THESIS

DEVELOPMENT AND APPLICATION OF ELECTROCHEMICAL DITHIOTHREITOL (DTT) ASSAY FOR ANALYSIS OF PARTICULATE MATTER

Submitted by

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ABSTRACT

THE DEVELOPMENT OF ELECTROCHEMICAL DITHIOTHREITOL (DTT) ASSAY FOR ANALYSIS OF PARTICULATE MATTER

Particulate matter (PM) in air pollution, known to have a negative impact on biological systems, is regulated in many countries across the globe. The generation of PM from energy and mining industries is monitored in an effort to minimize its contributions to diminished human health. And although quantifying total PM generation (mass and number) and exposure can help track health risks, ultimately there exists a need to develop rapid, efficient, accurate methods for analyzing PM composition and health effects. Leading hypotheses over the last decade have theorized that PM, once absorbed into bodily tissues, generate reactive oxygen species (ROS), leading to oxidative stress, thus catalyzing cellular damage. A recently developed analytical electrochemical protocol has shown great potential for investigating the potential for PM to cause oxidative stress. The work discussed within this thesis focuses on the development of the electrochemical assay and its application to real world PM samples.

Herein, the development of an electrochemical version of the well-studied dithiothreitol (DTT) absorbance spectroscopy assay is presented as a platform for the analysis of PM in air samples. Flow injection amperometry was used as the primary electrochemical method. Amperometry was performed using a CH Instruments potentiostat and commercially available DropSens modified carbon screen printed electrodes (SPE's) with a DropSens impinging jet flow cell, granting the assay flexibility to be performed in any lab with commercially available components. Previous examples used homemade components restricting accessibility to the field. The ability to use inexpensive, purchasable components eases trouble shooting, training, and

allows for the potential to make the assay more mobile. The use of a flow cell also allows for the possibility for linking the assay to other analytical methods to further analyze PM which may not be reactive in the assay.

Assay development focused on optimizing the assay temporally, as well as investigating its precision, detection limits, fluid dynamics, reproducibility, and relative accuracy. The electrochemical assay uses shorter reaction times and avoids the need for additional chemical quenching agents used in the absorbance assay, allowing for batch processing. Assay performance was compared to literature with a model oxidant quinone, 1,4-naphthoquinone, and trace metal, Cu(II). The assay was then applied to real exposure samples collected in Fresno, California and Honduras. Data from these samples were correlated against data obtained by the traditional DTT assay to investigate accuracy. Analyses of the Honduras samples will be correlated against health data as part of the Honduras Cookstove Project, moving one step closer to directly connecting PM reactivity to health effects. Although the traditional absorbance DTT assay is the standard in assessing PM reactivity in air samples, and has been used for the last 15 years, the electrochemical assay is a robust, quick, and precise alternative method that can be readily performed using readily available components.

ACKNOWLEDGMENTS

This work would not have been possible without the support and contribution of many people. The majority of the credit rests on my advisor, Chuck Henry, who has always been honest, been willing to take a chance on me, and has forged a formidable group of diverse, talented researchers that I am grateful to have known for the past several years.

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Finally, Tom Siller, thank you for taking the time to have me sit in your offices, tell you about my life, receive your sage advice, and for all of the dark chocolate you shared over the years. Your perspectives on life, on living, and on education have shaped me into a ferocious supporter of educators like you. And that will never change. You should also know that I came to Colorado State University because of you and I don't think I ever told you. I can still remember the two recruitment events I attended when I was in high school. You were at both and I remember feeling intimidated, but inspired and reassured. I knew I wanted to be at a school which recognized the

value of a person like you. Of all people acknowledged here, you are probably the most responsible for me being at Colorado State for the last six years, for me lasting this long, and for me never losing faith in my ability to succeed.

I thank Colorado State University, the Walter Scott Jr. College of Engineering, and the Department of Chemical and Biological Engineering for being my home(s) for a long six years and for providing the means to complete not one, but now two degree programs. Integral in that, of course, is the amazing gift Claire Lavelle has also proven to be. She has been a steady voice of reason in my six years here and I love her for that.

I also want to set aside space here for the second family I have found at Rocky Mountain Student Media. What started out as a fun, musical hobby for the weekend quickly grew into one of the most formative, constructive experiences I have ever enjoyed in my entire life. I was given the opportunity to gain management experience and, though I'm thankful for the boost to my resume, I have learned so much more about conflict, about people, and about creative production than I could have ever hoped for. When I think back on the best parts about my time at CSU, a great many memories will be from my time at student media. Not everyone in science understands my life-long love for music and its nuances…but my radio family does. They've opened me up to being an entirely new person and I'll be eternally grateful. More than this, they have helped me to see exactly the types of people science will need in order to remain resilient and relevant in our society. Media people are our bridge to the future and I'm so grateful to know some good ones.

This may be unique, but before closing this section, I want to extend thanks to Damon Larson, John Kissingford, Judy Murray, and Izzy Perez. It's probably unusual for a graduate student to choose to thank high school teachers, but I was a very lucky student in high school. I may be far removed from that time in my life, but I could not ignore or forget the influence these

four have had on me, even as I have grown past who I was back then. They each nurtured a deep love and passion for literature, expression, chemistry, and biology. They encouraged me to be myself, to persevere with integrity, to pursue the great mysteries of life, to dare to disturb the universe, and to never be afraid to be a strong voice. Those lessons took time and I want them to know I have become a person they can be glad they helped to influence, shape, and to raise. I know they'd be proud.

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CHAPTER 1. ELECTROCHEMICAL ASSAY DEVELOPMENT FOR EVALUATING OXIDATIVE STRESS FROM PARTICULATE MATTER

1.1 Chapter Overview

Small-scale electrochemical detection methods have shown considerable promise for point-of-care, point-of-need, and environmental diagnostics due to numerous advantages including: [1] small reagent volumes, increased capacity for portable field testing, as well as increased precision and accuracy. While electrochemical methods, both analytical and material, have been developed in academic research, a considerable gap still exists between their development in laboratories and their use in field applications. Most of the burden associated with developing electrochemical detection methods capable of being employed outside of an academic setting lies in validating that the methods perform as good as or better than traditional analytical counterparts, such as absorbance spectroscopy, with real samples.

One area of growing interest in the field of analytical electrochemistry is the analysis of particulate matter (PM) in air pollution. Air pollution, including diesel fumes and cook stove smoke generated within households, is of concern globally, as it is known to have serious, long-lasting consequences on biological systems and healthy development. [2,3,4] Such consequences have already produced a push for monitoring PM generation. Thus, increased awareness and regulation of PM generation have yielded an increased focus on developing the capability to rapidly, cheaply, and accurately analyze PM and its potential to cause harm. However, it has been recognized that simply tracking PM amount is not enough; understanding composition and reactivity are critical for understanding and then mitigating impacts on human health.

Within this thesis, an optimized analytical electrochemical assay, an adaptation of the well-studied DTT absorbance assay, [3,4] is presented as a solution for monitoring PM reactivity. Development of this assay focuses on the use of commercially available components, protocol reproducibility, relative accuracy, and application. There is also an investigation of the fluid dynamics inherent in the use of flow cells in electrochemical protocols. In this chapter, the necessity for tracking PM risks, the formative work of others, as well as the development of the electrochemical DTT assay using commercially available components are discussed. Casey Quinn and Kevin Klunder contributed absorbance data and statistical analyses used for validating this work.

The second chapter focuses on the application of the electrochemical assay to analyzing real samples as part of the Honduras Cookstove Project. The third chapter presents a fluid dynamics study adapted to better understand the methodology behind standardizing redox reactions in flow systems, as flow conditions and design parameters within a flow cell have a direct effect on the electrochemical response of analytes. The electrochemical DTT assay developed in the first chapter serves as the subject of the fluid dynamics study.

1.2 Particulate Matter and Reactive Oxygen Species

Particulate matter (PM) in air pollution, known to have an impact on human health and mortality, may have this effect by a theorized mechanism involving absorption into bodily tissues, generation of reactive oxygen species (ROS), and oxidative stress, thus inducing tissue damage. [3−7] Epidemiological research has proven links between airborne particulates and serious health issues ranging from respiratory and cardiovascular diseases to premature death, with

particular focus on fine particle sizes of less than 2.5 µm in diameter (PM2.5). [4,8–10]. Further, ROS can be generated by chemical compounds found in PM including quinones, organic aromatic compounds such as polycyclic aromatic hydrocarbons (PAHs), and transition metals such as Fe, Cr, Mn, Co, Ni, Cu, and Zn. [4,11–12] Further, people and animals exposed to any variety of air pollution are not exposed to any single metal, PAH, or quinone. Rather, studies suggest PM exists as a mixture of compounds, compounding their capacity for causing tissue damage. [5] And though the exact mechanism by which PM causes oxidative stress is not perfectly understood, ROS generation associated with PM does cause damage to lipids, protein, DNA, and mitochondria, which indicate oxidative stress in cell tissues. [3,4,8,9,12] PM may also be responsible for inflammatory responses in living tissues. [7,12–15] Normally, biological systems are equipped to balance ROS generation via endogenous antioxidants. [16] However, when ROS levels within biological systems exceed cellular antioxidant capacity, a cascade of events is triggered, causing inflammation and cellular damage. [17,18] While these associations have become better understood in a laboratory setting, being able to apply them in a real world setting has suffered from slow growth due to the lack of easy-to-use, low-cost, field-ready sensing methods.

Because compounds found in diesel exhaust particulates (DEP), industrial exhaust, and cook stove fumes have a high potential for catalyzing disease and ill health, [3] governments around the world have begun to focus on quantifying such exposures. Special attention is paid to PM with a diameter less than 2.5 µm (PM2.5), because this size exists in higher quantities and is more likely to cause cell damage. [3] In major United States cities such as New York City and Los Angeles, real-time public data estimates that daily PM2.5 levels in the air exist somewhere in the range of 8-15 µg/m^3 and 25-100+ µg/m^3, respectively. [19,20] To put these numbers in perspective, the Environmental Protection Agency (EPA) marks an acceptable average level of annual PM2.5

exposure at 12 $\mu g/m^3$. [19] The World Health Organization (WHO) limit is lower, at 10 $\mu g/m^3$. Although the EPA, WHO, and even OSHA have each attempted to regulate and/or enforce the generation of PM2.5 over the last few decades within the United States, they have been recently met with resistance, most importantly from policy makers. Though technology exists for quantifying particulate exposure, quantifying oxidative activity efficiently is another matter. Thus, there exists a demonstrable need to rapidly evaluate just how much of a risk air pollution may pose on human health and well-being.

Various approaches to measuring oxidative activity of PM2.5 have been proposed and developed, often focusing on redox-specific chemicals, chromatography, and fluorescence. [2,6,10,16,21−30] Several works in the past have established that oxidative activity can be accurately measured by taking advantage of the well-studied dithiothreitol (DTT) activity assay, giving use of the dithiol precedence. [2,3,5,13] The goal of the assay is to use the oxidation of a dithiothreitol to measure the capacity of a given PM2.5 sample for inducing oxidative stress.

One of the important founding groups to study the effect PM has on biological systems is that of Yoshito Kumagai at the University of Tsukuba, Japan. Prior to their first execution of the DTT assay, their work and the work of others helped to reveal that quinones found in DEP were reduced by one electron by NADPH-cytochrome P450 reductase. DEP reduction leads to increased production of superoxide, a reactive oxygen species (ROS) which can form hydrogen peroxide (HOOH) and hydroxyl radical ($^{\bullet}$OH), showing that quinones can catalyze the generation of ROS within biological systems. [31,32] A few years later, their studies indicated that when macrophages were exposed to organic DEP extracts, which contain quinones, apoptosis resulted, suggesting that quinones could cause cell death. In addition, an increase in the gene expression of the protein heme oxygenase-1 (HO-1) within the macrophages was observed (HO-1 protein expression is induced

by oxidative stress). [33,34] These results suggest a relationship between quinone exposure, ROS generation, and cell death/tissue damage. Since these studies were conducted, few other groups have attempted to re-approach the analysis of particulates found in air pollution, like quinones, and their capacity to induce oxidative stress.

Addressing the need to efficiently evaluate health risks from PM, an electrochemical alternative assay, based on the same core reaction as the traditional assay, was reported in 2012. [2] Its redevelopment and optimization, presented in this chapter, is a reproducible protocol with a calibration limit of detection (LOD) of 8.45 μM DTT and average reproducibility of 94.54%, indicating a high degree of long-term precision. In applications, y-residuals yield LOD's of 0.11 nmol DTT lost/min, 63.2 ng/m^3 1,4-naphthoquinone, and 12.6 ng/m^3 Cu(II) with an average reproducibility of 94.74%. Further, the electrochemical assay is capable of running one sample, in triplicate, in just under 15 minutes and can run non-stop for several hours. This rate is comparable to the preliminary development of the electrochemical assay and is more rapid than the traditional DTT assay, which is capable of running 1 sample in triplicate in about an hour. [2] To the best of our knowledge, the electrochemical DTT assay has the fastest time resolution for an aerosol oxidative activity assay. The speed of the electrochemical assay could contribute to future work analyzing how PM affects human health during short-term exposures.

1.3 The Traditional DTT Assay

The importance of evaluating and tracking risks to human health as a result of exposure to PM2.5 and ROS has led to the development of protocols for gaining information ranging from particle number quantification to PM sample reactivity and composition. For evaluating sample reactivity, two key protocols have been developed: the traditional and the electrochemical DTT

assay. The traditional, or absorbance, assay is discussed in this section; the newer electrochemical assay, the next.

To address and investigate whether or not quinones lead to cell death via oxidative stress rather than by covalent modification, the Kumagai group recognized that the oxidation of cysteine residues in proteins is associated with decreased sulfhydryl status as well as changes in oxidative stress-mediated signal transduction. [32,36,37] This association led the Kumagai group to conclude that oxidative stress could be induced by a modification in sulfhydryls. And the capacity of quinones to cause sulfhydryl modification could be quantified by measuring the degree of the destruction of thiol groups resulting from, among other methods, a reaction assay of Phenanthraquinone (PNQ), N-Ethylmaleimide (NEM), and a dithiol compound, dithiothreitol (DTT), shown below. Results from this study showed that the destruction of thiols in the presence of quinones was consistent with a process by which quinones generate superoxide (ROS) in order to catalyze the oxidation of thiols and was also time-dependent. Through other methods conducted alongside the first DTT assay, they also concluded that quinone-catalyzed thiol destruction is not a covalent modification reaction, but a catalytic one, leaving the quinones intact. [32]

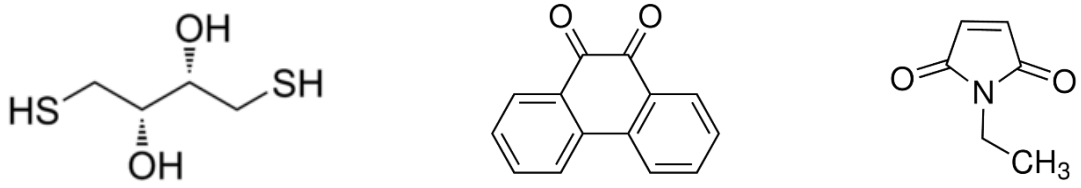

Figure 1.1. Structures of DTT (L), PNQ (middle), and NEM (R), used in the Kumagai study. The double bonded oxygens initiate the oxidation of DTT via one of the thiol bonds. [32]

The traditional assay reacts 100 μM (nmol) DTT with varying amounts of a standard oxidant, originally done with PNQ, or with a real PM2.5 sample in an incubated environment. Incubation occurs at 37 °C and its duration is typically arbitrary, but 45 minutes is common. Aliquots of the assay reaction mixture are extracted and tested at various time points over the course of the reaction in order to establish a kinetic reaction curve. Once aliquoted, the reaction mixture is mixed with 0.4M Tris-HCl, 20mM EDTA, and 10mM DTNB, a chemical quenching and developing step to stop reaction. This step produces a chromophore which is analyzed via absorbance spectroscopy and absorbs light at 412 nm. The rate of thiol destruction, or DTT "consumption", over time is proportional to the oxidative activity of a given PM2.5 sample or model oxidant and increased DTT consumption in the assay indicates increased capacity to induce biological oxidative stress. [2,3,5,32]

Of primary importance to the early development of the DTT assay were two questions: is a dithiol better to use in the assay than a monothiol and can quinones consume DTT independent of molecular oxygen and metals. To address the latter, the Kumagai group set up assays reacting DTT with different amounts of PNQ in aerobic and anaerobic conditions.

Figure 1.2 shows that the thiol groups of DTT are sensitive to dissolved oxygen under the reaction conditions, pointing to the dithiol's ability to reduce O_2 while the quinone acts as a catalyst (this was confirmed by ESR spectra).[32] However, even in an anaerobic experiment, the consumption of DTT appeared to be catalyzed by the model oxidant, PNQ, indicated by 100% DTT consumption by 1 nmol PNQ. Concerns that trace metals could be creating a falsely high reaction rate were addressed by the use of a chelated buffer. Results from the assay were correlated to data from another experiment involving reduction of cytochrome c, providing evidence for the mediation of sulfhydryl oxidation, *in situ,* by quinones.

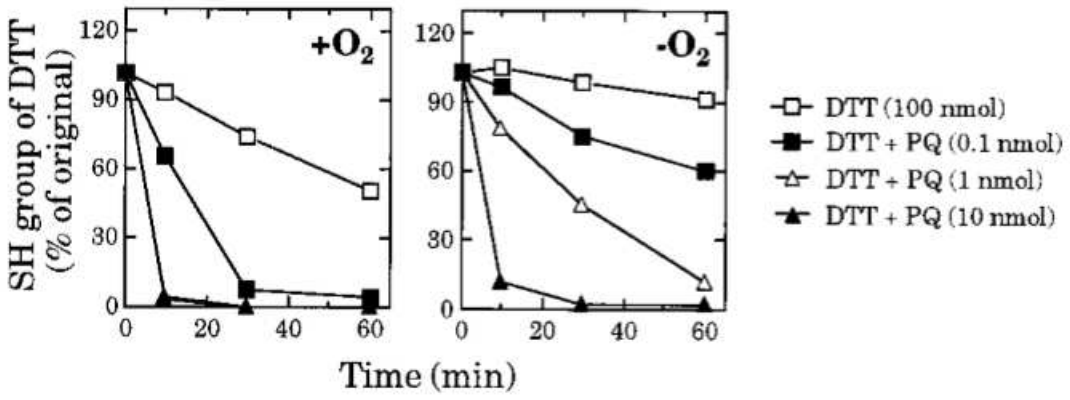

Figure 1.2. Percent consumption of thiol content in DTT during incubation with PNQ under aerobic and anaerobic conditions in chelated 0.1M PBS pH 7.5 carried out at 37 °C. Standard deviations were under 5%. [32]

In order to validate DTT's selective sensitivity to oxidation, the assay procedure was run using antioxidant monothiols and two other dithiols, 2,3-dimercapto-1-propanol (BAL) and 2,3-dimercapto-1-propane-sulfonic acid (DMPS). The experiments were run in aerobic conditions in order to focus on the thiol species' abilities to reduce oxygen. If DTT *is* selectively oxidized, the assay should work better with DTT than with monothiols. And if DTT is the most sensitive to molecular oxygen, its consumption in the assay should be stronger than other dithiols. The results are shown in the table below.

