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Analysis of ‘touch’ DNA recovered from metal substrates: an investigation into cfDNA-metal interactions and the efficacy of different collection techniques on DNA yield

Jessica E. Thornton, B.S.

Thesis submitted to the Eberly College at West Virginia University in partial fulfillment of the requirements for the degree of Master of Science in Forensic and Investigative Science

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Keywords: ATR-FTIR, transition metals, touch DNA, cfDNA, DNA collection, DNA extraction

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ABSTRACT

Analysis of 'touch' DNA recovered from metal substrates: an investigation into cfDNA-metal interactions and the efficacy of different collection techniques on DNA yield

Jessica E. Thornton, B.S.

While several improvements have been made in recent years to optimize the recovery of 'touch' DNA, relatively little research has been conducted to understand the relationship between 'touch' DNA and the binding affinity of that DNA to metal surfaces, specifically those with a significant copper presence. Furthermore, characterization of cell-free DNA (cfDNA) and its contribution to 'touch' samples and those cfDNA-metal interactions from objects commonly identified at crime scenes (cartridge casings, knives, doorknobs) have been lacking.

Research has identified the tendency of copper ions to intercalate with DNA helices, resulting in sample degradation among other damaging conformational changes; however, while these effects have been observed in aqueous solutions under controlled conditions, virtually no examples of this phenomenon exist out of solution. It is therefore of critical importance to first evaluate if similar interactions are taking place on copper containing surfaces once dried on the surface, as are the conditions of 'touch' DNA samples usually collected at a crime scene. Additionally, research pertaining to 'touch' DNA recovery from metals has focused on developing optimized mechanical recovery techniques to include the M-Vac® wet-vacuum DNA collection system. However, recovery remains problematic due to those approaches having been developed to retrieve as much cellular material i.e., intracellular nuclear DNA (nDNA) as possible when it has recently been suggested that circulating cfDNA comprises the majority of DNA in a 'touch' sample. Therefore, maximizing the amount of both cellular and cfDNA acquired from metal surfaces is critical to successful DNA profiling.

To generate an optimized workflow regarding collection and extraction procedures for 'touch' DNA from metal surfaces, the binding effects of metal ions with cfDNA, as well as the composition of 'touch' DNA samples, need to be further evaluated. As such, a three-fold study was designed with the following objectives: evaluate DNA-metal interactions at the surface level using ATR-FTIR, investigate efficacy of three collection methods (wet:dry double-swabbing method, tape-lifting, M-Vac® wet-vacuum DNA collection system) in recovery of 'touch' DNA from metal substrates, and test a centrifugal separation method for more efficient extraction of cellular and cfDNA. This study contributes to the ongoing research concerning the efficient collection of 'touch' DNA at the scene and from evidentiary items and provides investigators with a framework for which to collect and process such samples from commonly encountered metals.

Table of Contents

List of Figures v

List of Tables vii

Part I 1

1. Introduction 1

1.1 Problem Statement and Research Intent 1

1.2 Project Scope and Objectives 2

2. Trace DNA 3

2.1 Definitions 3

2.2 Variability in DNA Quantity 3

2.3 Transfer and Persistence 4

2.4 'Touch' DNA Sample Composition 5

2.5 Substrate Interactions 7

3. DNA-Metal Interactions 9

3.1 Transition Metals and Complexes 9

3.2 Metal's Crystalline Structures 9

3.3 Binding Motifs of Metal with DNA 11

4. Instrumental Analysis of M-DNA 12

4.1 M-DNA Observed in Solution 12

4.2 Infrared Radiation (IR) Spectroscopy and M-DNA 13

4.3 Spectral Imaging of M-DNA using ATR-FTIR 15

5. Trace/'Touch' DNA Collection and Extraction from Various Substrates 15

5.1 Collection Techniques for 'Touch' DNA 15

5.2 Extraction Methods for Cellular and cfDNA in 'Touch' Samples 18

6. Theoretical Considerations 18

6.1 Foundational Assumptions 18

6.2 Project Limitations 19

Part II 20

7. Evaluation of Trace DNA Recovery Techniques *(Preliminary Study)* 20

7.1 Materials and Methods 20

7.2 Results and Discussion 22

8. Evaluation of Trace DNA Recovery Techniques *(Primary Study)* 25

8.1 Materials and Methods 25
8.2 Results and Discussion 26
9. DNA-Metal Interactions 30
9.1 Materials and Methods 30
9.2 Results and Discussion 31
10. Centrifugal Separation 35
10.1 Materials and Methods 35
10.2 Results and Discussion 37
Part III 42
11. Recommendations for Future Practice 42
12. Concluding Remarks 42
13. Key Take-Aways 43
References 44
Appendix I – List of Abbreviations 50

List of Figures

Figure 1. Illustration of the layers of the skin during epidermal desquamation (adapted from Milstone, 2004 [31]).......... 6

Figure 2. Three primary metal crystalline structures (from left to right: HPC, FCC, BCC) where the blue spheres represent atoms (adapted from Iowa State University's Center for Nondestructive Evaluation [50]). 10

Figure 3. Illustration of site-specific binding of Cu2+ with nitrogenous bases in DNA, resulting in helical disruption (adapted from Shamsi and Kraatz, 2013 [56]).......... 11

