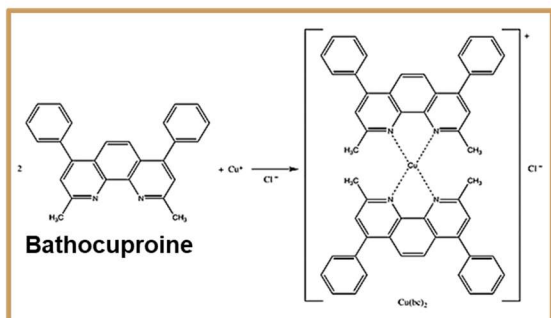
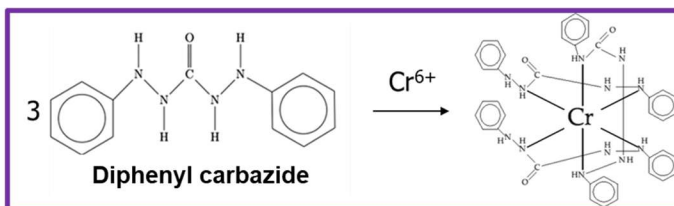
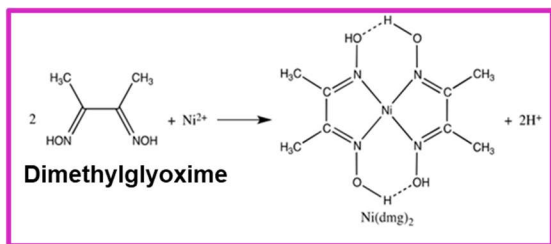


Figure S18. Reaction schematic for each heavy metal complexation.⁷⁻⁹ The color of the image lining represents the respective color of complexation.

References

1. Mentele, M. M.; Cunningham, J.; Koehler, K.; Volckens, J.; Henry, C. S., Microfluidic paper-based analytical device for particulate metals. *Analytical Chemistry* **2012**, *84* (10), 44744480.
2. Aryal, P.; Indrianingsih, A. W.; Henry, C. S., Smartphone-Enabled Green Anthocyanin Sensor for Fe (III) Sensing on Paper Using Capillary-Driven Microfluidics. *Green Analytical Chemistry* **2024**, 100091.
3. Mohamed, A. A.; Ahmed, S. A.; El-Shahat, M. F., Catalytic spectrophotometric determination of hexavalent chromium. *Journal of trace and microprobe techniques* **2001**, *19* (2), 297-311.
4. Kamnoet, P.; Aeungmaitrepirom, W.; Menger, R. F.; Henry, C. S., Highly selective simultaneous determination of Cu(ii), Co(ii), Ni(ii), Hg(ii), and Mn(ii) in water samples using microfluidic paper-based analytical devices. *Analyst* **2021**, *146* (7), 2229-2239.
5. Chakrapani, G.; Murty, D.; Balaji, B.; Rangaswamy, R., Spectrophotometric method for the determination of vanadium in uranium rich hydrogeochemical samples using pyridyl azo resorcinol (PAR). *Talanta* **1993**, *40* (4), 541-544.
6. Meredith, N. A.; Volckens, J.; Henry, C. S., based microfluidics for experimental design: screening masking agents for simultaneous determination of Mn (II) and Co (II). *Analytical Methods* **2017**, *9* (3), 534-540.
7. Godycki, L. E.; Rundle, R. E., The structure of nickel dimethylglyoxime. *Acta Crystallographica* **1953**, *6* (6), 487-495.
8. Hulthe, P., A bathocuproine reagent-paper for the rapid semi-quantitative determination of copper in the 1 to 70 p.p.m. range. *The Analyst* **1970**, *95* (1129), 351-355.
9. Sankar, R.; Ravisankar, P.; Sulthana, S.; Babu, P. S.; Afzal Basha, S. K.; Aswini, R.; Swathi, V.; Mahamuda Sultana, S. K.; Sri Lakshmi Prasanna, M.; Navyasri, N.; Thanuja, I. M.; Sessa Sai Sadan, S., Comprehensive review of important analytical reagents used in spectrophotometry. *Indo American Journal of Pharmaceutical Research* **2017**, (January 2017).