Table 1.1 Consumption of SH groups in monothiol and dithiol compounds. [32]

	consumption of SH group (nmol)	
compound	1 nmol	10 nmol
monothiol		
GSH	2.7 ± 0.5	1.8 ± 2.3
2-mercaptoethanol	0	0.3 ± 1.0
N-acetyl-L-cysteine	0	0.3 ± 2.0
dithiol		
DTT	103.0 ± 0.3	103.0 ± 0.0
BAL	43.9 ± 1.0	98.0 ± 0.6
DMPS	17.1 ± 2.1	89.1 ± 1.0

[a] The incubation mixture (1 mL) consisted of phenanthraquinone (1 and 10 nmol) and various thiol compounds (100 nmol) in 100 mM potassium phosphate buffer (pH 7.5). Reactions were carried out at 37 °C for 10 min under aerobic conditions. Each value is the mean ± SD of three determinations.

The summary data shows that thiol groups on dithiol compounds are selectively oxidized by PQN, but those on monothiol compounds are not. Due to the nature of redox reactions involving dithiols, this data also indicates that active quinones, like PQN, serve as catalysts for DTT oxidation as well as for the generation of ROS, which then goes on to further oxidize dithiols, rather than causing covalent modifications. Otherwise, PQN would have reacted with a monothiol

just as easily as a dithiol. This conclusion also means that the quinone, itself, is not consumed in the assay. As predicted, the strongest dithiol response in the assay belongs to DTT, which is why it was chosen for future executions of the assay. Further, DTT only exists as either a dithiol or disulfide; there is no stable half-state, like what is observed with glutathione oxidation. A proposed series of reactions for the DTT assay, shown below, were given by the Kumagai group. Though the aerobic catalytic mechanism can be observed in reaction 6, the anaerobic catalytic regeneration of the quinone after reaction 5 remains less clear.

$$\text{Q (phenanthraquinone)} + \text{R}-\text{SH} \rightarrow \text{Q}^{\bullet -} + \text{H}^{+} + \text{R}-\text{S}^{\bullet} \quad (1)$$

$$\text{R}-\text{S}^{\bullet} + \text{R}-\text{SH} \rightarrow \text{R}-\text{S}-\text{S}-\text{R}^{\bullet -} \quad (2)$$

$$\text{R}-\text{S}-\text{S}-\text{R}^{\bullet -} + \text{Q} \rightarrow \text{Q}^{\bullet -} + \text{R}-\text{S}-\text{S}-\text{R} \quad (3)$$

$$\text{R}-\text{S}^{\bullet} + \text{Q} \rightarrow \text{R}-\text{SQ}^{\bullet} \quad (4)$$

$$\text{R}-\text{SQ}^{\bullet} + \text{R}-\text{SH} \rightarrow \text{Q}^{\bullet -} + \text{R}-\text{S}-\text{S}-\text{R} \quad (5)$$

$$\text{Q}^{\bullet -} + \text{O}_2 \rightarrow \text{Q} + \text{O}_2^{\bullet -} \quad (6)$$

$$\text{O}_2^{\bullet -} + \text{O}_2^{\bullet -} + 2\text{H}^{+} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (7)$$

$$\text{Fe}^{3+} + \text{O}_2^{\bullet -} \rightarrow \text{Fe}^{2+} + \text{O}_2 \quad (8)$$

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^{-} \quad (9)$$

$$\text{Me}_2\text{SO} + \cdot\text{OH} \rightarrow \cdot\text{CH}_3 + \text{MeSO(OH)} \quad (10)$$

Figure 1.3. Reduction and oxidation reactions theorized by the Kumagai group to support assay activities and the idea that quinones are directly involved in inducing oxidative stress. [32]

One of the main groups exploring the assay in greater depth since its establishment is the Sioutas group at the University of California in Los Angeles. Through their work, they have been able to determine that the various sizes of PM (<1 µm, <2.5 µm, and <10 µm), collected in the Los Angeles basin, [38] directly correlate to their uptake in macrophages and epithelial cells as well as their ability to induce oxidative stress. [3] Building on top of what had been established by the Kumagai group before them, the Sioutas group determined that fine and ultrafine particles (< 2.5 µm) are the most prevalent (shown in **Figure 1.4**) and most potent toward inducing expression of cellular heme oxygenase (HO-1), which is a direct indicator of cellular oxidative stress.

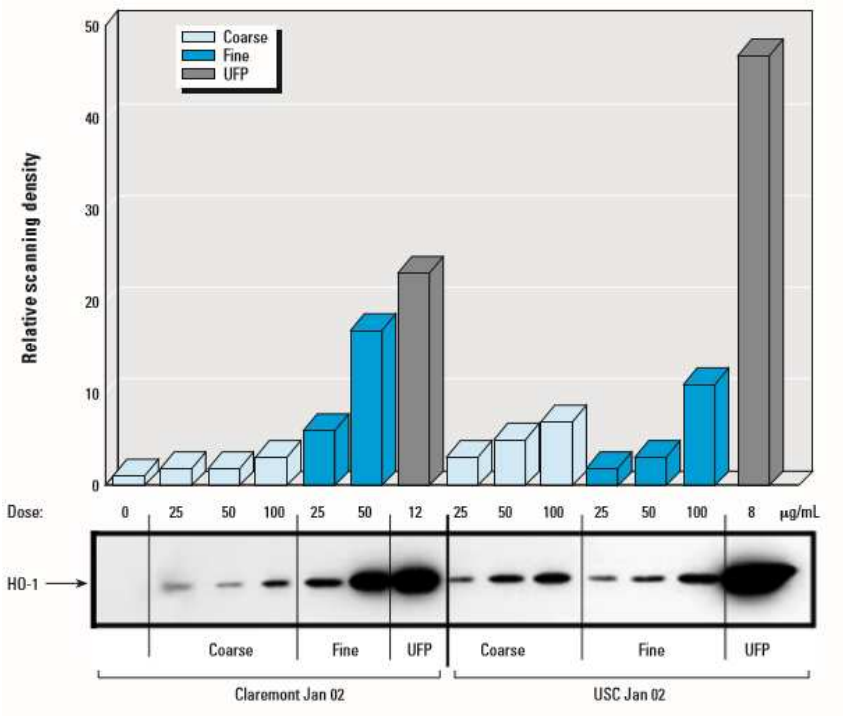

Figure 1.4. Particle counts and HO-1 expression data obtained from the Sioutas study showing particle size prevalence and toxicity in Claremont and on-campus at USC. Fine and ultrafine (UFP) particles are less than 2.5 µm in diameter. [3]

Data shown in **Figure 1.4** was the first to prove that fine and ultrafine particles induce HO-1 expression with a higher potency than larger counterparts. This data established the importance of studying PM2.5. However, that does not necessarily make the DTT assay a good metric for oxidative stress. The Sioutas group recognized the necessity in showing that HO-1 expression and DTT reactivity in the assay are proportionate. This proportionality allows DTT to be used as a metric for oxidative stress.

Shown in **Figure 1.5**, redox activity in the DTT correlates linearly to HO-1 induction. This data shows that, in the presence of PM, the assay serves as a dependable way to measure the ability of PM to cause oxidative stress. Thus, the DTT assay is the dominant PM analysis method, because it is capable of accurately mirroring oxidative activity in biological systems.

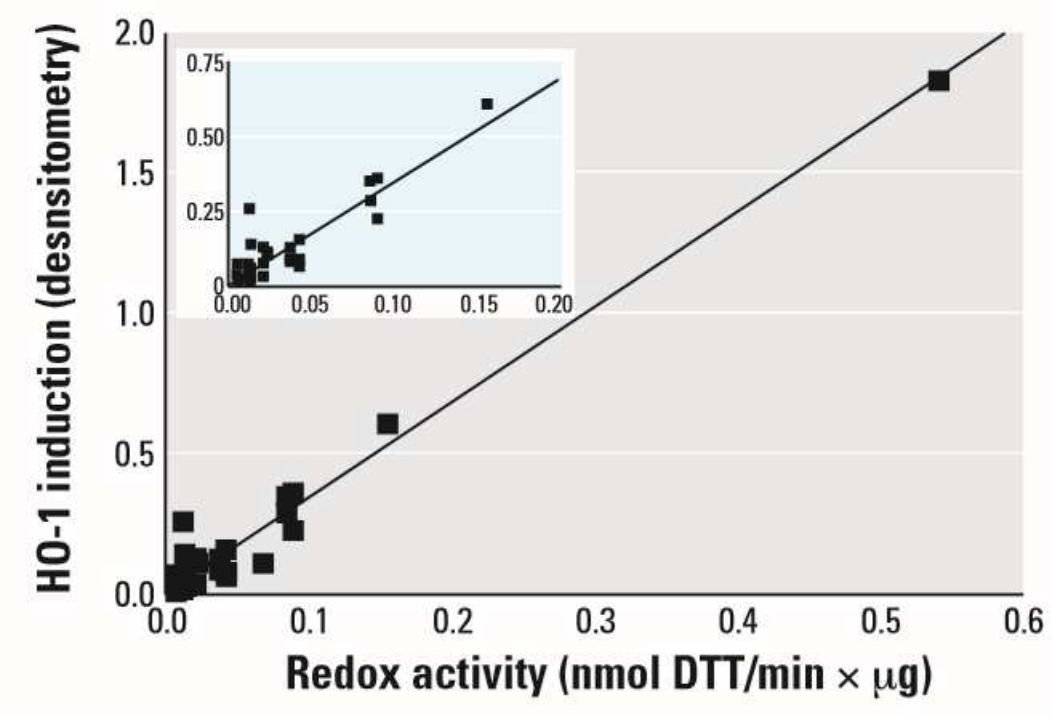

Figure 1.5 Regression analysis demonstrating positive correlation between redox activity in DTT assay and H0-1 induction (R^2=0.97). [3]

By testing different particle sizes in the assay, it was determined that PM<2.5 has the highest activity in the DTT assay (shown in **Figure 1.6**), directly correlating small particle sizes to higher potential for oxidative stress and high organic carbon content. A supplemental set of experiments treating cells with collected PM over the period of 16 hours were conducted to investigate the propensity of direct cell damage incurred by PM of various sizes. The resulting images are shown below. [3]

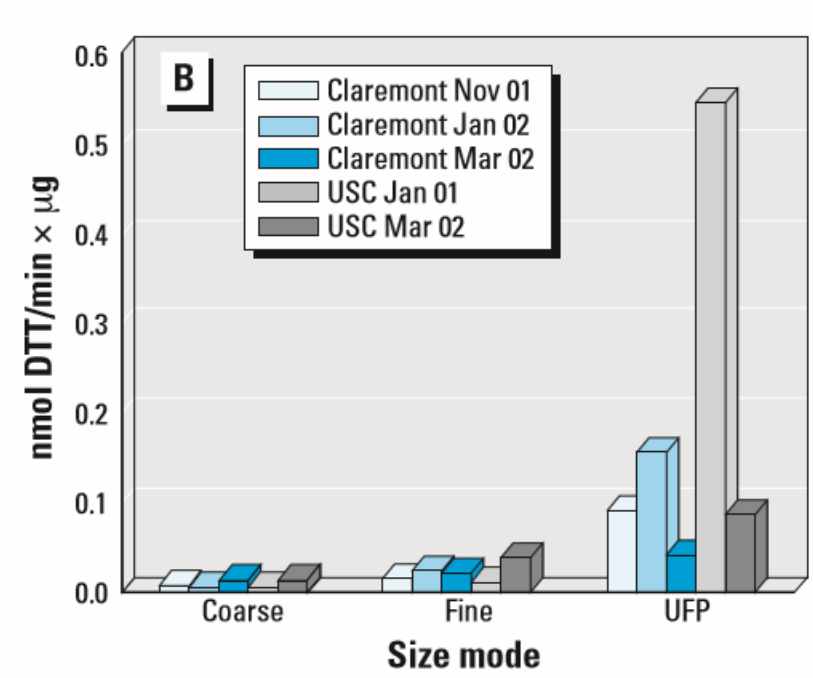

Figure 1.6 In vitro electron transfer capacity based off of a colorimetric DTT assay. Affirming other results, particles of 2.5 µm or less in diameter induce the greatest reactivity in the DTT assay. [3]

Demonstrated in **Figure 1.7,** small particle size increases the likelihood that PM<2.5 will infiltrate living tissues, penetrate cell walls, and damage mitochondria. This damage can be seen in the figure in E, F, G, and H, where PM was found within destroyed mitochondria after 16 hours of exposure. Because PM uptake into biological systems was established to cause damage by oxidative stress, PM penetration in organelles was concluded by the Sioutas group as a crucial step toward that damage. PM-induced destruction of mitochondria, the powerhouse of the cell, therefore contributes to oxidative stress within cells, leading to adverse health effects. This work highlights the need to rapidly and accurately analyze oxidative stress risk from PM exposures.

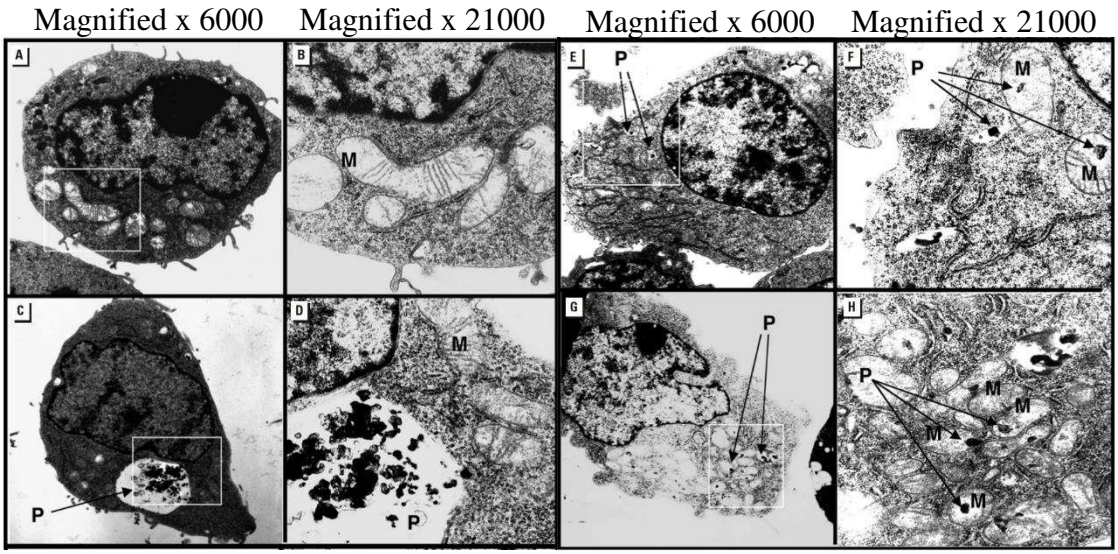

Figure 1.7. Electron micrographs showing effects of different particle sizes in cells treated with PM for 16 hours. (**A**) and (**B**) are untreated cells. (**C**) and (**D**) were exposed to large particles. (**E**) and (**F**) were exposed to fine PM2.5. (**G**) and (**H**) were exposed to ultrafine PM<2.5. (**P**) indicates particle locations and (**M**) indicates the location of the mitochondria. [3]

Similar to the Sioutas group, the Anastasio group in California has recently studied the application of the DTT assay in order to profile the reaction rates between DTT and standard oxidants including a formidable series of quinones and, more uniquely, soluble transition metals [5].

Early investigation of the DTT assay by the Sioutas group suggested that metals did not increase the rate of DTT oxidation in the assay, but this conclusion lacked strong evidence. Work from the Anastasio group has specifically focused on the fact that though correlation exists between levels of carbon-heavy species and DTT loss in the assay, correlation is not causation, particularly since particulate species can often be covariate. In addition to this, PAH's are not redox active, suggesting that their correlation to DTT consumption in the assay may have more to do with reactions between the PAH's and quinones found in DEP samples. Furthering Anastasio's point, there are also quinones known to *not* have the ability to oxidize DTT (i.e. 1,4-benzoquinone). [5,32] Additionally, other studies have suggested covariance between metals and carbonaceous redox-active species may exist. [32] Despite the fact that recent studies have indicated that DTT response in the assay is dominated by organic species rather than by metals, concrete conclusions on the reactive contributions of metals have been historically muddled enough to provide an opportunity for the Anastasio group to provide clarification. Thus, work done by the Anastasio group has been helpful for highlighting the benefit in chelating phosphate buffers used in the DTT assay, as well as identifying the individual reactivities of five quinones, three PAH's, and twelve transition metals. These reactivities, summarized below, provided a point of validation for the electrochemical assay.