Figure 4. Typical ranges whereby IR functional groups behaviors (i.e.: stretching, bending, etc.) might be observed (adapted from Wade, 2016 [62]). 14

Figure 5. Microbial wet-vacuum DNA collection system, alternatively referred to as the M-VAC® [72]. 17

Figure 6. Least squares mean plots of average quantitated trace DNA yields as recovered from brass, copper, and stainless steel. For all samples (N = 45), ~ 6 ng of mixed cellular and cfDNA was deposited in the form of saliva. 24

Figure 7. Least squares mean plot of average quantitated cfDNA yields as recovered brass, copper, and stainless steel. For all samples (N = 45), ~ 100 ng of cfDNA was deposited in the form of TaqMan Human Control DNA. 25

Figure 8. Least squares mean plot of average quantitated DNA yields as recovered using the M-Vac®, swabbing, and tape-lifting. For all samples (N = 135), participants firmly placed their thumb, index, and middle fingers onto the substrate for a total of 90 seconds. 27

Figure 9. Interactions effects between treatment and substrate material for all 'touch' deposits. For all samples (N=135), interactions were found to be insignificant ($p > 0.05$); however, similar trends can still be observed for all metal-types with swabbing and tape-lifting, as well as between brass and glass for the M-Vac® and swabbing approaches. 28

Figure 10. Participants effects for all 'touch' deposits (N = 135). For all samples, effects were found to be statistically significantly different using a Student's t-test ($p < 0.05$) for Participant #3, as observed by the outlying sample in the upper third of the figure; however, similar interactions can still be observed for all other participants involved in the study. 29

Figure 11. FTIR spectra of glass microscope slide both without (left) and with (right) extracted DNA (eluted in water) dried on its surface. The presence of the strong doublet in the spectra to the right at ~ 3000 cm-1 is indicative of C-H stretching, as well as those peaks may also be reflective of N-H stretching of the nitrogen residues of the nucleobases. Furthermore, those peaks in the spectra to the right appearing from ~ 1600 cm^{-1} and on into the fingerprint region provide further support that those peaks are originating from the DNA sample and not from a contaminant. 32

Figure 12. FTIR spectra of copper sheet metal both without (left) and with (right) extracted DNA (eluted in water) fixed to its surface. At ~ 3000 cm-1, a slight shift in the spectra can be observed when comparing the DNA containing sample (right) to the negative control (left). This may be indicative of either C-H stretching or N-H stretching of the nitrogen residues of the nucleobases, as observed in Figure 1; however, the relative intensity is such that no peaks can be reliably identified. Those peaks in the spectra to the right appearing at ~ 1000 cm^{-1} appear to be C-O stretching, indicating that those peaks originate from the DNA and not a contaminant. 32

Figure 13. FTIR spectra of glass microscope slide with initial DNA deposit (left), after rinsing with 200 µL nuclease-free water (center), and after rinsing with 200 µL 0.1X TE buffer (right). At ~ 3000 cm-1, a significant decrease in relative intensity of the peaks can be observed between the initial DNA deposit and the nuclease-free water rinse, thus indicating that a majority of the DNA was recovered from glass simply using water. In the spectra from the 0.1X TE buffer rinse, there are minimal changes in peak intensity at ~ 3000 cm^{-1} from the nuclease-free water rinse and the TE rinse. A slight shift in the peaks and their relative intensities for the initial DNA deposit and the remining two samples can be observed from ~ 1500 cm^{-1} to ~ 1000 cm^{-1}. 33

Figure 14. FTIR spectra of copper sheet metal with initial DNA deposit (left), after rinsing with 200 µL nuclease-free water (center), and after rinsing with 200 µL 0.1X TE buffer (right). At ~ 1000 cm-1, a slight decrease in relative intensity of the peaks can be observed between the initial DNA deposit and the nuclease-free water rinse; however, in the spectra from the 0.1X TE buffer rinse, the peak has almost entirely disappeared. This is indicative of a displacement reaction occurring between the buffer and the substrate, effectively removing the DNA. 34

Figure 15. Total contribution of cellular and cfDNA to allelic profiles as observed in samples (N = 20) collected using nuclease-free water (left) and PBS (right). 39

Figure 16. Concentration of recovered DNA from saliva samples (N=18) as measured using Quantifiler™ Trio Quantification Kit following 30 min centrifugation at increasing speeds. All samples were collected from a single donor. Centrifugal separation was conducted in triplicate and the averages used to evaluate trends. For both cellular and cfDNA fractions, no significant increase in cfDNA yields at higher centrifugal forces are observed, illustrating that few, if any, cells were artificially ruptured, and their contents transferred to the supernatant. 40

Figure 17. Proposed decision matrix for the collection of 'touch' DNA based on both the present findings as well as those previously published in the literature, to consider optimal collection of both cellular and cfDNA components, from non-porous substrates. Underlined recommendations are those which were evaluated in this thesis. 42

List of Tables

Table 1. Summary of select research pertaining to the recovery of touch DNA from metal substrates [14, 37, 39–44]. 8

Table 2. Brief selection of published research pertaining to M-DNA in an aqueous environment [10, 12, 57–61]. 12

Table 3. Summary of cfDNA recovery (completed in triplicate) from copper substrates with nuclease-free water and 0.1X TE buffer to moisten sampling matrix. All samples (N=6) were extracted with the addition of 1 µL carrier RNA and eluted to a final volume of 25 µL. 35