Figures 1.8 and **1.9** show thorough analyses of quinones, PAH's, and metals known to be in common sources of PM2.5. Among these, the most reactive are PQN, 1,2-Naphthoquinone (1,2-NQN), Cu(II), and Mn(II). Data also showed that PAH's do not independently show any reactivity with DTT. In **Figure 1.9,** there are linear and non-linear ranges of reactivity within the DTT assay. Of primary importance in developing and applying the electrochemical DTT assay, quinone reaction rates tend to increase linearly with increased quinone concentration, but it is not clear if

this trend continues at extremely high concentrations. The strongest metallic reaction rates, however, increase according to a partial reaction order. [5]

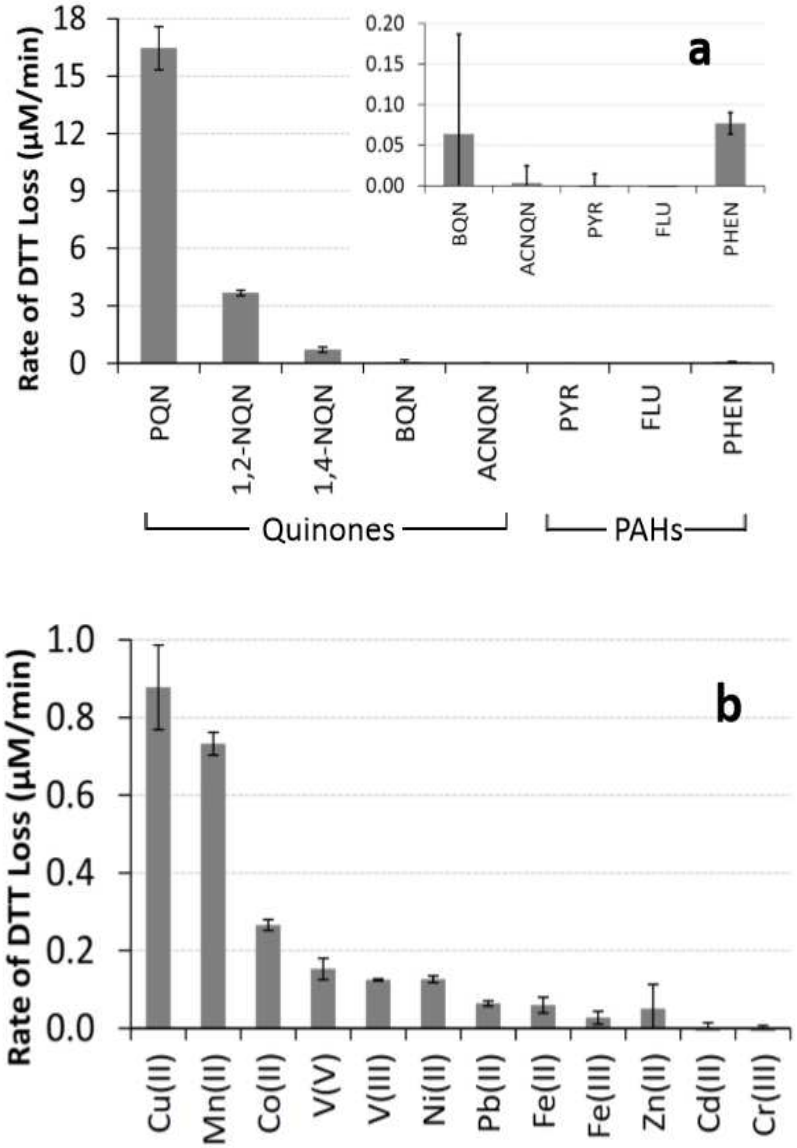

Figure 1.8.Blank corrected DTT loss rates from five quinones and three PAH's (**A**) and 12 transition metals (**B**) tested by the Anastasio group. The concentration of each species was 1 uM, except for 0.93 uM BQN, 1.21 uM NQN, and 5 uM Cd(II). 100uM DTT used in each case. [5]

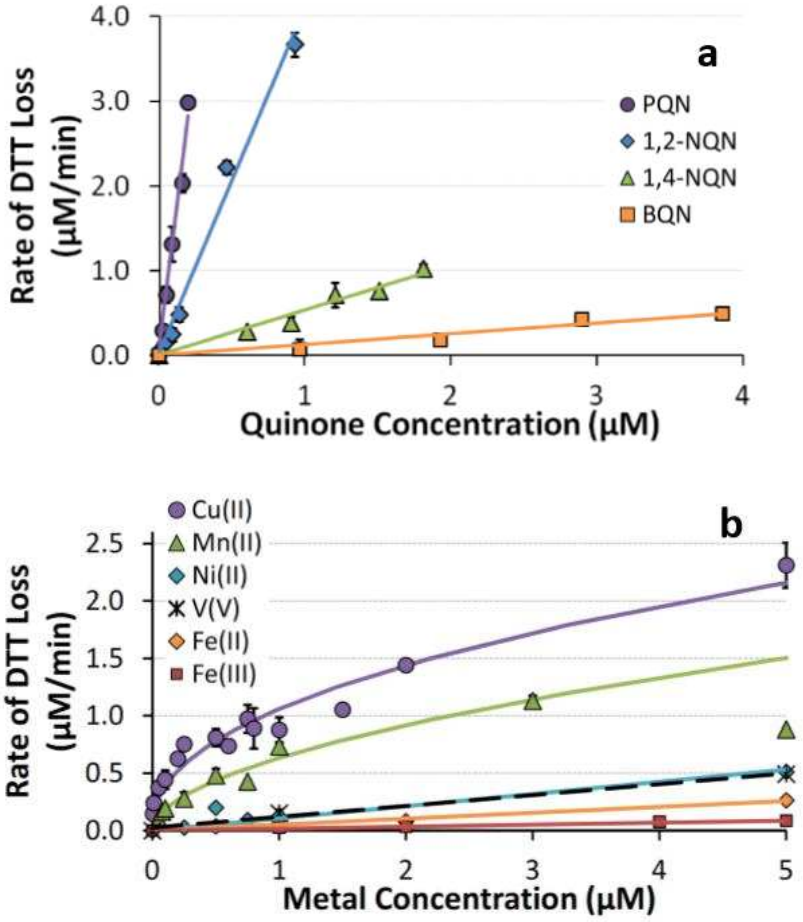

Figure 1.9. Blank corrected DTT loss rates as a function of oxidant concentration. Where quinone reaction rates are decidedly linear, the same is not necessarily the case for metals at higher concentrations. 100uM DTT used in each case. [5]

The traditional DTT assay, however, has its limitations. It relies on the addition of quenching and developing agents in order to be analyzed via absorbance spectroscopy, which typically results in sample dilution, a higher detection limit, and larger standard deviation. [2] In addition, the traditional assay focuses on the kinetics of DTT consumption *over time*, usually for 45 minutes or longer. In practice, this approach can limit experimental capacity to approximately one or two samples within the span of an hour (not in triplicate), severely limiting its ability to handle large sample sizes. Additionally, the ability to gain robust statistics is limited. In a recent study, the assay was automated, handling 500 samples without stopping. [39] Although the study solved capacity and contamination risk, the assay could only maintain a temporal capacity of one sample per hour. [39] If working through standard reactions or small sample sizes, temporal capacity is not a limitation. However, pollution and human health studies tend to employ a large scope and produce large sample sizes (>100 samples). Often, these larger sample sizes are entirely necessary for the fortification of statistical analyses and for providing a more detailed understanding of any given source or area of exposure. A sample size of 100 would require between 100 and 200 hours, at least, of simply processing the samples through the traditional assay. Adjusting for DTT's short shelf life in phosphate buffer, [2,3,5] requiring preparation and calibration each time that samples are evaluated, the traditional assay (and whomever performs it) has a strong likelihood of being overwhelmed. Furthermore, the current paradigm shift in detection-focused analytical chemistry has more recently focused on the development of point-of-care or field-based detection. An assay requiring the use of a spectrophotometer and several additional chemical compounds is an obstacle to this paradigm shift.

Thus, a more efficient assay should have the ability to function without additional chemical agents, which removes a risk for contamination and/or lack of reproducibility. An improved assay

should also cultivate the ability to run samples in a batch setting over a shorter period of time, which increases sampling rate. In addition, an optimized protocol should approach the ability to perform precise, reproducible, and accurate field analyses. These goals shaped the development of the electrochemical DTT assay.

1.4 Considerations for the Electrochemical DTT Assay

The idea of using well-studied redox reactions, such as the ferri/ferrocyanide couple, to characterize the electrochemical response of a material is a mainstay in analytical electrochemistry, but the adaptation to use a specific redox reaction in order to characterize oxidative stress from PM2.5 is a relatively new concept. The electrochemical DTT assay was first proposed in 2012 by the Henry group at Colorado State University, but was found to be difficult to reproduce. [2] The traditional DTT assay can be modified such that flow injection chronoamperometry replaces absorbance spectroscopy as the chosen analytical technique.

Pictured below in **Figure 1.10**, reduced DTT is electrochemically active and oxidizes to its disulfide form in a one electron process, which catalyzes the loss of the second electron and avoids the formation of a dimer. Because of this, DTT oxidation is considered a one electron process. The assay reaction mixture is maintained at a ratio of 25 µL DTT: 975 µL 0.1M phosphate buffer (pH 7.4) with the addition of model oxidant or PM2.5. If reduced DTT is reacted with PM2.5 or a model oxidant, just as what has been done in the traditional assay, some of the starting DTT will oxidize, but some of the starting DTT will remain in its reduced form if the reaction is stopped before completion. This remaining reduced DTT can be detected by electrochemical oxidization via chronoamperometry as its oxidation produces a current. DTT oxidation occurs at the surface of an appropriate electrode (i.e. cobalt(II)phthalocyanine modified carbon (Co-PC)). The assay

reaction mixture is plug-flowed with phosphate buffer over the electrode via an impinging flow cell, producing peak currents over time.

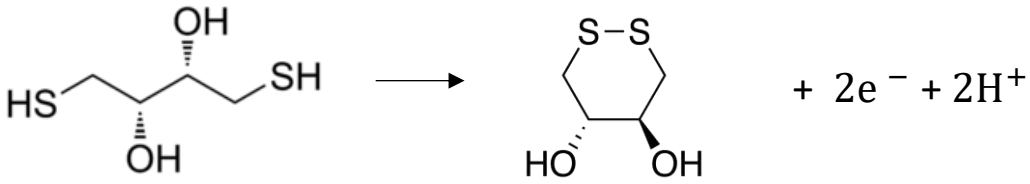

Figure 1.10. Oxidation reaction for DTT.

The first positive aspect of the assay is that, rather than quenching the reaction in order to isolate the remaining reduced DTT, the electrochemical assay relies on chilling the reaction to -2 °C to halt it.[2] Where the traditional assay struggles to process samples quickly, chilling the reaction means the electrochemical assay can process samples as a batch, *en masse*, introducing the potential for rapid field testing. However, the strength of the traditional assay rests in the fact that the status of DTT oxidation in the assay can be evaluated immediately. Chilling without chemical quenching may introduce the risk that, given enough time (i.e. several hours), the reaction mixture will continue to react slowly, or remaining DTT will degrade, causing the assay itself to lose accuracy. However, the ability to quickly process large quantities of field samples is a major advantage over the vast amount of time spent when using the traditional assay. But, the electrochemical assay is deceptively simple and has obstacles the traditional assay does not.

Absorbance spectroscopy is a *static* process, but flow injection amperometry, as its name would suggest, is not static, presenting a challenge. The traditional assay used reaction mixtures with a final volume of 1mL which were divided into three or four aliquots over the course of a 45 minute reaction. This volume can be adjusted to be much smaller in order to accommodate final reaction concentrations of real PM2.5 samples in the assay. The logistics associated with a flow

system, in practice, restrict the ability to cultivate robust statistics from aliquots less than 300µL, in an ideal case. This restriction is due to the fact that precision is measured by injecting an aliquot no less than three times through the assay (one injection in this work requires 70-90µL). If a final reaction volume is adjusted to under 300µL, there is a clear issue. Further non-ideality occurs if air bubbles make their way into the system, risking data point loss or wasted aliquots. To counter this, the electrochemical assay uses an end-time point reaction rate approximation. The end-time point is an arbitrary reaction time (typically 20-40 minutes). Thus, final volumes can range between 400µL and 1.5 mL and still be able to provide robust statistics. Statistics gained by the end-time point electrochemical assay can only focus on method precision and reproducibility whereas statistical error analysis for a longer reaction has the ability to estimate error in the reaction rate itself. To justify using an end-time point approach, both reaction methods were conducted when developing the electrochemical assay.

1.5. Development of the Electrochemical DTT Assay

The electrochemical and the traditional DTT assays are cousins. They follow the same general protocol with major differences appearing after the reaction is incubated. When designing the development of the assay, the following characteristics were focused on and are discussed herein:

1. Dilution effects from chemically quenching the reaction
    a. This was addressed by eliminating quenching and developing agents all together and cooling the reaction instead.
2. Obstacles to easy troubleshooting and mobility of the assay
    a. These were overcome by employing readily available, inexpensive materials. Specifically, a DropSens impinging flow cell and Co-PC SPE's were used.

3. Restricted sample capacity and temporal resolution
    a. This was addressed by validating the use of an end-time point reaction as opposed to a long-term kinetic reaction. Combined with the chilling technique, samples can be processed as a batch.
4. Lack of reactivity reproducibility
    a. Reproducibility of the electrochemical assay was never proven. Reproducibility, and precision by proxy, were studied in this work by repeating model experiments over time.
5. Results and detection limits comparable to results from Anastasio et al.

Rendering the assay inactive via temperature change was justified in the preliminary development of the electrochemical assay. [2] At different oxidant concentrations, if the assay is kept cold at -2 °C, no reaction occurs and no change in chronoamperometric current is observed, demonstrated in **Figure 1.11**, below.

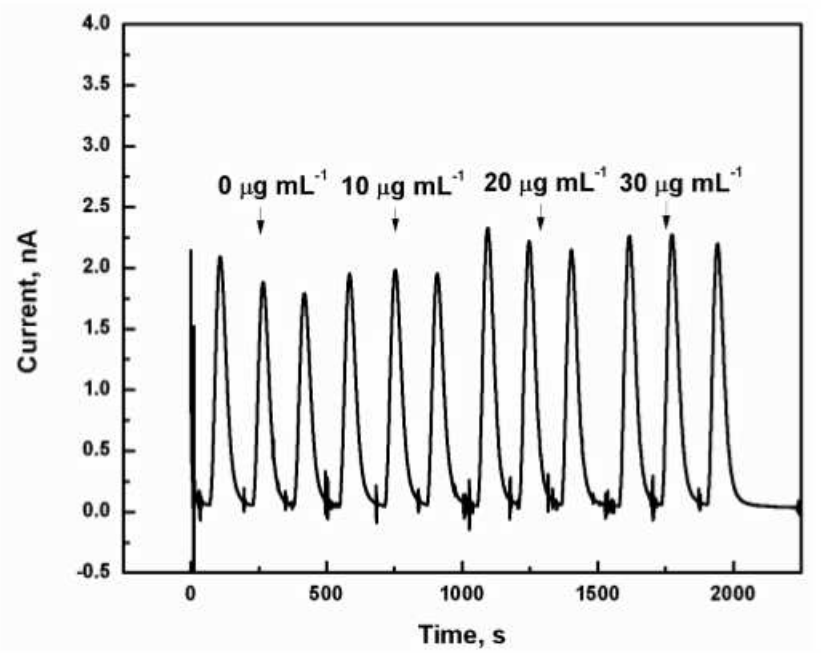

Figure 1.11. Chronoamperometric data showing a lack of assay reactivity, even with increased oxidant concentration, after 20 minutes at -2 °C. [2]

Where the traditional assay produces a kinetic reaction curve, taking multiple data points over time, the electrochemical assay employs a batch approach and produces a single end point reaction curve, taking multiple data points at the start and the end of the reaction. Both assays will use this information to produce a measurement of DTT loss over time, which is referred to as the reaction rate of DTT loss in the assay. If reaction rate is normalized to parameters such as collection rate, PM2.5 mass loading, or whole filter particle rate, reactivity of the oxidant/sample is reported, instead.

When developing the assay protocol, calibration curves are generated to provide a reference peak current as a proxy for DTT concentration. These curves come from the electrochemical response of DTT's oxidation by a manufactured Co-PC modified carbon SPE (DropSens 410). Based off the linear sweep and hydrodynamic voltammogram (HDV) in **Figure 1.12**, +0.3V vs Ag/AgCl was chosen as an appropriate redox potential for DTT.

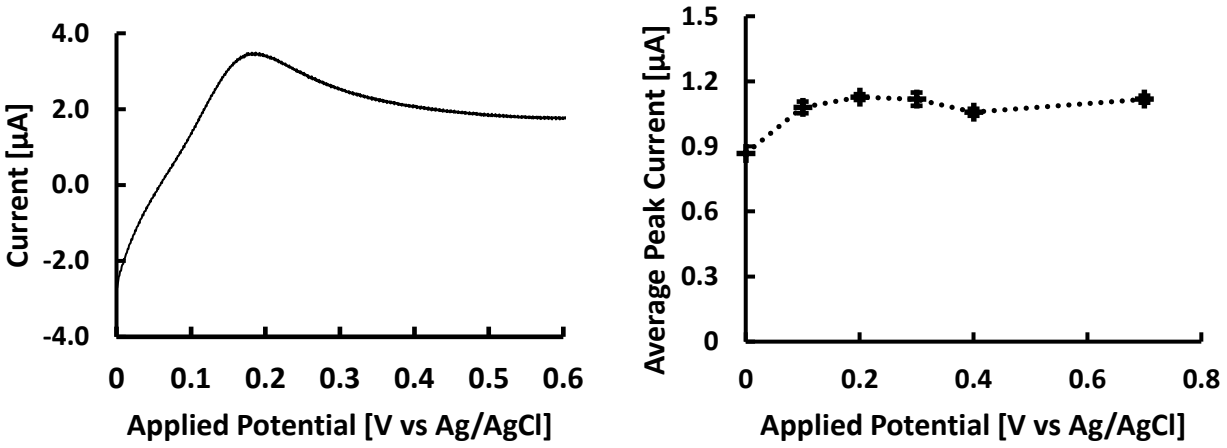

Figure 1.12. Linear Sweep Voltammogram and Hydrodynamic Voltammogram of 100 µM DTT taken on Co-PC carbon DropSens electrode. Redox potential shows where expected, close to +0.2 and +0.3 V vs Ag/AgCl.

While phosphate buffer is constantly flowed through a wall jet flow cell, impinging onto an electrode, plug flow injections of the analyte solution are sent through, creating injection currents over time. Flow injection chronoamperograms, shown below, provide the necessary data. The peak currents from the chronoamperograms directly correlate to concentration and were used to develop a DTT calibration. Injections through the flow system were staggered in order to avoid residual effects when switching between DTT concentrations.

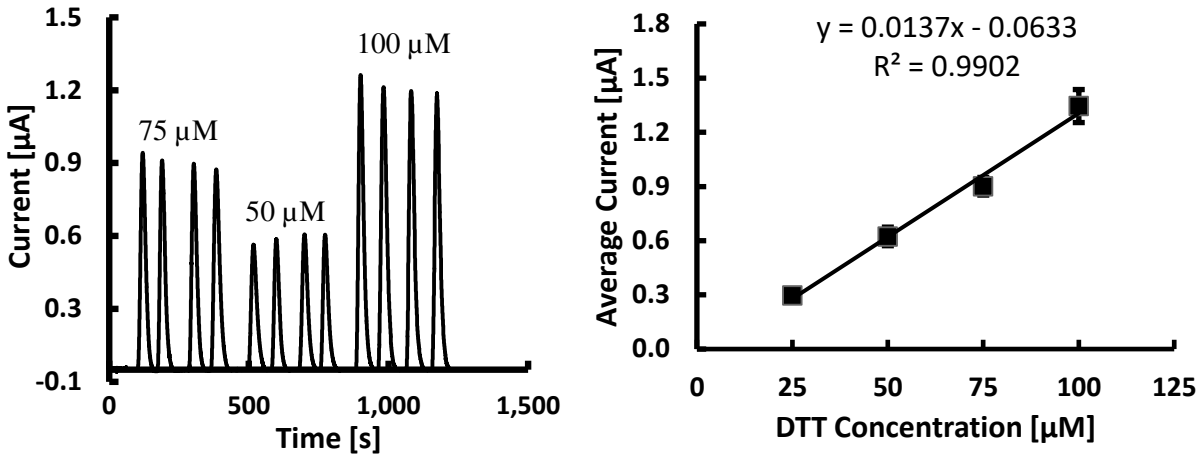

Figure 1.13. Chronoamperograms (L) from a calibration curve and summary data in a calibration curve representing over 20 different curves taken over a month (R). Slope error for the calibration curve, calculated using y-residuals is 6.2E-04 µA/µM. LOD = 8.45 µM DTT.

The data in the calibration figure above is the summary of approximately 20 different calibration curves taken over the course of one month. Also, chronoamperometry data does not have a consistent or strong bias, indicating the data is likely Gaussian; a linear calibration curve can be generated from calibration averages. The x-intercept of the calibration curve is 4.6 µM DTT, and though there is very little noise above the nano-amp range, using y-residuals yields a LOD of 8.45 µM (or 8.45 nmol) DTT. Error bars in the calibration curve come from standard deviations between injection peak currents across all experiments. Using standard NIST reproducibility standard deviation algorithms,[40] the calibration curve has a long-term average reproducibility of 94.54%, indicating excellent precision.

Once a calibration curve is established, model oxidants are analyzed in the electrochemical assay to validate the data against that reported for the absorbance assay. DTT consumption in chelated buffer is tracked over time (chelation, as established by the Anastasio group, is used in an attempt to remove trace metals in the buffer) [5] as a reaction blank. 1,4-NQN and Cu(II) are used as a model oxidants. To establish single time point capability for linear reactions, two aliquots of

the same reaction mixture of 75µM DTT and 1µM 1,4 NQN were analyzed over time. The first of these was analyzed in the traditional way of taking aliquots from the incubating reaction mixture over 45 minutes. The second reaction mixture was cold quenched after 20 minutes incubation, allowing for a 5 minute cool down period for the reaction mixture to go from 37 °C to -2 °C, and was electrochemically analyzed after.

Shown in **Figure 1.14**, data from the shorter reaction curve, taken three weeks after the kinetic curve, agrees very well with its counterpart. This agreement strongly indicates precision and reproducibility even when using single time point analysis. Error in the reaction rate (slope) itself was determined to be 0.025 µM/min which is less than error reported for the absorbance assay data used in this work, which averages from 0.066 to 0.083 µM/min. A summary schematic of the assay is shown below in **Figure 1.15**.

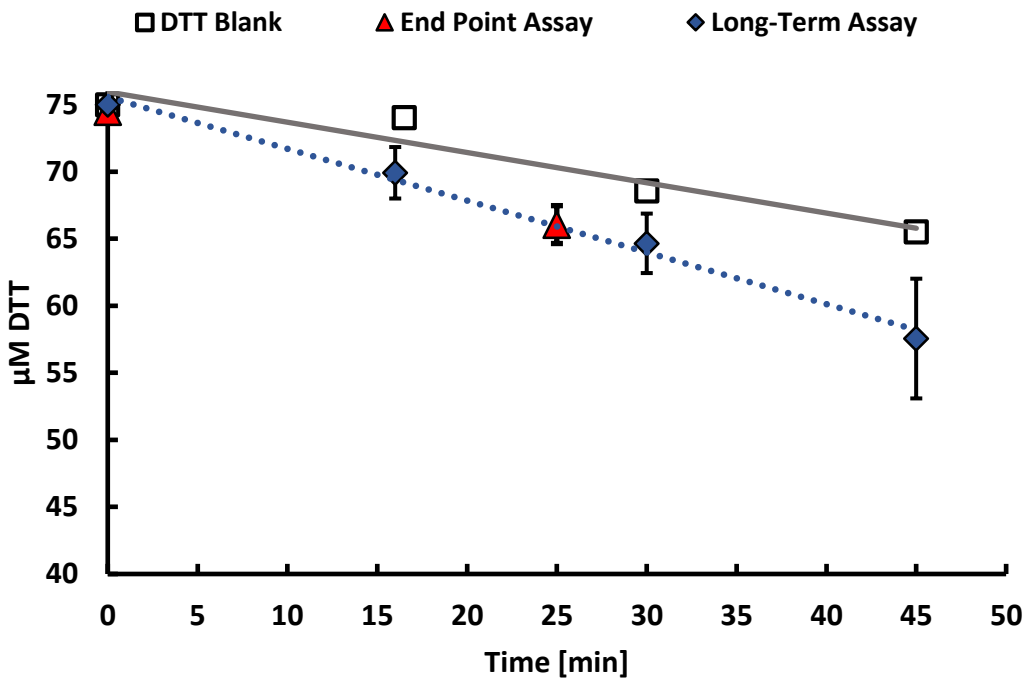

Figure 1.14.Reaction curves of a blank and 75uM DTT with 1uM 1,4-NQ . A long-term kinetic curve is shown overlaid by a short-term single time point reaction curve. Error on the kinetic curve reaction rate (slope) is 0.025 µM/min. Error bars are standard deviations from triplicate injections of a single sample.

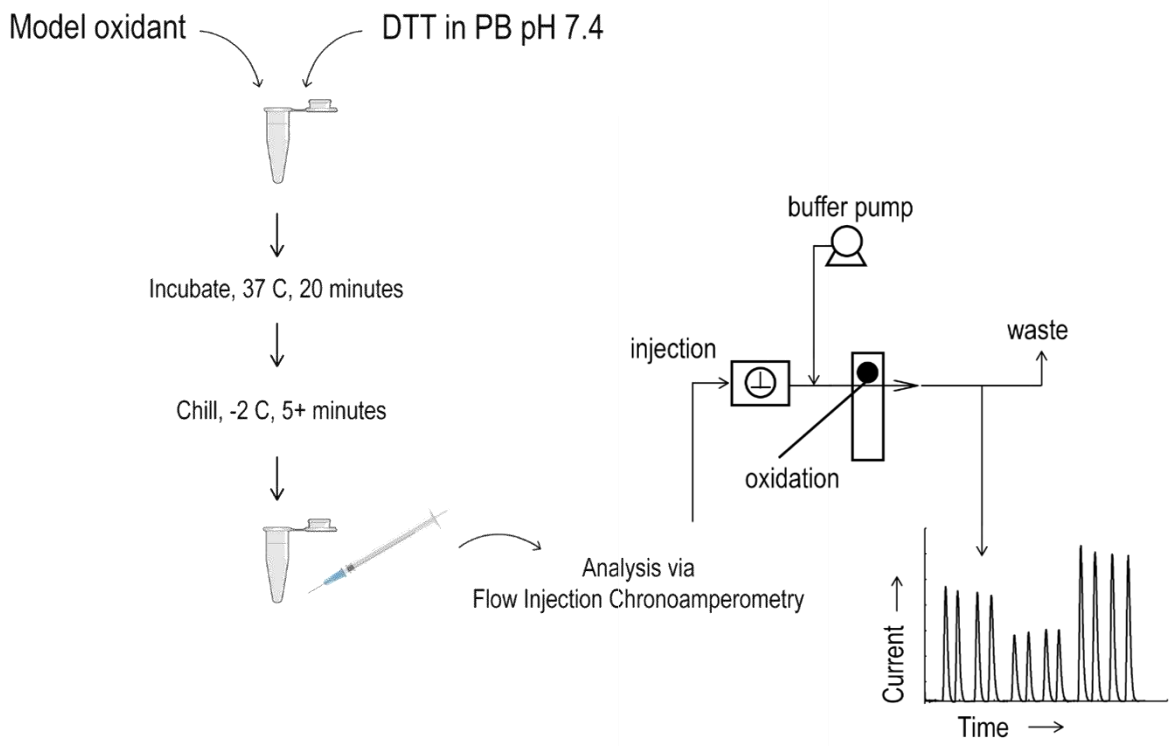

Figure 1.15. Schematic of electrochemical assay protocol.

The establishment of the viability of an end-time point analysis allows protocol development to move forward to testing how assay reactivity changes as model oxidant concentration is increased. This change in reaction rate should be linear for quinones unless a threshold is passed such that DTT oxidation is so rapid that additonal oxidant makes little difference. For metals, however, this is not the case and it is likely that the reaction mechanism is more complicated and the reaction itself is a partial order. The reaction data is seen in **Figure 1.16** and **Figure 1.17**.

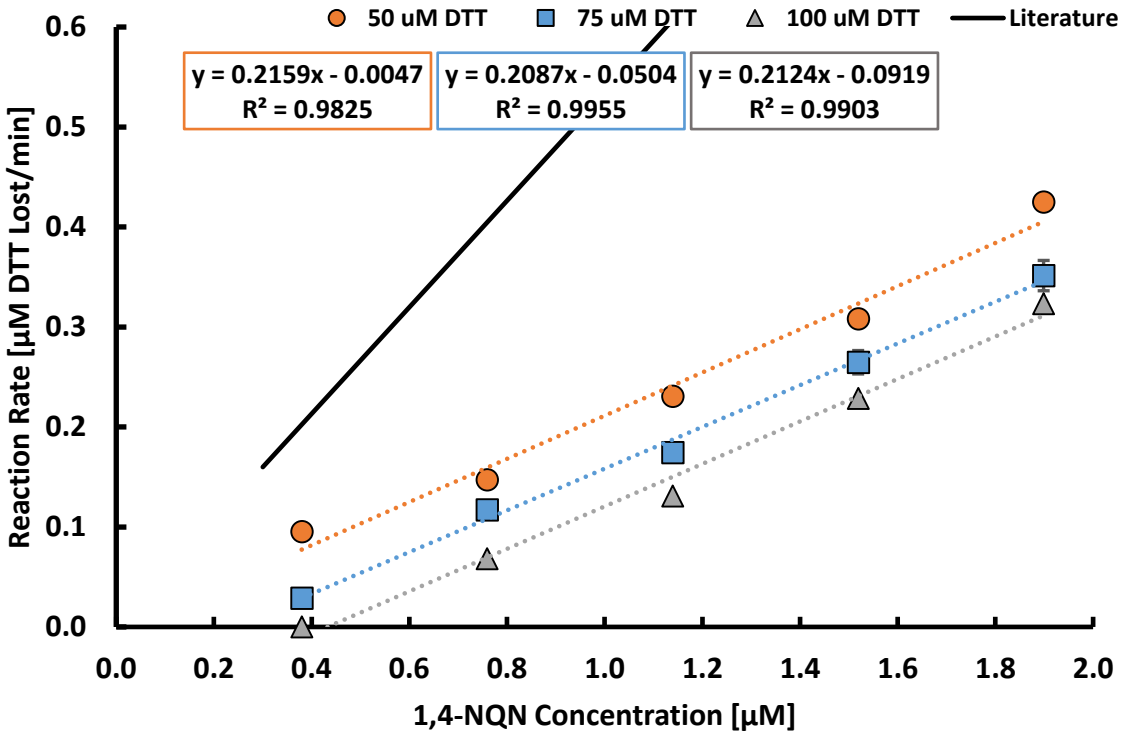

Figure 1.16. Blank-corrected reactivity data; summary over the course of a month (n=6 for each 50µM DTT point, n=9 for 75µM DTT, n=3 for 100µM). LOD, calculated using y-residuals, is 0.4 µM 1,4 NQN. Reaction rate LOD is 0.11 nmol DTT lost/min. Average reproducibility is 94.74%.

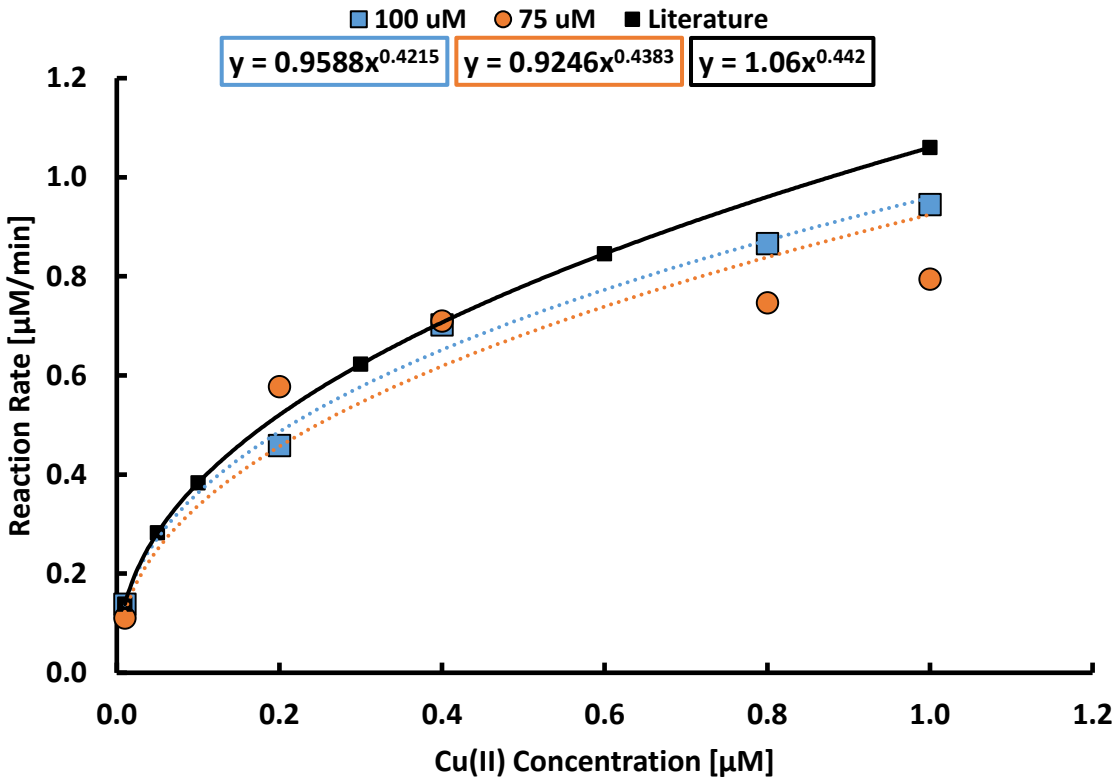

Figure 1.17. Blank-corrected reactivity data for Cu(II). LOD is 0.1µM Cu(II) (n=3). The reactivity of Cu(II) in the assay has a partial reaction order of approximately 0.4.

Reporting the oxidant results in this way shows that increased quinone concentration in the assay will cause a linear increase in reactivity, reflecting similar conclusions as works using the traditional assay, though the reaction rates are not what has been reported in the past. [2,5] The data also suggests that the reaction rate between DTT and quinones is pseudo-first order, echoing early results from the Kumagai study. [32] The DTT + quinone reaction is likely pseudo-first order, because reactivity (change in reaction rate) depends on one reagent more than the other. In this

case, the dominant reagent is the quinone. For the metals, however, reaction rates reported in literature are fit to a power model and the assay aligns well with the previously established rate. This behavior indicates the DTT + metal reaction mechanism likely depends on concentrations of both reagents, suggesting a possible second order reaction.

The final step in developing the electrochemical assay is directly correlating its data against that of its predecessor, the traditional absorbance assay, using identical PM2.5 samples. Both methods were employed in evaluating PM2.5 samples collected in Fresno, CA, providing the best opportunity to simultaneously test the application of the electrochemical assay and its accuracy relative to the absorbance assay. Data from the two assays was compared using a Deming regression and variance comparison.

The Deming regression in **Figure 1.18** shows good agreement between the two assays when processing PM2.5 samples, where higher variability is expected. The general trend suggests the electrochemical assay may underestimate reactivity relative to the absorbance assay. Error bars reflect variance in precision for both methods. Variance represented in those error bars is compared by an f-test, shown in **Figure 1.19.** The f values were calculated by comparing sample variances between the absorbance and electrochemical methods. F values are far enough above 1.0 to indicate that variance in the absorbance assay is characteristically greater than in the electrochemical assay. High variance in the absorbance assay could also indicate that the absorbance assay is more sensitive to sample materials, such as Pallflex filters, which were not filtered out of the assay. Thus, in both a model setting and in an application setting, the electrochemical assay is an excellent alternative to the absorbance assay. A summary of data obtained in the assay development is in the table, below.

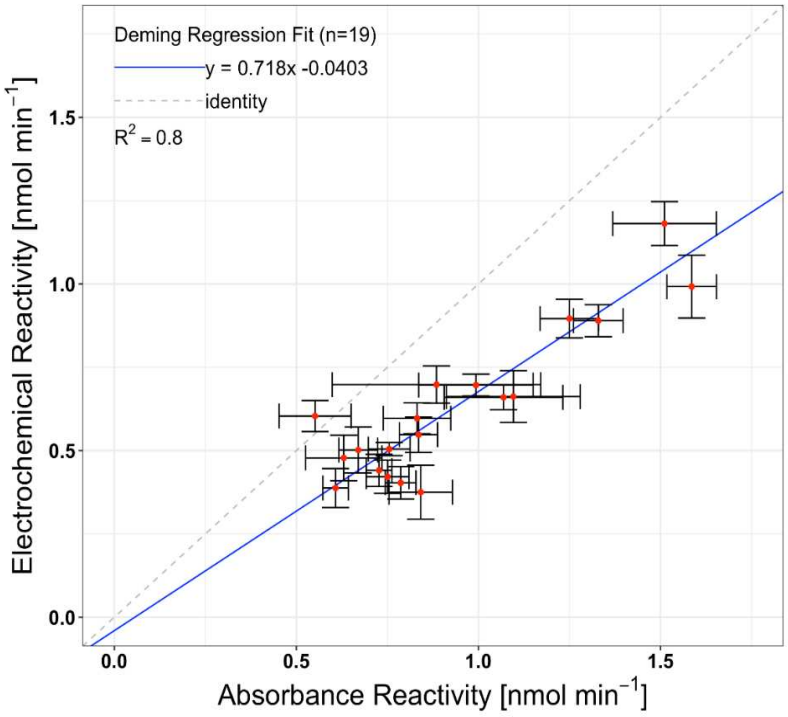

Figure 1.19. Deming regression of absorbance and electrochemical assay data for same PM2.5 samples collected in Fresno, CA in 2016. Error bars for the electrochemical assay are standard deviations from five measurements from one sample, representing precision.

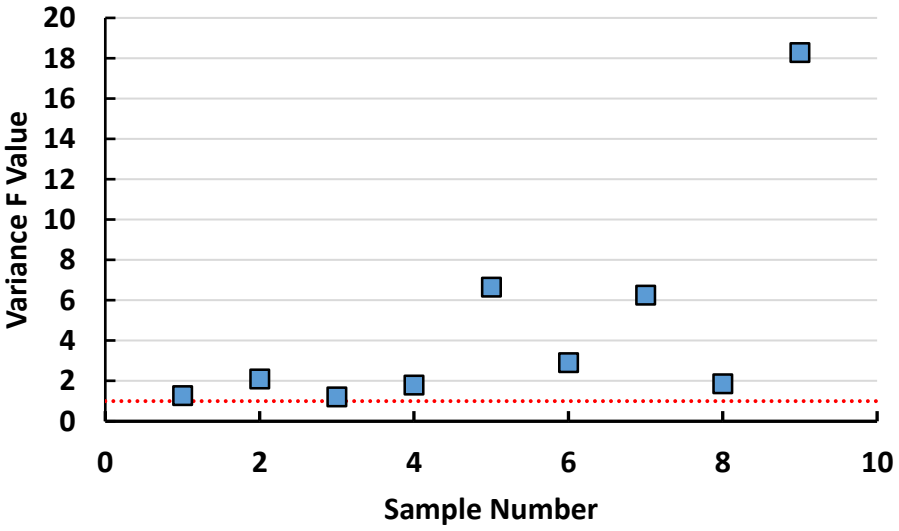

Figure 1.18. F values for 9 Fresno samples. F is taken as absorbance variance over electrochemical variance, as it is typically larger. Variances much larger than 1.0 indicate, here, that one assay has a reduced variability versus the other.