Table 4. Summary of average DNA recovery from glass substrates with nuclease-free water and PBS to moisten sampling matrix (N=20). Net yields for all samples remained lower than reported values after centrifugal separation. All samples quantitated in half-reaction volumes (10 µL) with quantification thresholds as low as 0.005 ng/µL [78]. 38

Table 5. Summary of called allele for both cellular and cfDNA fractions of touch deposits (N=20). This data indicates that the cfDNA in touch samples, while understood to be more highly fragmented (140-200 bp [8]), contributes a greater proportion of genomic data to STR profiles than the more commonly target cellular fraction. 39

Table 6. Summary of p-values of pairwise comparisons for all sample types and RCFs. At $\alpha = 0.05$, no significant changes were observed for any RCF. These data indicate that elevating centrifugal force did not result in artificial cell lysis. 40

Part I

1. Introduction

1.1 Problem Statement and Research Intent

The recent influx in knife and firearm-related crime throughout major cities in the United States poses a significant threat to the stability of civil society and contributes to the already disproportionate number of open versus closed cases across police departments throughout the nation. In the past several years, the number of homicides, violent crimes, and aggravated assaults resulting from the use of knives or firearms has reached the highest percentage since the 1960s [1]. Moreover, according to the Federal Bureau of Investigation's Crime Data Explorer, the number of murder victims in 2020 killed via handguns, firearms (type not stated), knives or cutting instruments alone totaled 82% of all homicides in the United States. This does not account for non-fatal violent crimes or aggravated assaults in which the weapon used was a knife or firearm, nor does it include those murders committed using rifles, shotguns, or other firearms [2]. This striking number highlights the need for research into collecting evidence from items of this nature, as well as identification of those perpetrators and assailants who have yet to be identified.

With any crime, moving beyond the scope of simply knife and firearm-related incidents, identifying the source of an evidentiary sample, be it material, chemical, digital, or biological, is the foundation upon which an investigation lies. As such, human identification has persisted as a vital element of the investigation process, beginning with the first use of fingerprint identification in the 19th century to the now commonly employed DNA analysis technologies like STR profiling. With advancements in DNA collection and testing technologies, from the microbial vacuum (M-Vac®) wet-vacuum DNA collection system and Bardole modification to the highly sensitive GlobalFiler™ PCR Amplification Kit, human identification has become even more powerful allowing items that may have been touched or handled to successfully samples to generate DNA profiles.

These 'touch' or trace DNA samples, not necessitating the presence of a bodily fluid [3–6], are commonplace in property and violent crimes whereby investigators seek to use shed cellular material to identify individuals present at the scene [5, 6]. For over two decades, the collection of trace DNA has been evaluated from a variety of surfaces, including but not limited to clothing, wood, and plastics [4]. However, the challenge with these recommendations is that the majority are mechanical-based approaches which fail to characterize the composition of the sample with which they are attempting to recover. In 2015 and 2021, researchers at Virginia Commonwealth University and King's College London reported that the composition of what is referred to as 'touch' DNA was primarily of acellular origin rather than shed nucleated cells [7, 8]. Furthermore, the acellular DNA left behind in touch samples, colloquially termed "cell-free DNA" (cfDNA), appeared in an Organization of Scientific Area Committees (OSAC) research needs assessment form when it was stated, "Evidence collection on touched objects also needs to consider the presence of cell-free DNA" [9]. The presence of cfDNA implicates the recovery of 'touch' DNA in that, if the majority of DNA deposited is not protected by a cellular structure, then its interactions and viability on different substrates must be examined to effectively optimize its recovery.

Reconsidering the issue of knife and firearm-related crimes, as well as including those counterterrorism investigations whereby explosive devices have been confiscated, it stands that items manufactured or constructed from metals must be included in the evaluation and optimization of DNA recovery. Furthermore, the interactions between unprotected, cfDNA and metal ions need to be further characterized to recover ‘touch’ DNA more effectively from items such as metal wires, knives, cartridge casings, and firearms. Throughout the literature, the complexation of metal ions to the DNA backbone and nucleobases has been demonstrated in aqueous solutions under optimal binding conditions [10–12]. Damaging conformational changes to the DNA helix have been frequently visualized *in vitro* via x-ray crystallography, circular dichroism (CD), and nuclear magnetic resonance (NMR) imaging [10, 11, 13]; however, this phenomenon has yet to be explicitly demonstrated out of solution. It has been posited that these binding interactions may still be occurring on dry, metallic surfaces because while trace amounts of DNA can be recovered from small metal objects (i.e.: cartridge casings and projectiles), DNA recovery has been shown to be more difficult on certain transition metals and alloys thereof (i.e.: copper and brass) [10, 12–14]. Furthermore, DNA recovery from large metal surfaces has remained a cumbersome task for investigators due simply to the size and irregular shapes of these objects.

These factors related to ‘touch’ DNA, DNA-metal interactions, and the dimensions of the evidentiary item have made it of critical importance to evaluate what constitutes ‘touch’ DNA and challenges unique to these sample types, how metal ions have been problematic in sample recovery, and what implications follow for DNA recovered from metal substrates. The findings detailed in this thesis will hopefully impact the forensic science community by proposing a decision matrix for collection of ‘touch’ DNA from metal surfaces. By comparing three metals (i.e.: copper, brass, stainless steel) and three collection techniques (i.e.: swabbing, tape-lifting, M-Vac®), investigators can better evaluate the nature of the evidentiary items left behind at the scene of a crime to determine the approach best suited for maximum DNA recovery. Additionally, this study will evaluate a centrifugal separation method for cellular and cfDNA components of ‘touch’ samples to further characterize each fraction’s influence in downstream processing such as amplification, STR separation and detection, and subsequently, profile interpretation. The proposed workflow for each sample and metal type will be applied to mock evidentiary samples, taking into consideration the nature of the biological material deposited and its relationship to the substrate, therefore aiming to increase DNA recovery and STR profile quality for those typically more challenging evidentiary items thought to contain trace amounts of DNA. As a result, investigators may be able to acquire probative information to be used to guide an investigation or potentially serve as evidence in court.

1.2 Project Scope and Objectives

The primary aim of this study was the optimization of collection and extraction methods of touch DNA from those metallic surfaces and objects which bind to unprotected, cfDNA left behind during deposition, beginning with the evaluation of those DNA-metal interactions at the surface level. To achieve this objective, the following tasks were completed:

1. Evaluate the collection efficacy of swabbing, tape-lifting, and the microbial vacuum (M-Vac®) wet-vacuum system on recovering cellular and cfDNA from metal substrates, as well as from mock evidentiary ‘touch’ samples.

2. Identify the conformational changes to cfDNA when bound to copper-containing surfaces, as demonstrated via ATR-FTIR spectroscopy.

3. Develop a separation method for cellular and cfDNA fractions of 'touch' samples for more effective extraction and purification.

4. Provide practical recommendations and/or considerations as a framework for investigators as they collect and process 'touch' DNA from commonly encountered metals.

The remainder of Part I in this thesis will focus on establishing the theoretical framework from which this project was developed. This study began with preliminary experimentation, as detailed in Part II, by evaluating the binding effects of common transition metals and alloys thereof, with cfDNA, as well as characterizing the contribution of cellular and cfDNA components in 'touch' samples. Using the findings from the preliminary testing a more effective DNA processing workflow will be proposed, particularly concerning the collection and extraction steps. From there, the application of the optimized methods to mock evidentiary 'touch' samples will be examined. Lastly, Part III of this thesis summarizes the overall findings from both the preliminary and primary studies, as well as details the project's principal conclusions. Recommendations for future optimization as it pertains to the recovery of 'touch' DNA from metal substrates and considerations for subsequent data interpretation will also be discussed.

2. Trace DNA

2.1 Definitions

Throughout the literature, trace DNA has been defined as low levels of DNA, colloquially termed low-template DNA (LT-DNA), that are not directly associated or identified to originate from a specific serological fluid [4, 15]. It is a broader classification established to characterize any DNA present in minute quantities. It has since been expanded to include DNA deposited upon direct and indirect contact between individuals and/or objects. Trace DNA describes that DNA that was speculatively collected and of which the mode of transfer not clearly defined [4].

Within the classification of trace DNA both 'touch' DNA and 'transfer' DNA are included, whereby touch DNA implies the action of direct contact with an individual or surface, and transfer DNA is indicative of primary, secondary, or tertiary transfer [4]. It should be noted, however, that while a standardized definition for trace, touch, and transfer DNA have yet to be published [4], there is general consensus throughout the community to define these sample types as "any sample where there is uncertainty that it may be associated with the crime itself – so that it is possible that transfer may have occurred before the crime event or after the crime event" [3].

2.2 Variability in DNA Quantity

The variable, and oftentimes, low levels of DNA common when working with trace samples have proven challenging when employing the commonly used forensic approach for DNA analysis, beginning with DNA collection and extraction up until profile interpretation. Throughout this process, a great deal of DNA can be lost, proving problematic when working with an already

limited quantity of trace DNA. [15]. Therefore, attention has turned to improving the community's understanding of initial quantity of trace DNA deposited on a surface, the collection of that DNA, and STR typing technologies so to increase instrument sensitivity.

When considering trace DNA, the idea of donor shedding status and its relationship with the amount of deposited DNA has been researched [6] along with the extent to which an item has been handled [4, 6]. Shedding is the concept that there is variability in the number of cells individuals leave upon surface contact, and subsequently, variability in the quantity of DNA available for recovery [4, 6]. It has been postulated that an individual's shedder status can be correlated to their sex and age, and statements to these effects have even appeared in courtroom testimonies [6, 16]. While this concept has been widely cited in the forensic literature, there persists a significant lack of evidence to characterize an individual's shedder status, notwithstanding the concept itself is founded upon the assumption that the majority of trace DNA originates from shed cells despite recent data to the contrary [7, 8]. There is inherent variability in DNA deposition with 'touch' samples, and the amount of DNA recovered from trace samples ranges widely from 0 to 170 ng [4]. Trace DNA, as the name suggests, persists in low quantities upon initial deposition [4], not accounting for the potential of transfer or binding of the DNA to the deposition substrate, both of which further result in a lower net quantity of recovered DNA [4, 17].