Table 1.2. Regression equations and detection limits for DTT loss from correlated assay data from the Anastasio study [a]

Species	Range of Concentrations (μM)	Regression Equation	N	LOD	Reproducibility
Calibration	0-100	$y = 0.0137x - 0.06$	>20	8.44 nmol	94.54%
1,4-NQN [b]	1.0	$y = 0.386x + 74.33$	1	0.11nmol/min	n/a
1,4-NQN	0-2.0	$y = 0.2124x - 0.09$	3	63.2 ng/m^3	94.74%
1,4-NQN [c]	0.5-1.5	$y = 0.533x$	6	83 ng/m^3 [d]	-----
Cu(II)	0-1.0	$y = 0.958x^{0.421}$	3	12.6 ng/m^3	n/a
Cu(II) [c]	0.005-5	$y = 1.06x^{0.442}$	14	0.32 ng/m^3 [d]	-----

[a] For each equation, y represents loss in DTT at a rate of μM DTT/min and x is the concentration of metal or quinone in the assay. All reactivity rates are based on 100 μM DTT.
[b] This data is a single reaction rate of 75 μM DTT versus 1.0 μM 1,4-NQN.
[c] The regression data reported by Anastasio [5]
[d] This limit comes from the lowest reported concentration levels from the Anastasio study [5]

1.6 Conclusions

An electrochemical DTT assay has been developed with a focus on eliminating dilution effects, reproducibility, using readily available components, batch processing, and accuracy relative to the absorbance assay. Cold quenching the assay was used to avoid diluting the reaction with quenching agents, improving precision and risk of contamination. Manufactured components like electrodes, flow cells, and syringe pumps contribute to easier trouble shooting and can help make the assay mobile. They also help to contribute to precision as the variability in homemade electrodes and flow cells is avoided entirely. The next step in development investigated whether or not the assay can be shortened to a single time point. The combination of cold quenching and shortening the assay reaction means that samples can be processed in batches, rather than one at a

time, making the electrochemical assay the fastest reported method. Finally, assay reactivities were correlated using standard oxidants and real samples with good agreement between the absorbance and electrochemical assays. These results establish the electrochemical DTT assay as a viable, robust alternative to the traditional assay.

For future consideration, the DTT assay is often used in conjunction with other, more precise methods, such as ICP-MS, which can help to reveal metal compositions in pollution samples, including metals not highly reactive in the DTT assay, like Cd(II), Fe(III), and Zn(II). [5] Due to the facts that the electrochemical assay is flow based, that DTT oxidation is catalytic, and that not all PM components in air pollution react with DTT, flowing waste from the assay could be linked to other analytical tests which may illuminate the full nature of collected PM.

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CHAPTER 2. ANALYSIS OF PM2.5 IN HONDURAS COOKSTOVE SAMPLES USING THE ELECTROCHEMICAL DTT ASSAY

2.1 Chapter Overview

The electrochemical DTT assay is presented here as a robust, precise analytical method for measuring the oxidative reactivity of PM2.5 samples collected in Honduras. This study may be incorporated into the Honduras Cookstove Project, whose goal is to track domestic exposures during the transition from inefficient wood-fired cook stoves to clean-burning cook stoves. The aim is to coordinate reactivity data to biological data obtained from study subjects and determine if health can be directly correlated to domestic PM2.5 exposure. Over 200 samples were tested with one half representing personal exposures and one half representing area exposures. The electrochemical DTT assay utilized readily available components and batch processing in order to process the samples in a short period of time.

Linear reaction rates were investigated for the samples so that end-time point analysis could be used with the DTT assay, enabling rapid batch processing. Next, linear reactivity rates with increasing PM2.5 reaction concentrations in assay samples were established. Results from these experiments allow for streamlined preparation. Personal exposures showed relatively high reactivity with an average of 12.6 nmol DTT lost per minute with a maximum reactivity of 39.8 nmol DTT lost per minute and a minimum reactivity of 2.2 nmol DTT lost per minute. Area exposure reactivity was almost double at an average of 20.3 nmol DTT lost per minute with a maximum of 68.8 nmol DTT per minute and a minimum of 0.9 nmol DTT per minute. Because PM in this study was collected based on size (<2.5 μM) and not on composition, there does not appear to be a correlation between whole filter particle weight (total mass of particles collected

per sample) and reactivity, but this may be due to a limited range of collected particle weights. Results from this study suggest that study subjects were exposed to varied, multivariate chemical mixtures of carbonaceous and metallic compounds. Kevin Klunder, Luna Martinez, and Casey Quinn contributed data correlation and analyses for this work.

2.2 Introduction

Rapidly and accurately evaluating oxidative reactivity levels in aerosol particulates has been an area of interest for the last 15 years. Most publications in that time have focused on characterizing dithiol oxidation catalyzed by compounds often found in air pollution (i.e. quinoids, polycyclic aromatic hydrocarbons, and metals). Dithiol oxidation can mirror oxidative stress in cell tissue and has been studied using a well-studied absorbance DTT assay. [1–3] It is believed that oxidative stress is the precursor to respitory diseases and premature death. [4–7] The absorbance assay relies on chemical quenching and long reaction times, which can contribute to a risk of inaccuracy, lack of repeatability, and slow sample processing. To address these issues, an electrochemical assay has been developed, as was discussed in the previous chapter.

The electrochemical assay utilizes flow injection chronoamperometry, measuring current as a function of time while passing analyte injections over an electrode surface. [8] Peak currents generated reflect dithiol oxidation in dithiothreitol (DTT), the main compound in the assay, used as a metric for reactivity in the assay. Academic works centered on electrochemistry typically use homemade carbon paste, carbon screen print, or even BDD powder electrodes, but characterizing and normalizing these electrodes for large sample sizes is problematic and inconsistent. Discussed in greater depth in the previous chapter, the electrochemical assay counters this concern by using readily available, inexpensive electrodes and flow cell. The electrochemical assay is temporally

efficient, capable of handling formidable sample sizes, and more precise than the traditional assay while maintaining relative accuracy.

The electrochemical DTT assay was used to analyze the oxidative capacity in 220 personal and area exposure samples from various households in Honduras. The overarching project is focused in Honduras and is specifically concerned with the use of inefficient cook stoves. Although regulations for cleaner air and water have been present in developed countries, this is not always the case in developing countries. This does, however, present a helpful opportunity to investigate 1) if inefficient cook stoves can contribute to negative health and 2) whether or not negative health effects can be correlated to collected pollution samples from these cook stoves.

2.3 Experimental

Materials and equipment

In this study, manufactured carbon screen printed electrodes modified with cobalt phthalocyanine, with Ag/AgCl reference and a coordinating clam shell wall jet flow cell (DropSens 410 SPE, Metrohm USA) were used. Solid DTT powder and trifluoroethanol (TFE) were purchased from Sigma Aldrich, sodium phosphate dibasic anhydrous and sodium phosphate monobasic hydrous were purchased from Fischer Chemical. Chelex 100 mesh was purchased from Sigma Aldrich and buffer was chelated appropriately. [3] All reagents were used as received. DTT was prepared at 75 μM for all samples. Electrochemical measurements were conducted using a CH Instruments Potentiostat (model number 812). Samples from Honduras were received frozen, sorted, massed, and categorized from Dr. Maggie Clark and they remained frozen at -20 °C until processing. The PM2.5 samples were collected on Pallflex 25mm quartz filter papers. Filters were handled with plastic forceps and ceramic scissors to avoid metal contamination.

Electrochemical DTT Assay Protocol

A general schematic of the assay protocol is shown, below in **Figure 2.1**.

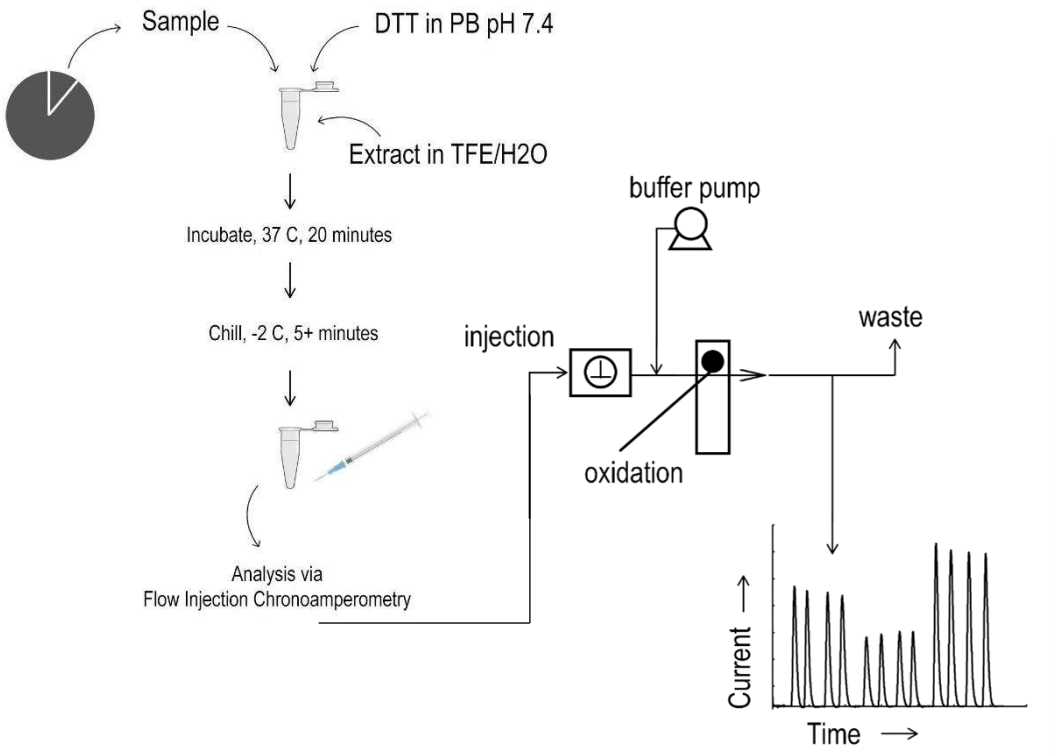

Figure 2.1 Schematic of the electrochemical assay protocol, including filter sample extraction. After incubation and cooling, remaining DTT in samples is oxidized and analyzed by flow injection chronoamperometry. Starting DTT concentration chosen as 75 μM.

To prepare filter samples for the DTT assay shown in **Figure 2.1**, sample mass after and before collection are needed. According to the equation below, these yield **whole filter particle weight (WFPW)** and **particle mass loading (PML)** for each sample:

$$\text{WFPW} = \text{mass after collection} - \text{mass before collection} \quad (1)$$

$$\text{PML} = \frac{\text{WFPW}}{\text{mass after collection}} \quad (2)$$

The mass loading value is integral in determining final PM2.5 **reaction concentration** the assay by the following equations:

$$\text{PM2.5 mass in reaction} = \text{filter piece mass} * \text{PML} \quad (3)$$

$$\text{reaction concentration} = \frac{\text{mass in reaction}}{\text{reaction volume}} \quad (4)$$

Filter samples are cut into small pieces, in triplicate, and massed. If a filter sample is recorded to have a **PML** of 12µg/mg, a cut weighing 1mg will yield a filter piece with 12µg of particle mass on it. The desired **reaction concentration** would be, say, 15µg/mL with a **reaction volume** of 800µL of 75µM DTT. When processing filters for the electrochemical DTT assay, it is best to stay between 0.5 and 1 mL final volume. Other literature recommends aiming for assay reaction concentrations as close to 10µg/mL as possible, but this is debatable. [3] If the relationship between sample reaction concentration and reactivity is linear, the reaction concentration is arbitrary.

Once prepared, filter pieces were placed into separate microcentrifuge tubes and, unless being processed right away, were stored in a -20 °C freezer. DTT concentrations were prepared, the system was calibrated, and microcentrifuge tubes were filled with the DTT and phosphate buffer reaction mixture, sans sample, for tracking blank decay rates in the assay. Samples were incubated at 37 °C for 30 minutes. Incubation time is arbitrary, but was long enough to allow sample reaction rates to be distinguishable. After incubation, samples were placed in a -2 °C fridge or refrozen until they could be processed. Lab blanks (blank filters cut in the lab, but never transported to Honduras) were extracted and processed in the same manner as the samples and used for every batch of processed filters. For analysis, samples were intermittently injected in to a laminar flow of buffer (85 µL/min), through a wall jet flow cell, and over an electrode, producing

peak currents over time (+0.3V vs Ag/AgCl). Lab blanks were used frequently to track DTT consumption as a result of filter materials present in the assay. Averaging 4 injections per sample, sample processing rates averaged 4 or 5 samples analyzed in an hour, in triplicate.

2.4 Results and Discussion

The electrochemical DTT assay evaluates PM2.5 reactivity, as a whole. The assay does not, however, provide a composition profile. It was expected that, at lower assay concentrations, assay reaction rates would be linear over time and that reactivity would correlate linearly to PM concentration. Linear reactivity rates are seen in the DTT assay when analyzing quinones and less reactive metals, like Fe(II). Additionally, because real PM samples are often made up of more than one compound, it was not expected that a strong correlation would exist between sample particle weight and sample reactivity. But, a positive correlation should be present, to some degree.

2.4.1 *Establishing Linear Reaction Rates*

To determine if reaction rates for the Honduran samples were linear, a random selection of samples were run in both long-term kinetic curves and a shortened end-time point curve, an example of which is shown below. This experiment was done to establish that the shorter, end-time point analysis is capable of accurately estimating sample reaction rates. End-time point analysis of sample reaction rate(s), applied to large sample sizes, allows for batch processing and more time efficient analyses.

Shown in **Figure 2.2**, end-time point analysis with real samples provides an excellent approximation of sample reaction rate. Error bars show standard deviations between peak currents, indicating reproducibility and precision. Validating the use of end-time point analysis allows samples to be processed through the assay rapidly. Once end-time point analysis is adopted,

incubation takes less time, samples can be incubated as a batch such that sample turn-over rates are n = 3-5 per hour, in triplicate. The use of end-time point analysis also allows for smaller reaction volumes, as samples are injected at the time of evaluation, rather than multiple times throughout the reaction.

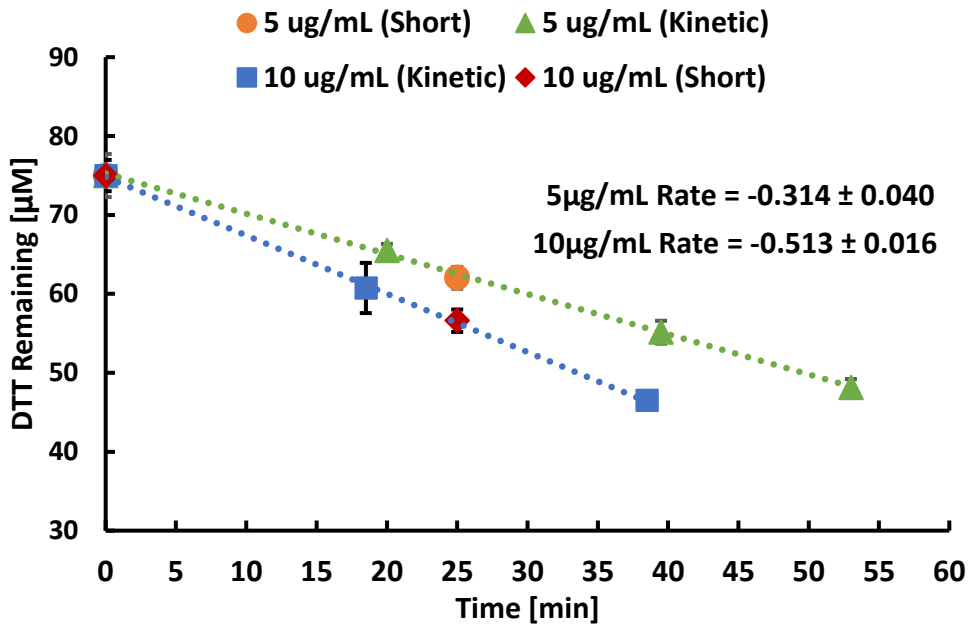

Figure 2.2 The reaction rates averaged from kinetic and end-time point curves are shown for two different reaction concentrations from the same Honduran filter. Two different concentrations for H004 are shown. Starting currents taken as the calibration value. Longer, kinetic reaction curves were done twice per concentration (n=6). 5µg/mL rate error = 0.040 µM/min; 10µg/mL rate error = 0.016 µM/min.

2.4.2 *Establishing Linear Reactivity*

To establish the behavior of sample reactivity rates (change in reaction rate as a function of oxidant concentration), filter pieces were cut from 30+ randomly selected samples, adjusted to different concentrations, processed, and a representative set are plotted below. If a linear reactivity rate is established, it indicates that a single reaction concentration can be chosen for a filter sample

and the reactivity can be estimated by dividing the resulting reaction rate by reaction concentration, as in the equation below.

$$\text{Reactivity} = \frac{\text{Reaction rate}}{\text{Reaction Concentration}} \quad (5)$$

Linear reactivity rates, seen in **Figure 2.3,** reveal that the Honduran samples are linear, with some deviation. Reactivity data also show a variety of rates (slopes), indicating that different filters may contain different particulate compounds in different compositions. The reactivity curve for F020, however, suggests that, though the reactivity itself can be reasonably estimated from a linear regression, a non-linear trend may appear at higher concentrations. Moving forward, to estimate reactivity, only one reaction concentration was chosen for each filter sample. Any possible correlation between WFPW and reactivity was also investigated by multiplying sample reactivities by the WFPW, producing a normalized reactivity. The results for both personal and area exposure samples are presented below.