2.3 Transfer and Persistence

In 1997, the first study was published that evaluated the nature of the transfer of trace DNA between intermediaries [18], and again in a 1999 paper the nature of primary and secondary transfer was examined [19]. It was concluded that profiles can be generated from primary transfer, but the ability to interpret genotypes from secondary transfer of DNA was not supported [19]. However, in the nearly twenty-five years since those studies were published, more recent research pertaining to secondary and tertiary transfer in conjunction with the increase in sensitivity of STR DNA profiling kits has repeatedly demonstrated the ability to produce interpretable DNA profiles. Moreover, these profiles have been resultant of direct and indirect transfers that occur from routine activities including handshaking, washing clothes, as well as moving past other individuals [4].

It has been reported that after a single transfer, the majority of DNA collected was the transferred DNA rather than the original persisting DNA [4]. These findings have been supported by additional studies concluding that the major contributor of a sample was the last person to handle an item [4]; yet, exceptions persist, and considerations also need to be made to account for the possibility of the major contributor profile being generated from unrelated, background DNA [4]. In more recent reports, the transfer of DNA to clothing was evaluated, and it was concluded that DNA was acquired on an individual's clothing through hugging (i.e., direct transfer), as well as simply by occupying an individual's office space (i.e., indirect transfer) [20]. Additional studies have further demonstrated indirect transfer of trace DNA between an individuals' clothing and vehicles [21], as well as aerosolized, non-contact transfer of DNA from dried biological materials (i.e., blood, semen, saliva, etc.) [22]. Furthermore, that transfer by examiners from gloves or fingerprint powder brushes has been reported [23–25] to occur, but the extent of unwanted transfer is still not clearly defined. Because limitations in our characterization and understanding of trace DNA transfer exist, if not addressed, they could lead to erroneous conclusions regarding the hierarchy of propositions (i.e., sub-source, source, activity, and offense level propositions) [3].

The persistence of a sample after deposition (to include direct, indirect, and innocent transfer) for an extended period of time is significantly influenced by the conditions to which it is

subjected. Exposure to contaminating or degrading environments (i.e.: high humidity, UV, heat, etc.) has extensively been shown to reduce the amount of trace DNA recovered [26]. Furthermore, the time for which trace DNA persists on a surface, excluding consideration of damaging environmental factors, has resulted in a reduced amount of DNA able to be recovered in as little as a week. While there is research to be conducted concerning all the possible environmental factors and time intervals that could influence trace DNA samples, there is sufficient evidence that details common enzymatic and environmental factors that degrade DNA (not necessarily trace DNA) [4, 26]. Studies have further evaluated the storage of trace DNA samples in standardized lab conditions (i.e.: dry, cool, sterile), and findings have revealed that while exposed samples see a significant decline in DNA recovery in as little as a week, properly stored trace DNA samples can persist for months [4].

The transferability and persistence of trace DNA poses challenges with DNA profile interpretation [3]. With an influx in the generation of profiles from objects directly handled, consideration must be given not only to the primary transfer of DNA to a substrate but also to the transfer between people and objects. Once DNA has been deposited, be it direct, indirect, or innocent transfer, it is important to evaluate the resulting profile under the consideration of the unbiased potential modes of transfer as detailed previously to mitigate errors in interpretation or inadvertently overstating the value of that DNA sample as evidence [3]. As such, the relationship between activity-level propositions, transfer and persistence of trace DNA, and the conclusions made in trace DNA interpretations are increasingly being evaluated.

2.4 'Touch' DNA Sample Composition

Throughout the day, individuals are constantly touching parts of their face and neck, as well as touching a variety of objects and surfaces. For years, it was believed that the DNA deposited via 'touch' was primarily comprised of shed skin cells, as research has demonstrated that thousands to millions of cells can be shed in a 24-hour time span [4, 27]. However, recent studies have presented data to the contrary, suggesting a great majority of those shed cells are not nucleated epithelial cells, but corneocytes, which have lost their nuclei and presumably nuclear DNA (nDNA) during the cornification process [27]. Understanding whether the biological material and thus DNA, deposited in a 'touch' sample was generated by the hands (i.e. shed nucleated cells) or from elsewhere (i.e.: exogenous cellular material) within the body is important as it affects sample recovery and processing for forensic casework. In recent years, studies have revealed that as STR technologies have progressed, so has the ability to generate profiles from almost a single cell (~0.01 ng DNA) [4]. Consequently, recent research has shifted its attention toward the composition and origins of DNA from touch deposits, particularly concerning cfDNA, as its presence is evident on and throughout the human body [4, 7, 8, 27, 28].

2.4.1 Anucleate Corneocytes

The majority of cellular material left in 'touch' deposits was previously presumed to be shed epithelial cells from people's hands; yet recent work has indicated that instead, terminally differentiated keratinocytes, otherwise known as corneocytes, are what is primarily left behind [29, 30]. These cells are anucleate as a result of the cornification process, and consequently, have been presumed to lack DNA therefore not contributing to the DNA deposited in a 'touch' sample [29].

The *stratum corneum* consists of layers (typically 15-20 cell layers) of keratinocytes (Fig. 1). These keratinocytes are formed in a process referred to as differentiation whereby keratin filaments aggregate, replacing the epithelial cell membrane with an envelope of cross-linked proteins referred to as desmosomes. Following initial keratinization, the final differentiation step is initiated – epidermal desquamation. During epidermal desquamation, desmosomes are rearranged in the *stratum corneum* to the edges and are ultimately subjected to hydrolytic enzymatic digestion, resulting in the loss of their nuclei [31]. These anucleated corneocytes, as they approach the outer surface of the epidermis, are stacked on top of one another until they are either passively or actively shed [31].

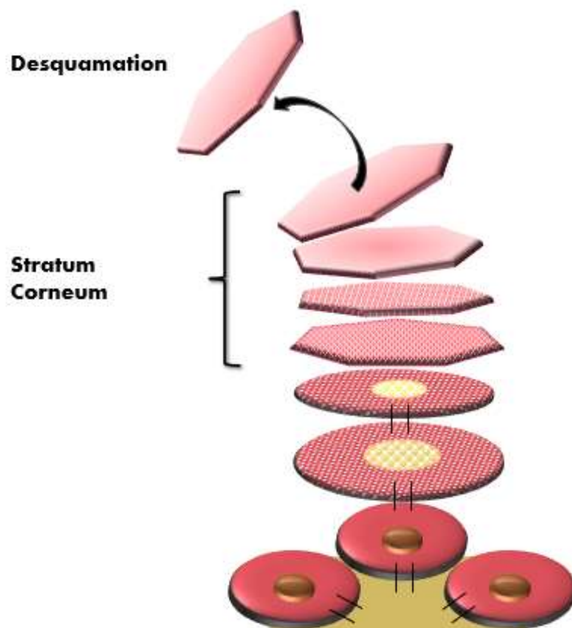

Figure 1. Illustration of the layers of the skin during epidermal desquamation (adapted from Milstone, 2004 [31]).

There are a limited number of studies that have explored the DNA content in corneocytes. It has been postulated that the structure of the cornified envelope makes extracting DNA from keratinized cells difficult for traditional DNA lysis methods [29]; however, advancements in DNA recovery from keratinous material in hair have resulted in enhanced lysis methods. As a result, it was found that if the anucleate corneocytes were treated with a reducing agent containing lysis buffer and extended incubation period, greater yields of DNA were recovered [29, 30]. However, while DNA was able to be recovered from these cell types previously assumed to lack DNA, it remains unclear whether any of the DNA from touch deposits actually comes from these corneocytes, or if it originates from another source, such as nucleated epithelial cells or cfDNA.

2.4.2 Nucleated Epithelial Cells

Although the majority of shed cells deposited in 'touch' samples are corneocytes; nucleated epithelial cells are also deposited, in lesser quantities, and do serve as a primary source of DNA. There is an existing body of literature on the subject, from studies evaluating the correlation between cell count and recovered DNA quantity, as well as fluorescent imaging of nucleated cells

in fingerprint evidence [4]. Furthermore, reports of approximately 2.6 cells and 5.6 cells have been observed on the palms and fingerprints respectively, an hour after handwashing without any additional contact [4]. The quantity of shed nucleated epithelial cells observed, however, were lower than the anucleate corneocyte cells [4]. Research has sought to optimize STR profile quality by separating the nucleated epithelial cell fraction, and presumably the nDNA, from other components of the sample including but not limited to corneocytes, fragmented cfDNA, and proteins [4].

2.4.3 cfDNA

Due to the ambiguity in the origin of cfDNA, many research projects have begun to venture into determining where this DNA comes from within the body and how it remains intact without protection from cellular membranes. It was first discovered in blood plasma and serum in 1948 and has since been discussed in cancer research and diagnostics due to its elevated presence in cancer patients as a result of rapid cellular turnover [4]. However, even in healthy individuals, cfDNA has been measured anywhere between 1.8-44 ng/mL of blood [4].

Necrosis and apoptosis have been discussed as likely sources of cfDNA since during those processes, DNA fragments (typically 150-200bp) are released from the cell [4, 32]. From there, they circulate through the body and have been documented in semen, saliva, urine, sweat, and even tear fluid [33–36]. It is thought that the cfDNA found in touch samples is accumulated on the hands through sweat, and this is supported by research where the levels of recovered cfDNA from participants' hands averaged 11.5 ng/mL of sweat [4]. The abundance of cfDNA in 'touch' samples has been evaluated and recently demonstrated as the majority contributor (84-100%) of DNA [7]; however, due to the short fragment lengths and fragile nature, the STR loci able to be amplified are limited and inconsistent [28]. Aiming to improve the typing proficiency of cfDNA, a few studies have focused on separating the cellular and cfDNA fractions of 'touch' deposits, demonstrating that cfDNA, once separated and purified, provided useful information for those smaller STR markers [8]. More research is needed to optimize the separation of cellular and cfDNA fractions, and to further evaluate the downstream implications of such a step.

Finally, because cfDNA is not housed in a protective membrane, the extent of degradation and evaluation of the quantity of these sample types cannot be performed in the same manner as cellular DNA [4]. 'Touch' DNA has been documented to be degraded, presumably due to environmental conditions; however, there is an increasing body of literature which suggests that the DNA is enzymatically fragmented inside the body during necrosis [26]. As such, understanding the persistence of cfDNA after deposition is critical to prevent further sample degradation.