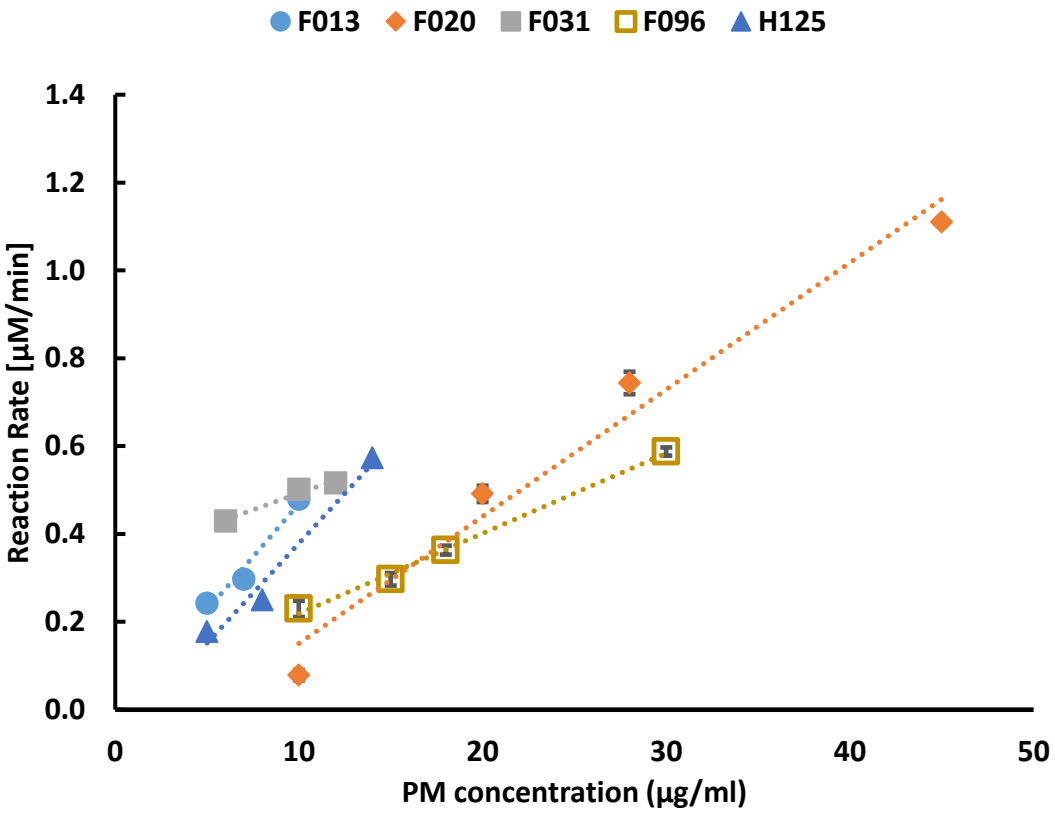

Figure 2.3 Five filters, chosen at random, were processed at multiple reaction concentrations, generating linear reactivity curves as a function of reaction concentration. Their linearities and reactivity errors $\frac{(\mu M\ mL)}{(min\ \mu g)}$ are as follows: F013 (R^2=0.9654; Err=0.002); F020 (R^2=0.9718; Err=0.003); F031 (R^2=0.9688; Err=0.003); F096 (R^2=0.9958: Err=0.001); H125 (R^2=0.9742; Err=0.007)

2.4.3 *Reactivity of all Samples*

Once linearity has been demonstrated for reaction rates and for reactivities, the rest of the Honduras sample reactivities, summarized below, were calculated using the following algorithm:

1. Filters were massed, final volume and concentration are calculated and archived (typically 1 to 2 times the mass loading) (equations 1-4).
2. DTT loss over time was calculated by subtracting the final peak current of the sample from the starting point (taken to be the pre-incubation peak current of a lab blank). This loss was

divided by incubation and cooling time (in this study, 35 minutes) to generate the reaction rate. Units are reported in μM/min.

$$\text{Reaction rate} = \frac{I_p(t_0) - I_p(t_f)}{\text{incubation time} + \text{cooling time}}$$

3. The reaction rate was divided by the final concentration, generating a reactivity rate. Reactivity rate was reported in units of (nmol DTT lost * μg)/min.

$$\text{Reactivity rate} = \frac{\text{reaction rate}}{\text{reaction concentration}}$$

4. Sample reactivity rate was multiplied by whole filter particle mass (mass reported in units of μg) and a plot was generated based on the resulting normalized reactivity rates. Normalized reactivity rates are reported in units of nmol DTT lost/min.

$$\text{Normalized Reactivity rate} = \text{Reactivity rate} * \text{WFPW}$$

Summarizing the normalized reactivity rates of the Honduran samples, two figures are presented below. Error bars are standard deviations between injections from three separate cuts from the same filter (n=9 per data point). Small error bars show that the electrochemical system is self-precise and that the protocol results are reproducible.

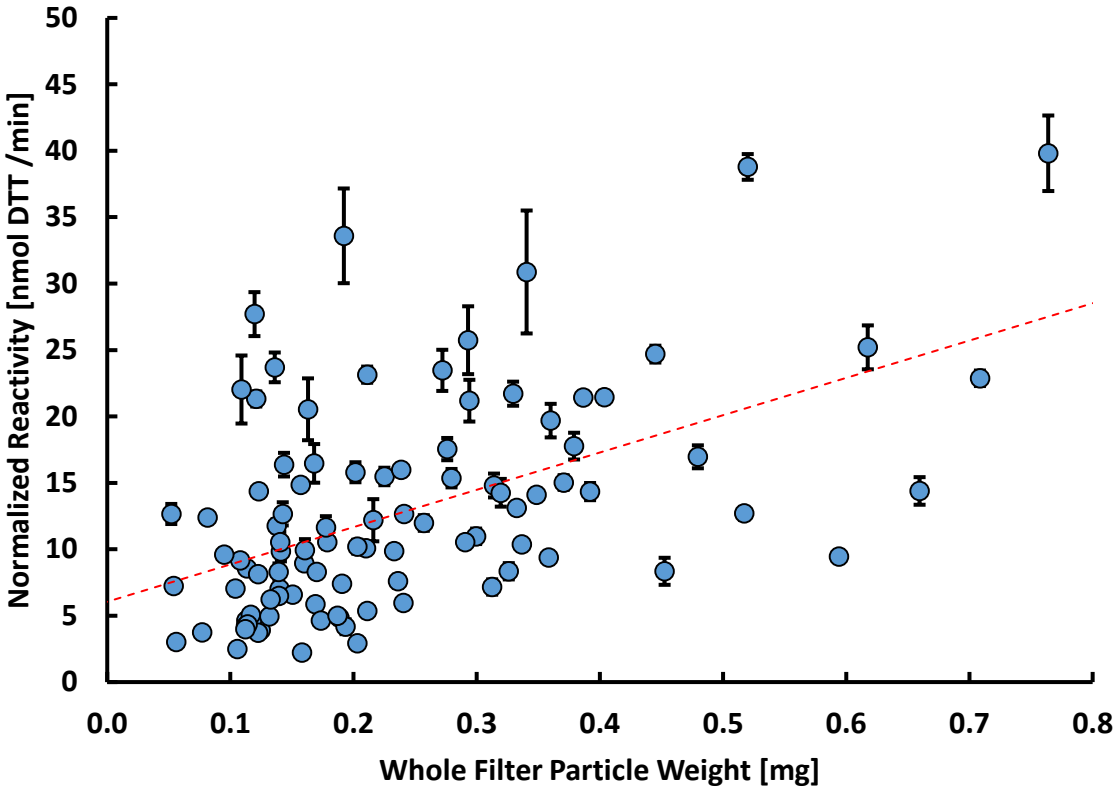

Figure 2.4 Data from approximately 110 **personal** exposure samples. A weak, positive correlation between particle weight and reactivity can be seen ($R^2 = 0.27$).

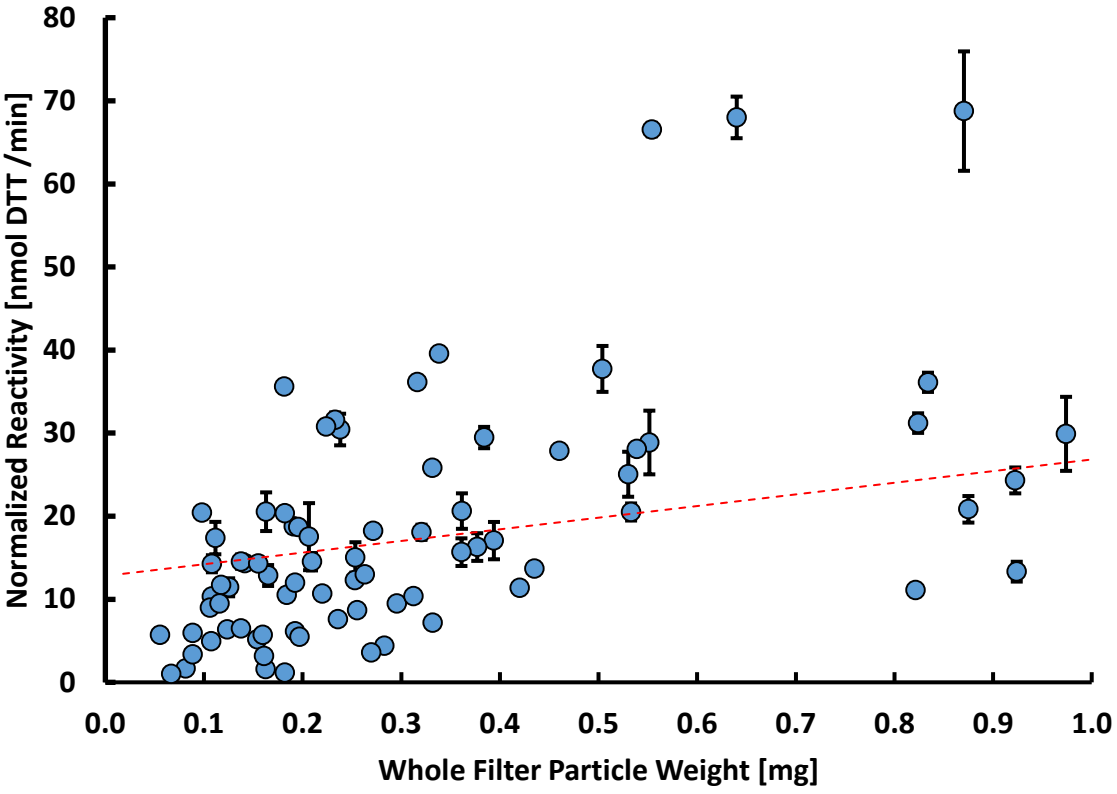

Figure 2.5. Data from approximately 110 **area** exposure samples. Again, like with personal exposure, a weak positive correlation can be seen ($R^2 = 0.33$). There are three outliers, which were not included in the final reactivity summary in **Table 1**.

Though weak positive correlations exist between WFPW and reactivity, data suggest that PM2.5 composition is more crucial to determining dangerous levels of exposure than PM2.5 weights or concentrations, alone. Personal exposures were collected from study subjects whereas area exposures were collected from fixed points near the wood-fired cook stoves. Although the data from a few area exposure samples contained more reactive and heavier particles, it apparently makes little difference being near to or away from an inefficient cook stove. It should be noted, however, that particle weights for personal exposures versus area exposures were nearly half, on average. Considering particle quantities alone, the World Health Organization recommends an annual exposure averaging 10µg/m3. [9] The Honduran samples represent, on average, 168 $\mu g/m^3$ for personal exposures and 392 $\mu g/m^3$ for area exposures in a single day. When considering reactivity from these particles, relative to other examples, the data is best presented in the following table.

Table 2.1 Summary of Honduran reactivity data compared to known reactivities.

Species	Average Reactivity [nmol DTT lost/min]	Exposure
Honduras Personal [a]	12.736	168 μg/m^3
Honduras Area [a]	18.695 [1]	392 μg/m^3
1,4-Napthoquinone [b]	0.334	300 ng/m^3
1,2-Naphthoquinone [c]	4.090	158 ng/m^3
Phenanthraquinone [c]	2.820	42 ng/m^3
Cu(II) [b]	0.945	80 ng/m^3
Fe(II) [c]	0.260	360 ng/m^3

[a] These species were analyzed in the electrochemical DTT assay and are discussed in this chapter.

[1]The average Honduran area exposure reactivity does not take three outliers into account.

[b] Species analyzed during the development of electrochemical DTT assay protocol, discussed in Chapter 1.

[c] Species analyzed by the Anastasio group using the absorbance DTT assay. [3]

These data demonstrate that the potency of real samples collected in Honduras, considered as a whole, is complicated when considering what has been previously reported in model DTT assays involving quinones and metals. It is highly likely that the Honduras samples contain multiple quinones and metallic species. It is also possible that the samples contain other materials like polycyclic aromatic hydrocarbons or other quinones and metals which are non-reactive with DTT (i.e. Acenaphthoquinone, Pyrene, Cr(III)) which may still be reacting with compounds in the samples to produce reactive covariate species. [3]

2.4.4 *Correlation to Absorbance Assay*

The final step was validating the electrochemical DTT assay results against reactivities in the absorbance assay for identical samples. All samples were processed in the electrochemical assay in triplicate; all but a few of the samples in the absorbance assay were run in triplicate. The data was correlated using a Deming regression (**Figure 2.6**) and variances were compared using f tests (**Figure 2.7**), shown below.

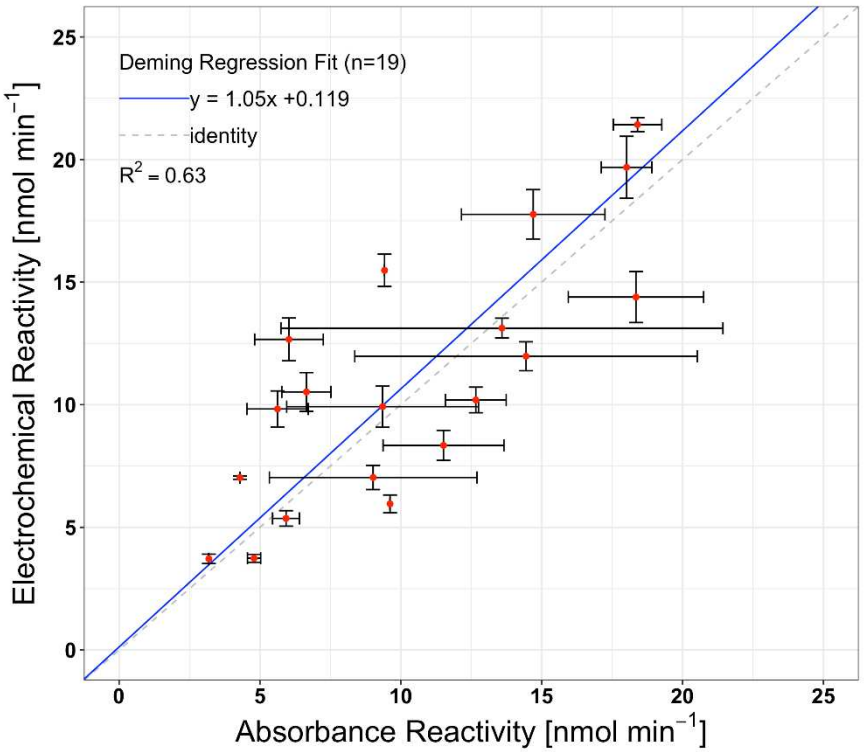

Figure 2.6 Deming regression analysis comparing absorbance assay reactivity to electrochemical assay reactivity for identical samples. Error bars represent repeatability between reactivities for separate pieces of the same filter sample.

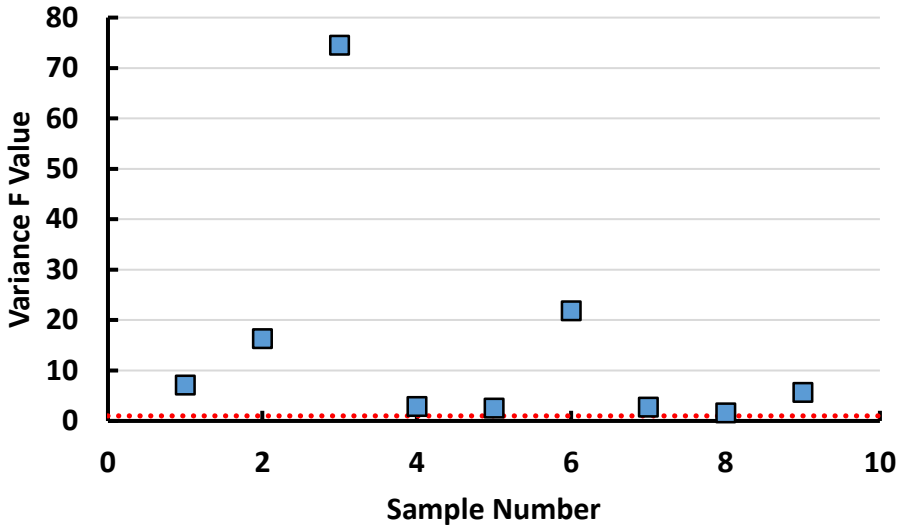

Figure 2.7 Variance analysis comparing standard deviations in absorbance assay vs electrochemical assay. Not pictured: an f value of 144 and 497.

Evident in the Deming regression in **Figure 2.6,** there is decent correlation between the two assays, confirming confidence in electrochemical assay accuracy. Additionally, error bar size suggests the reproducibility of real sample reactivity rates is improved in the electrochemical assay. Variance between samples was compared, in **Figure 2.7**. F values well above 1.0 indicate a characteristic difference in precision between the two methods. In this case, the electrochemical assay is more precise than the absorbance assay. Exceptionally large variation in the absorbance assay, however, may depend more on filter material than initially anticipated, as filter pieces are left in the assay. This is one aspect that clarifies that the stronger assay to use when analyzing samples, say on Pallflex quartz coated Teflon, would be the electrochemical assay.

2.5 Conclusions

For the first time, the electrochemical DTT assay was applied to a study with a large size of real samples (n>100). The assay has the advantages of high reproducibility, high precision, rapid processing rates (n=3-5 samples per hour, in triplicate), while maintaining accuracy relative to the absorbance DTT assay. The electrochemical assay also relies exclusively on readily available, purchasable electrodes and flow cell. Significance of this achievement lies in the potential to apply the electrochemical assay to field studies which may be time sensitive.

High reactivities, collected over a period of just 24 hours, from the Honduran samples suggest that PM2.5 exposures from inefficient cook stoves are capable of inducing ROS generation and oxidative stress in biological systems. Solid conclusions on the ability of these samples to cause DNA damage or disease will require health and DNA tests of the study subjects in Honduras. Though, it is also worth noting that a positive correlation between reactivity in the DTT assay and capacity to induce cellular damage has been firmly established. [10] Due to limitations of the electrochemical DTT assay, it is not clear the exact species present in the samples, but these findings should prove important for bolstering PM2.5 regulations and improving manufacturing standards for wood-burning cook stoves.

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CHAPTER 3. ESTABLISHING ELECTROCHEMICAL PERFORMANCE AND FLUID DYNAMICS IN IMPINGING JET FLOW CELLS

3.1 Chapter Overview

An electrochemical DTT assay that makes use of commercially available components was developed and optimized, as discussed in the previous chapters. This chapter focuses on the fluid dynamics in the analysis portion of the assay with the goal of providing better insight into its accuracy. Fluid dynamic considerations in this study are concerned with the use of an impinging flow cell, or wall jet flow cell. The design features an inlet centered at a 90° angle over a working electrode, an outlet off-center from the working electrode, often at an angle, and fluid being pumped into the flow cell from the inlet, flowing radially across the electrode. A picture and a side schematic of the DropSens flow cell used in this work are shown in **Figure 3.1**. The flow is assumed to be laminar, perpendicular to the electrode surface, and not impeded by the cell design.