2.5 Substrate Interactions

The sample type (i.e., shed nucleated cells, cfDNA, etc.) largely influences the overall quantity of DNA able to be recovered from a surface, and consequently, the surface on which the DNA was deposited plays a vital role in the retention and potential transfer of DNA. Previous research has demonstrated that as the roughness and/or porosity of the substrate increases, the amount and ease with which the DNA can be retrieved increases as well [37]. This in part could be due to the rough surfaces resulting in more sloughing of cells thus increasing the nDNA yield, the porous surfaces absorbing more cfDNA from 'touch' deposits, or any combination thereof. Successful DNA typing has been conducted from 'touch' samples on a variety of substrates

including wood, fabrics, plastics, and glass [4, 37, 39–44]. From these studies, wood has been shown to have the greatest retention of DNA, with materials such as glass and plastic falling behind [43]. Nevertheless, additional reports have in recent years demonstrated the ability to generate a DNA profile from a variety of surfaces including paper, plastic shopping bags, lipstick, and cyanoacrylate-fumed fingerprints [4]. While there has been increasing success in 'touch' DNA recovery from an abundance of substrates, one material remains exceptionally challenging regarding the acquisition of the DNA from its matrix – metals.

Metals and metal alloys are abundant in a variety of environments, from jewelry to door handles and weapons. Touch DNA left behind on door handles and metal window frames from crime scenes could serve as additional surfaces for analysts to sample. The issue, however, is that metal, unlike plastic, fabric, and glass, is thought to bind to unprotected, cfDNA, rendering traditional collection techniques ineffective [45].

Previous research has been performed in regard to retrieving DNA from metal substrates (Table 1) and has primarily looked at altering swab types or using soak and sonicate methods for smaller sample sizes, such as cartridge casings and projectiles [37, 39–44]. However, there are much larger metal surfaces that that cannot be soaked and the DNA is too tightly bound to the substrate to collect simply by means of traditional swabbing or even tape-lifting. Therefore, Section 3 of this thesis will focus on the molecular ways in which metals bind to DNA.

Table 1. Summary of select research pertaining to the recovery of touch DNA from metal substrates [14, 37, 39–44].

Substrate(s)	Collection Method(s)	Sample Type	Comments and/or Conclusions	Author and Publication Year	Reference
PVC pipe, 9V battery, electrical tape, copper wire	Cotton, nylon, foam swabs; Six different moistening agents	Trace DNA	No single best swab or moistening agent for all substrates	Phetpeng et al., 2015	[44]
Plastic, metal, glass	Nylon flocked swabs, cotton swabs, mini-tapes	Touch DNA, seeded swabs (DNA solution)	<2% DNA recovered from metal cables	Wood et al., 2017	[37]
Fired and unfired cartridge cases (brass, nickel-plated, aluminum, steel)	Wet:wet swabbing, wet:dry swabbing, soak and sonicate, soak and vortex, tape-lifting	Touch DNA	Optimized pairing and simple modifications of existing DNA processing can improve profile generation	Danielson et al., 2019*	[39]
Fired and unfired cartridge cases (brass and nickel)	Swabbing, tape-lifting, vacuum filtration, direct PCR	Trace DNA (saliva)	Swabbing yielded greatest amount of DNA followed by tape-lifting and vacuum filtration, no statistical difference between swabbing and tape-lifting	Prasad et al., 2020	[40]
Aluminum, brass, stainless steel, plastic	Isohelix™ swab with isopropyl alcohol, Rayon swab with water	Touch DNA	Improved recovery using Isohelix™ swab with isopropyl alcohol	Bonsu et al., 2021	[41]
Fired and unfired cartridges (brass)	Swabbing, tape-lifting, soaking	Touch DNA	Tape-lifting yielded greatest amounts of DNA followed by soaking and swabbing; firing found to decrease DNA recovery	Prasad et al., 2022	[42]
Stone, wood, plastic, metal (knife blade, aluminum can), glass, latex, cotton, synthetic carpet	Seven different swab kits	Touch DNA, blood, saliva, semen	Highest DNA yield with 10μL water for all swab types, DNA yield improved by increasing touch frequency	Seiberle et al., 2022	[43]

Fired and unfired cartridge casings (brass, nickel-plated)	Soaking, rinse and swab	Exogenous DNA, HEKa cells	DNA extracts did not appear to have PCR inhibitors, both methods yielded equivalent DNA quantities	Elwick et al., 2022	[14]

*Not published by the U.S. DOJ. Made publicly available through the Office of Justice Programs' NCJRS.

3. DNA-Metal Interactions

The majority of metals which can be found in households and crime scenes are the transition metals that are used in the production of alloys, as seen on hardware, kitchenware, and jewelry. These compounds within the alloys have unique coordination geometries which allow for the binding of nucleic acids. Additionally, the electrochemical properties of these metals enhance the attraction between the metal and DNA [46], making it more difficult to separate DNA from substrate. Research has demonstrated that there are different binding patterns between metals and nucleotides which alter both the strength of the bond, as well as the extent of the effect on the structure and stability of the DNA helix [47]. These metal-DNA complexes have been colloquially termed M-DNA and the way in which each complex forms presents unique challenges that need to be considered when trying to optimize recovery. Metals and alloys of interest in forensic casework include but are not limited to the following transition metals: copper, brass (copper & zinc), and stainless steel (iron & chromium) due to their prevalence throughout the environment, and thus, they will remain the focus of this thesis.