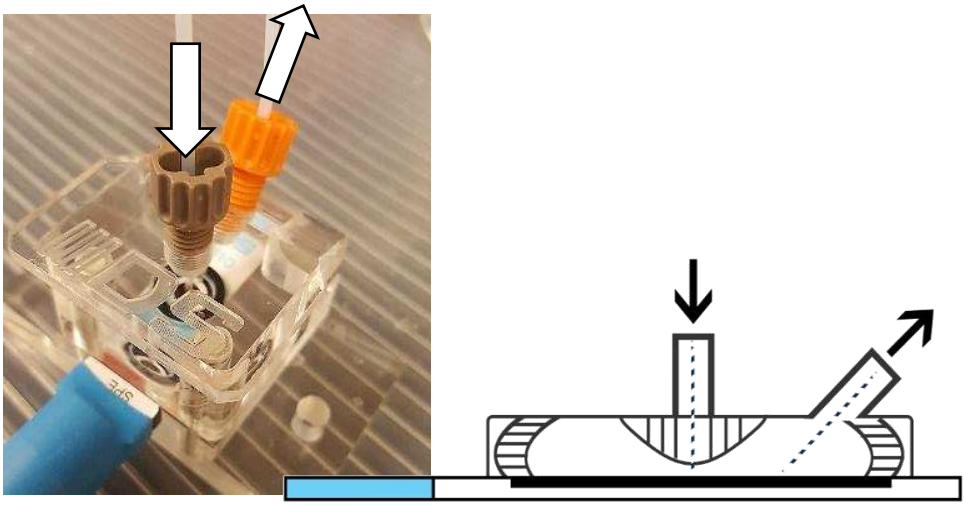

Figure 3.1 DropSens clear PMMA clamshell impinging flow cell used in this study and a side-on schematic of the inner flow in the cell. An SPE can be seen inside the cell.

To explore how flow cell fluid dynamics are observed in practice compared to theory, three species were investigated: potassium ferrocyanide ($K_4Fe(CN)_6$), (ferrocenylmethyl)-trimethylammonium hexaflurophosphate ($FcTMA^+$), and dithiothreitol (DTT). Ferrocyanide and $FcTMA^+$ were paired to a carbon screen printed electrode (SPE) while DTT was paired to a cobalt(II)phthalocyanine (Co-PC) modified carbon SPE as Co-PC modification should catalyze the DTT oxidation. A series of flow injection amperograms revealed that both $FcTMA^+$ and DTT adhere to theoretical predictions, within a 10% tolerance. Peak currents obtained from ferrocyanide, however, deviated from theoretical values, which may be due to its inconsistent inner/outer sphere characteristics. Peak currents taken from DTT experiments also provide a precedence for calibration curves when performing the DTT assay. Results from these experiments show that it is imperative to pay attention to whether or not the electrochemical performance of a chosen analyte is obeying fluid dynamics theory and that the methodology presented here should be adopted as an initial experiment when developing electrochemical protocols using flow systems.

3.2 Introduction

The recent push for more efficient and accurate ways to analyze air pollution's adverse health effects has resulted in the use of analytical techniques such as chromatography, absorbance spectroscopy, and inductively coupled mass spectrometry (ICP-MS) to perform such analyses. [1,2,3]As often happens, paradigm shifts and political climates have induced renewed enthusiasm for further optimizing well established protocols, particularly those meant for rapid, accurate analysis. Within these paradigm shifts, engineering and chemistry are reconciled, paving the way for optimizing intelligently. Fluid dynamics, in particular, is a powerful engineering tool that has

been employed in optimizing detection systems, such as those used in microfluidics and even paper based diagnostic systems. [4]

The protocol which served as the subject for this study was the dithiothreitol (DTT) assay, which has proven to be a formidable analytical technique for analyzing air pollution toxicity over the last 15 years. [2,3,5] Traditionally, this assay has relied on absorbance spectroscopy, however this approach is relatively slow and suffers from high errors leading to poor detection limits. [1,3,6] To address these issues, an electrochemical assay was developed, which utilizes flow injection amperometry and an impinging flow cell. The incorporation of fluid flow in this protocol presents an important opportunity to investigate the relationship between fluid dynamics, electrode materials, analyte species, and electrochemical performance.

Flow cell fluid dynamics theories are well studied and most of the founding papers were published in the 1970's. For an impinging flow cell like what was used in this study, peak current, Ip, is given by the following equation. [7,8] It was first derived by J. Yamada and H. Matsuda: [8]

$$I_p = 1.597nFC^{\infty}kD^{2/3}v^{-5/12}V^{3/4}a^{-1/2}R^{3/4} \quad (1)$$

Where 1.597 accounts for circulation constants in the flow cell, **n** is the number of electrons transferred in an oxidation or reduction, **F** is Faraday's constant (96485 coulombs/mol), $\mathbf{C}^{\infty}$ is bulk analyte concentration, **D** is analyte diffusivity (cm^2/s), **v** is kinematic viscosity (cm^2/s), **V** is volumetric flow rate (mL/min), **a** is inlet diameter (cm), **R** is working electrode radius (cm), and **k** is an empirically derived dimensionless momentum flux constant (usually taken to be between 0.86 and 0.9, specific to the flow cell). This equation assumes laminar radial flow across the working electrode surface, that the electrode is wide enough to allow for efficient signal processing across its surface, and that the inlet jet is not impeded. [7–10]

This theoretical equation dictates that the peak current in an analyte's chronoamperogram is going to be dependent upon the number of electrons transferred in the redox reaction, Faraday's constant, species-specific parameters such as kinematic viscosity, concentration, and diffusivity, sizing parameters such as electrode radius and inlet diameter, and, finally, a flux factor and volumetric flow rate. Optimization of the electrochemical flow system requires manipulation of the non-fixed variables, for example increasing **n**, the ratio between **R** and **a**, **D**, and **V** should increase **Ip** linearly. Whereas increasing **v**, the opposite trend should be observed.

The most consequential sizing parameter is the empirical dimensionless momentum flux factor, **k**. The fact that it is a *momentum* flux factor, as opposed to magnetic, heat, or mass flux, directly correlates it to flow cell design. If an impinging flow cell does not achieve a flux factor aligning with equation (1), electrochemical performance of an analyte will be hindered. In practice, the independent variable is chosen to be flow rate, as this can be easily and accurately changed using a pumping system. Though analyte concentration can also, technically, be chosen as the independent variable, it is a less convenient and less accurate parameter to choose. This equation, therefore, is often used to characterize custom-made wall jet flow cells, using well characterized redox reactions on catalytic electrodes such as platinum. [10,11] It is less common, however, to use this fluid dynamics theory to characterize a *reaction* using a manufactured flow cell.

It should be noted that there is not a parameter addressing electrode material variance. This leaves a gap in the theory—if an electrode has poor kinetics or has too much resistance across its surface, this equation does not quantify how that may affect electrochemical response. This discrepancy is understandable, because the original experiments as well as the vast majority of other studies employing the flow cell mass transfer theory, were conducted on a platinum disk electrode. [7,8,10] If resistance can be assumed to be low across an electrode, electrode material can

be left out of the theory, entirely. But, modern-day pushes for convenient, efficient, and accurate electrochemical methods employed as detection systems present an unforeseen obstacle to this flow cell theory. If cheap, manufactured electrodes like carbon SPE's are used, ohmic drop and poor electrode kinetics can lead to erroneous electrochemical response in these flow cells. While this phenomena is documented in literature, the impinging flow cell theory equation (1) does not account for it.

Beyond the question of electrode material, a question regarding the analyte itself also needs to be considered: is it inner sphere or outer sphere? For any electrochemist hoping to work on a novel protocol to do electrochemical detection in a flow system, this question is important and is addressed in this study. In the original studies on flow cell fluid dynamics, the only hint toward accounting for inner sphere behavior is that data analysis specifically aimed to correlate to reported diffusion coefficients for a given species. [2,10,11] But, this still does not fully account for inner sphere behavior from redox species. For example, the ferri/ferrocyanide redox couple is one of the most commonly used in electrochemistry, usually employed to characterize electrodes. Its popularity may be due to the fact that it's just what everyone uses and has always used, it is a stable redox couple, it's inexpensive and easy to prepare, and its behavior is consistent. It does *not*, however, consistently display outer sphere characteristics, suggesting that its electrochemical performance may be surface sensitive. [12,13] $FcTMA^+$, a ferrocene derivative, however, has been firmly established as an outer sphere species, making its electrochemistry less susceptible to changes in electrode material. [14] Comparing these two species on a carbon SPE is, therefore, an excellent way to probe how well the flow cell fluid dynamics theory holds when considering not only manufactured carbon electrodes, but also analytes which may be sensitive to electrode surfaces.

Herein, I explore the methodology originally used to derive equation (1) and use it to characterize the electrochemical performance of ferrocyanide and $FcTMA^+$ on a carbon SPE, and, finally, DTT on a Co-PC modified SPE. All electrodes and flow cells are manufactured and readily available. The choices of ferrocyanide and $FcTMA^+$ on carbon offer the possibility of exploring how inner sphere and outer sphere species will perform with an electrode material known to have poor kinetics, [15] which the Yamada theory (equation 1) does not account for. The redox species respective behaviors directly apply to understanding how lesser-studied compounds, like DTT, may perform when paired with similar electrode materials. DTT's performance in this study, then, will directly influence the accuracy of the newly developed electrochemical DTT assay, mentioned above. Through experimenting with these three species-electrode pairings, the importance of using fluid dynamics as a tool to better understand electrochemical performance is made clear. Methods like this one should be employed when first establishing a benchmark for calibration curves involving lesser-studied redox reactions.

3.3 Experimental

Materials

Academic work centered on electrochemistry typically uses homemade carbon paste, carbon screen print, or even BDD powder electrodes. [12,15] When processing large sample sizes, however, characterizing homemade electrodes can become problematic. In this study, premanufactured carbon screen printed electrodes, both unmodified and those modified with cobalt(II)phthalocyanine, with Ag/AgCl reference and a coordinating clam shell wall jet flow cell (DropSens 410 SPE, Metrohm USA) were used, instead. Solid DTT powder was purchased from Sigma Aldrich, potassium ferrocyanide was purchased from Mallinckrodt Chemical Works, concentrated nitric acid was purchased from EMD, sodium phosphate dibasic anhydrous and

sodium phosphate monobasic hydrous were purchased from Fischer Chemical. $FcTMA^+$ was synthesized in-house, according a previously described procedure.[16] Chelex 100 mesh was purchased from Sigma Aldrich and buffer was chelated accordingly. All reagents were used as received. All electrochemical measurements were conducted using a CH Instruments Potentiostat (model number 812).

Where equation parameter values are concerned, **n** is 1 electron transferred for all reactions tested, the radii of electrodes are 2mm, flow cell inlet diameter is 3/100. Species-specific parameters are as follows: ferrocyanide in KCl has a kinematic viscosity of 0.0088 cm^2/s and diffusion coefficient of 6.20 ($10^{-6}cm^2/s$); $FcTMA^+$ in KCl has a kinematic viscosity of 0.009 cm^2/s and diffusion coefficient of 6.70 ($10^{-6}cm^2/s$); DTT in PBS has a kinematic viscosity of 0.009 cm^2/s and diffusion coefficient of 2.83 ($10^{-6}cm^2/s$).[17]

Method

In order to explore the fluid dynamics theory, the method described below, adapted from the original work was used.

1. Ferrocyanide was prepared at 2mM and diluted to 50µM in 0.4M KCl. $FcTMA^+$ was prepared at 1mM and diluted to 100µM in 0.4M KCl. DTT was prepared at 4mM in 2mM nitric acid and then diluted to 100µM in 0.1M phosphate buffer at pH 7.4.
2. For each analyte, a linear sweep (static analysis on the electrode surface) and a hydrodynamic voltammogram (dynamic analysis of analyte injections through the flow cell) were performed to best identify which applied potential would coordinate to the redox potential of the species.

3. When the redox potential is established, this potential is applied as a constant for chronoamperometry. Flow rate is varied from 50µL/min to 300 µL/min and the resulting peak currents are plotted against the flow rate to the ¾'s power.
4. The slope from step 3 is compared to that predicted by theory. Theoretical data, itself, was adjusted to account for variations in mass from when the analyte concentrations were prepared. This was done, because some of the concentrations were prepared such that inherent mass balance error was over 10%.

If the data resulting from this method align well with theory, it can be concluded that the species and electrode material can be used together in a flow protocol. This method will also establish a benchmark for calibration curves for different concentrations of analyte, increasing accuracy in the protocol. If the data does not align, the opposite is the case and a different analyte-electrode pairing should be chosen.

3.4 Results and Discussion

3.4.1 *Ferrocyanide Oxidation on Carbon SPE*

Ferrocyanide, pictured in **Figure 3.2**, was chosen because a history of displaying mixed behavior with regards to it being inner and outer sphere and this remains contentious in the literature. [12,13] If it *does* display inner sphere behavior, that should translate into higher sensitivity to the surface material of an electrode and less adherence to values predicted by theory. Thus, pairing it to a carbon SPE, an electrode material known to have slower kinetics than, say, platinum disk electrodes could reveal this inner sphere behavior. But, carbon SPE's also remain one of the most affordable and available manufactured electrodes on the market. Thus, applying the method

to a ferrocyanide-carbon SPE pairing should help to show the importance of considering fluid dynamics when working with species and electrodes which may not behave ideally.

Following the original methodology, the experiment begins by determining the redox potential for 50μM ferrocyanide in 0.4M KCl by conducting a linear sweep and a hydrodynamic voltammogram (HDV) run at 200 μL/min. Although an HDV provides to most accurate

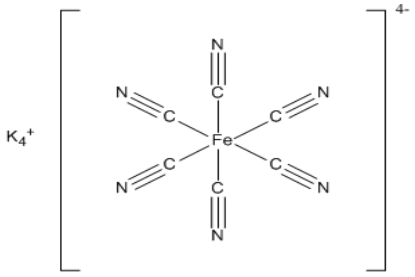

Figure 3.2 Ferrocyanide structure. This will oxidize to ferricyanide.

measurement of the applied potential coordinating to the best peak current, conducting both will illuminate any unexpected behavior in the analyte when switching from static (linear sweep) to flow (HDV). Once an appropriate applied potential is selected from **Figure 3.3** (in this case, +0.27V vs Ag/AgCl), it is used as a constant in chronoamperometry while **V** is varied (shown in **Figure 3.4**). The data is compared to ideal data with k=0.86, the momentum flux factor historically chosen when conducting this method.[7,8] When comparing the actual data to the theoretical prediction, it is important to understand that because the theory does not account for poor kinetics in electrode material and was originally developed on platinum electrodes, theoretical data will trend through zero. This is not the case for the actual data, because poorer electrode kinetics will induce a slight resistance to electron transfer, producing a positive y-intercept.

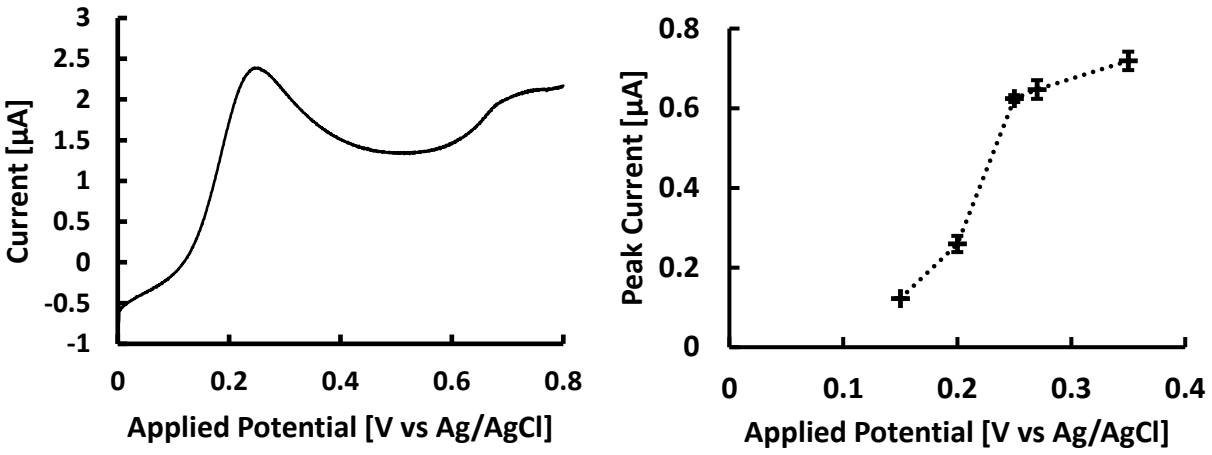

Figure 3.3 Linear sweep (L) and HDV (R) of ferrocyanide oxidation. Applied potential is chosen as +0.27V vs Ag/AgCl, aligning well with a ferrocyanide oxidation potential of +0.44V vs SCE.

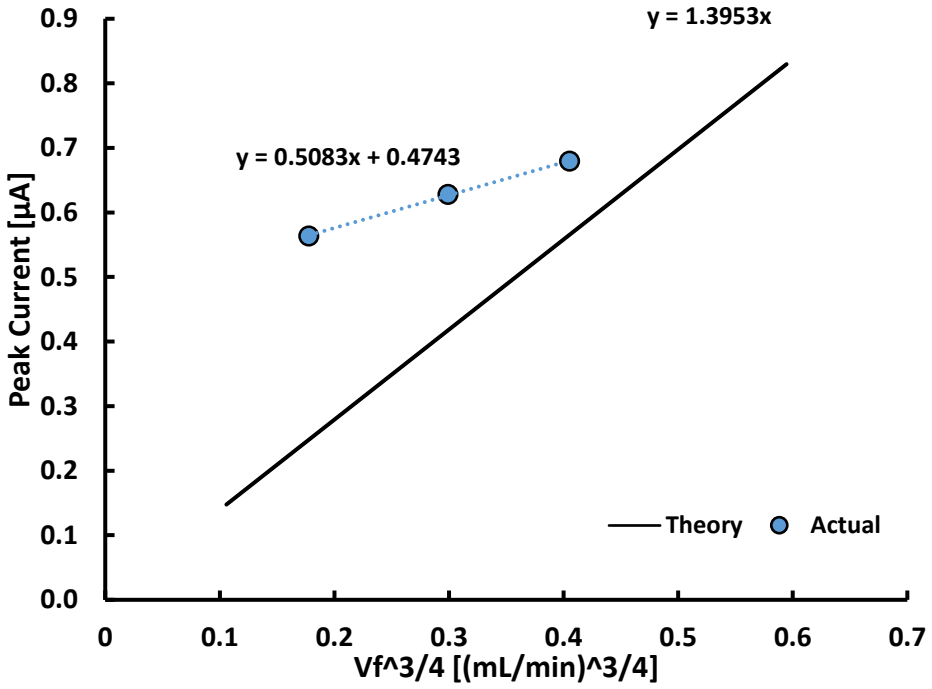

Figure 3.4 Chronoamperometry data showing the actual and theoretical data for ferrocyanide oxidation across a DropSens carbon SPE.

Seen in **Figure 3.4**, ferrocyanide oxidation does not occur favorably in an impinging flow cell paired to a carbon SPE. This behavior is most likely the result of a combination of slow kinetics across the electrode as well as inner sphere behavior from the ferrocyanide. As flow rate is increased, the electrode itself is unable to transfer enough electrons to induce the expected current. For volumetric flow rates higher than 300 µL/min, a plateau effect on peak current begins to take shape, furthering this conclusion (data not shown).

3.4.2 *$FcTMA^+$ Oxidation on Carbon SPE*

$FcTMA^+$ is another well characterized redox species and is pictured below. $FcTMA^+$ is an outer-sphere species, which, according to Marcus theory, means that its rate of electrochemical oxidation occurs via tunneling, without the need to bind or absorb onto the electrode surface. As such, its electrochemical oxidation is not dependent on the catalytic activity or kinetics of the electrode. Thus, any deviation from theory would be the result from resistance effects in the electrode or poor flow cell design. This makes $FcTMA^+$ an excellent candidate for characterizing electrodes. Being an outer sphere species should also mean that, upon increased mass transport to the surface of an electrode, peak current will increase without having to change concentration, making $FcTMA^+$ an ideal species for characterizing the fluid transport of impinging flow cells as its electrochemical response is decoupled from electrode surface kinetics.

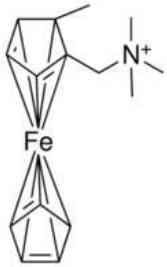

Figure 3.5 $FcTMA^+$ structure. The Fe exists in a +1 state and can be oxidized to its 2+ version. [18]

As before, the method begins with establishing the redox potential of $FcTMA^+$ with a carbon SPE. Linear sweep voltammetry (static) and hydrodynamic voltammetry (dynamic) are used in conjunction with each other, shown below in **Figure 3.6**.

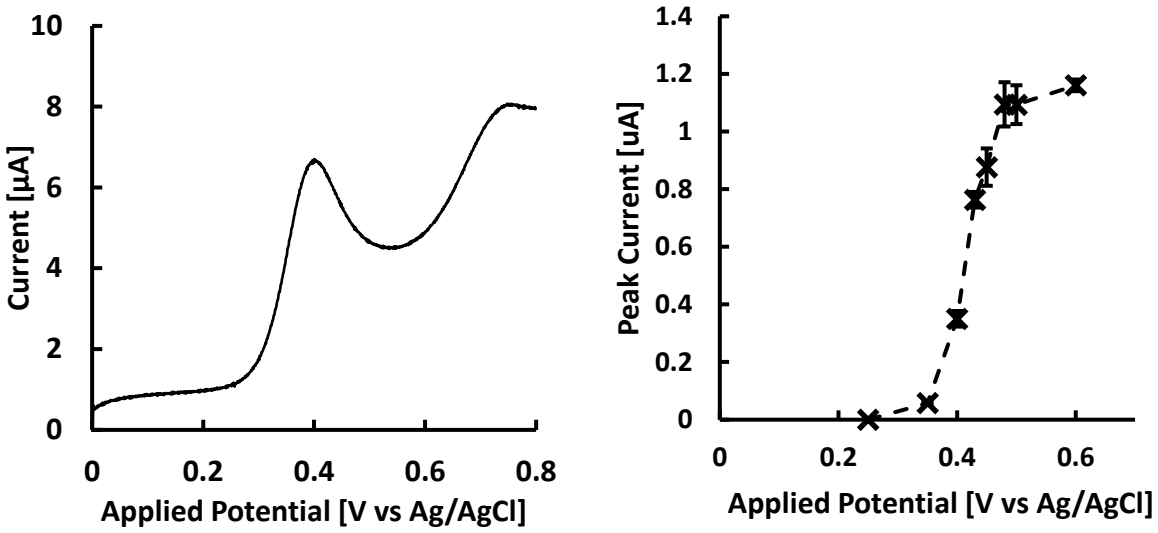

Figure 3.6 Linear sweep (L) and hydrodynamic voltammogram (R) for 100uM $FcTMA^+$ oxidation on carbon SPE with Ag/AgCl reference at 200 uL/min. Redox potential is chosen as +0.48V vs Ag/AgCl.

Based off the linear sweep and HDV in **Figure 3.6** an applied potential of +0.48V was chosen. As before, a series of flow rates are chosen, and peak currents at the redox potential are recorded for each flow rate. The resulting data is compared to theoretical data with a momentum flux factor of, k=0.86.

Shown in **Figure 3.7**, actual data is compared to theoretical data, while also accounting for variations from mass balance error. In this study, mass balance error accounted for 15% of the analyte concentration. Rather than showing error bars, the low concentration variation is plotted separately to show how concentration error affects the slope, as it is the slope which is the point of comparison between theory and actual data. Given the low concentration theoretical gradient

(y = 1.81x) and actual gradient (y = 1.69x), it can be reasonably concluded that $FcTMA^+$ behaved as theory predicted within a 10% allowance and was likely a little under its intended concentration. Being an outer sphere species helps to explain its performance, despite being paired to a manufactured carbon electrode, as opposed to the characteristics displayed by ferrocyanide.

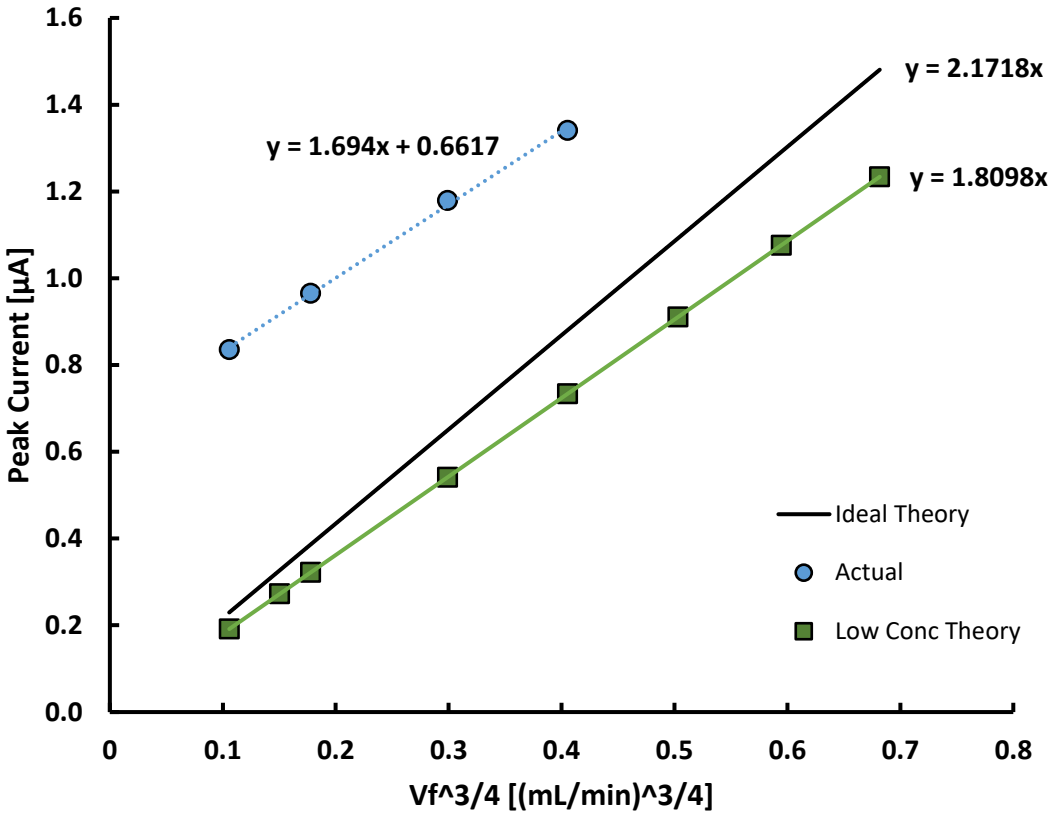

Figure 3.7. Summary peak current data from the $FcTMA^+$ chronoamperogram study. All error bars, representing standard deviation between flow injections, are below 3% and cannot be seen. N=4. Theory adjusted to account for low concentration to reconcile gradient.

3.4.3 *DTT Oxidation on Modified Carbon SPE*

Once the actual data for $FcTMA^+$ is acquired and is shown to align well with theory, manufactured carbon-based electrodes and the DropSens impinging flow cell can be used with reasonable confidence. The last investigation, therefore, is focused on validating the combination of DTT, in **Figure 3.8**, and a Co-PC SPE. Per the methodology, a linear sweep and an HDV, seen in **Figure 3.9**, are conducted to pinpoint an appropriate applied potential.

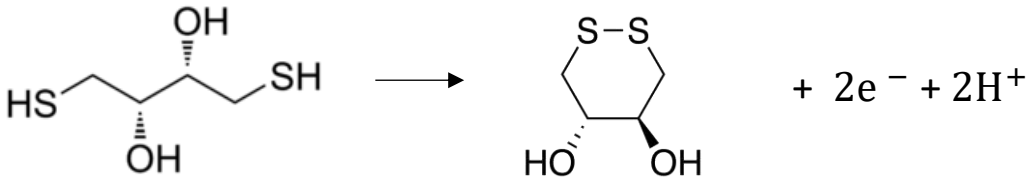

Figure 3.8. DTT is oxidized from its dithiol form to a disulfide. Though two electrons are lost, this reaction is a one electron process, as the loss of the first catalyzes the loss of the second.

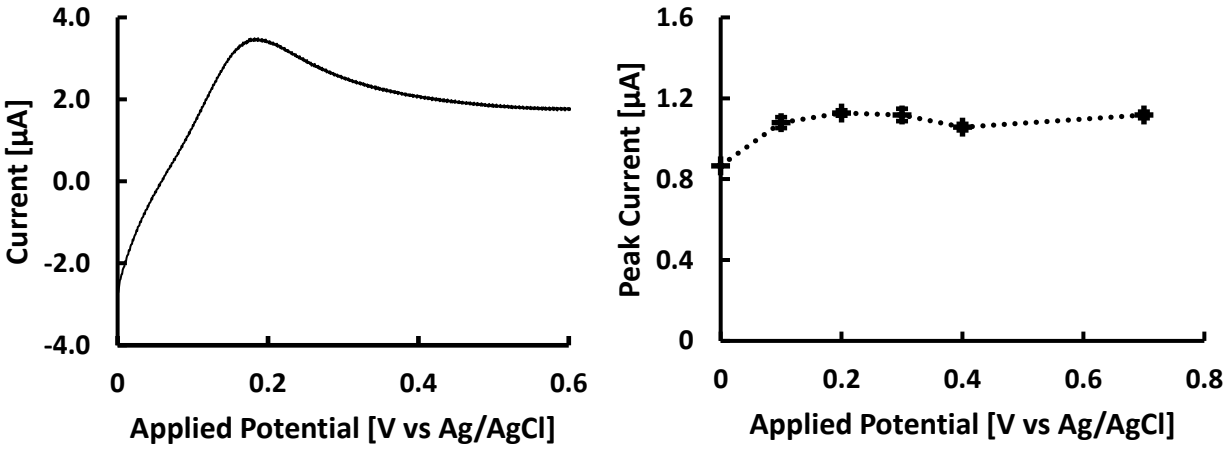

Figure 3.9 Linear sweep (L) and HDV (R) for 100uM DTT oxidation on Co-PC modified carbon SPE and Ag/AgCl reference. Redox potential of +0.3V vs. Ag/AgCl was chosen.

With a chosen applied potential of +0.3V, **V** is varied and chronoamperograms are recorded. It should be noted that the linear sweep and HDV for DTT are slightly unusual. Where previous linear sweeps and HDV's reported in this chapter feature resolved peaks and clear S-curves, respectively, the same peaks and curves are not seen in **Figure 3.9**. The same was also observed in a previous DTT study. [1] This data suggests that the interaction between DTT and the electrode is well catalyzed by the Co-PC modification. Comparison of actual chronoamperometic data to theoretical data is seen in **Figure 3.10**.

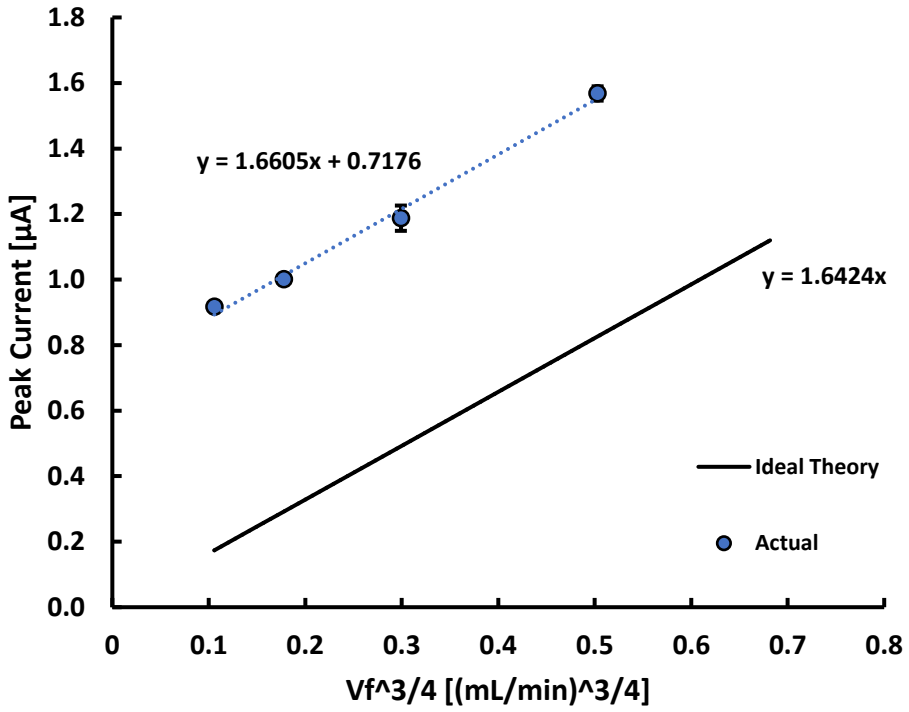

Figure 3.10 Summary showing actual DTT data compared to theoretical data. Error bars, representing standard deviation between flow injections, are all under 5%. N=4.

In **Figure 3.10**, actual data is compared to theoretical data (k=0.86). In the case of the DTT-Co-PC pair, theory predicted a gradient of $y = 1.642x$. Actual data produced a gradient of $y = 1.66x$. It can be concluded, therefore, that DTT behaved as theory predicted it to within 2% and pairing it to a Co-PC SPE is a good choice.

3.5 Conclusions

The method adaptation discussed in this chapter establishes that proper analyte-electrode pairings are important for maintaining accurate electrochemical responses and establishing these pairs can be made easier by understanding flow cell fluid dynamics. Flow cell dynamics were investigated by exploiting inner and outer sphere characteristics of common and under-studied redox species ferrocyanide, $FcTMA^+$, and DTT. It also provides an excellent way to establish a benchmark when conducting chronoamperometric calibration curves of novel redox-active species. With known parameters, uncertainty no longer rests on whether or not the currents are what they should be for various analyte concentrations or volumetric flow rates. The method also reduces the need to rely on validation of electrochemical flowing assays with other techniques, like absorbance spectroscopy. If electrochemical results align with theoretical ones, this method therefore provides its own validation and self-consistency. With regards, specifically, to the electrochemical DTT assay, reaction rates and reactivity rates are not dominated by DTT concentration. Those results don't invalidate the work of this chapter, because the DTT assay reaction and reactivity rates were impossible to establish accurately without first understanding DTT's electrochemical response as accurately as possible. Adopting the Yamada method to characterize reactions can only help to clarify future work and form the framework for other flowing electrochemical protocols.

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CHAPTER 4. CONCLUSIONS AND FUTURE WORK

Risk of disease and premature death as a result of sir pollution and PM2.5 exposure are of major concern for human and biological health, globally. [1,2,3] The ability to analyze PM2.5 inexpensively and efficiently, using readily available components, would allow for better understanding of the scope of PM2.5 exposures as well as the mechanisms between PM2.5 and adverse health effects. Better understanding would, in turn, allow for intelligent and better informed regulation and manufacturing standards. PM2.5 has been definitely proven to wreak havoc on animal cells and has cells exposed to PM2.5 from diesel exhaust have shown signs of undergoing oxidative stress and apoptosis. [2,4] Many techniques are already in use for analyzing PM2.5 and oxidative stress, the best-studied technique being the DTT absorbance assay, which was first developed 15 years ago. Since its inception, little has been done to optimize the assay, despite the fact that it is limited by dilution effects, slow sample processing rates, and has variable precision. [1,5]

This thesis provides the development, validation, and application of a flow-based electrochemical DTT assay as a formidable alternative to the absorbance assay. The electrochemical assay, in an effort to normalize reproducibility and take pollution analysis one step closer to field applications, exclusively uses inexpensive, readily available manufactured materials and uses chronoamperometry as its predominant electrochemical technique. The assay also takes advantage of shortened reaction times and cold quenching techniques to turn the assay into a batch process, quadrupling sample processing rates from one or two samples per hour (non-triplicate) to three to five samples per hour in triplicate. This optimization alone makes the electrochemical assay the fastest known assay for processing PM2.5 oxidative capacity. [1,5] When considering fluid dynamics in the development and validation of the assay, electrochemical response of DTT was

found to behave as theory predicts, allowing aspects of the assay (like chronoamperometric calibration curves) to be self-validating. Reactivities in the electrochemical assay were validated using coordinating reactivities in the absorbance assay. The electrochemical assay and was found to have improved precision and long-term reproducibility over the absorbance assay while maintaining a good level of accuracy, even when processing real PM2.5 samples (70-100% correlated to the absorbance assay). Additionally, reactivities in the assay when analyzing model oxidants were similar to what has been established in literature, furthering the conclusion that the electrochemical DTT assay works and works well. [1,6]

Future development involving the electrochemical assay could focus on taking advantage of the core reaction in the assay: a catalytic reaction between DTT and PM2.5 which consumes DTT, but leaves PM2.5 intact. Combined with the fact that the electrochemical assay is flow-based, waste generated from the assay could be passed through other tests including colorimetric tests, microfluidic techniques, etc. If the assay was automated, a series of accurate, precise analyses could be performed on large sample sizes rapidly, expanding the capacity for intimately understanding PM2.5 chemistry.

The assay was also applied to analyzing over 200 PM2.5 samples as part of the overarching Honduras Cookstove Project. Samples reflected PM2.5 exposures for study subjects and their homes. Reactivities from these Honduran samples indicated that PM2.5 coming from inefficient wood-burning cook stoves are highly reactive relative to reactivities reported for model oxidants like quinones and metals. [6] Future work pertaining to this study and others like it should focus on investigating the effect that PM2.5 has on human health. Studies could be done by comparing exposure reactivities from inefficient cook stoves to those from efficient cook stoves and correlating the results to medical data (i.e. DNA tests, general health, apoptosis rates in real cells).

More general future developments can simply employ the electrochemical DTT assay in environmental studies, perhaps even in the field, with confidence that sample processing can be done rapidly and efficiently.

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