3.1 Transition Metals and Complexes

Transition metals are those in which the *d* orbitals are progressively filled [48]. As such, these metals possess unique properties which allow for multiple stable oxidation states and the ability to form complexes with surrounding molecules or ions by coordination of a ligand to metal by means of a Lewis acid-base interaction [48]. The ligand that binds to the central metal atom in the complex is referred to as the donor atom, and there can be complexes with multiple donor atoms [48]. Those complexes with more than one donor atom are chelating agents [48]. These complexes have distinct physical and chemical properties different from those of the individually participating metal and ligand [48]. In case of DNA-metal complexes, the influence of the metal ions changes the stability of the DNA helix, and thus, the ability to recover the sample [11, 12, 45].

3.2 Metal's Crystalline Structures

Metals account for a majority of all elements and are comprised of atoms held together by strong, delocalized bonds. These bonds, when below a metal's melting temperature, produce a stable, crystalline structure by arranging atoms into compact geometries, allowing for different packing patterns to arise including hexagonal close packing (HCP), face-centered cubic (FCC) packing, and body-centered cubic (BCC) packing [49]. These different crystalline structures (Fig. 2) account for the majority of transition metal's geometries, and consequently, their properties such as ductility and binding affinity to organic and other inorganic compounds.

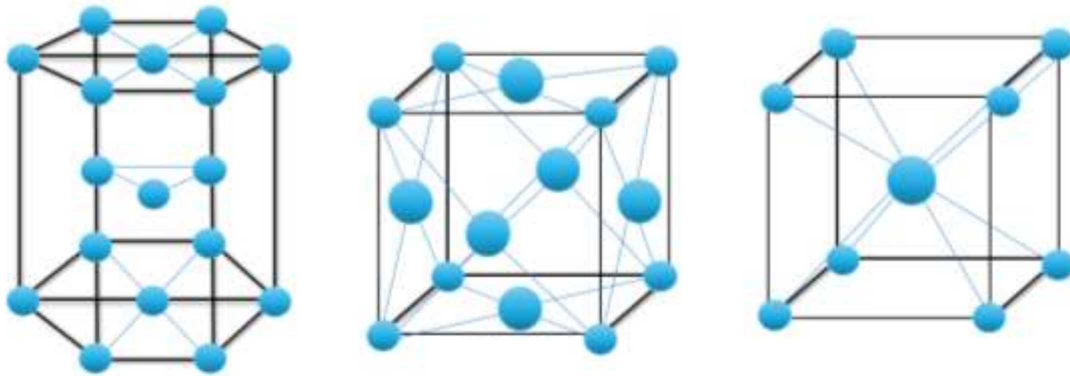

Figure 2. Three primary metal crystalline structures (from left to right: HPC, FCC, BCC) where the blue spheres represent atoms (adapted from Iowa State University's Center for Nondestructive Evaluation [50]).

In HCP, atoms are arranged in layers whereby the second layer of atoms nests in the hollows of the first layer of atoms, and so on. Similarly, FCC lattices form with atoms from one layer nesting into the gaps hollows of the adjacent layer. However, atoms in the FCC structure are located in each of the corners and centers of the cubic faces rather than hexagonally arranged. BCC arrangement does not allow for the close packing as is observed in the HCP and FCC nesting pattern, but instead has been observed to have atoms at each corner of the cube, as well as at its center [50].

3.2.1 Copper

Copper is a transitional metal with a characteristic crystalline FCC structure. This simple cubic structure results in copper's malleability, and thus, its ubiquity among household items. When considering pure copper metal grains (i.e., not an alloy), they are primarily composed of alpha grains, which are those FCC structures with a single carbon atom on their face. When copper is used to form alloys, those other metal atoms displace the copper atoms within the cubic lattice [51].

3.2.2 Brass

Brass is an alloy of primarily copper and zinc, with the presence of iron and lead reaching only up to 0.05% of its composition. When zinc binds with the cubic structure of copper, the zinc displaces those centered copper atoms, forming a BCC lattice. In this new structure, the zinc atom for that cube is not centered on the face, but instead centered within the cube with the displaced copper atoms now located at each corner. These beta grains found within brass play a large factor in the strength and ductility of the brass; however, the brass commonly used in manufacturing, be-it door handles or projectiles, consists primarily of alpha grains in a 70/30 copper-alpha/beta-zinc proportion [51, 52].

3.2.3 Stainless Steel

Stainless steel is an alloy of iron and chromium, with chromium comprising at least 10.5% of the alloy's composition. As copper binds in a BCC lattice conformation to zinc *(see 3.2.2)*, so

does chromium to iron within stainless steel [53]. For ferric stainless-steel samples, the alloy takes the form of a BCC lattice; however, upon introduction of nickel into the alloy, it is changed into an FCC lattice [53].

3.3 Binding Motifs of Metal with DNA

The structure of the metal and its inherent properties influence the way in which it binds to other organic and inorganic compounds. Reports have also documented that the geometries of the major and minor grooves of the typical DNA conformation, B-form DNA, (Fig. 3) possess specific structural differences, further influencing the binding motifs of DNA to metal cations [54].

3.3.1 Major and Minor Grooves

DNA is a double-stranded, right-handed alpha helix with both strands wound around the same axis. These strands are intertwined as a plectonemic coil whereby major and minor grooves allow for base stacking with rotation [55]. The coil's structure and the major and minor grooves' dynamics allow for DNA to bind to proteins, metals, and drugs [54]; however, the direct influence of certain cations results in the formation of metal-DNA complexes (M-DNA) that have the potential to disrupt the integrity of the helix [10].

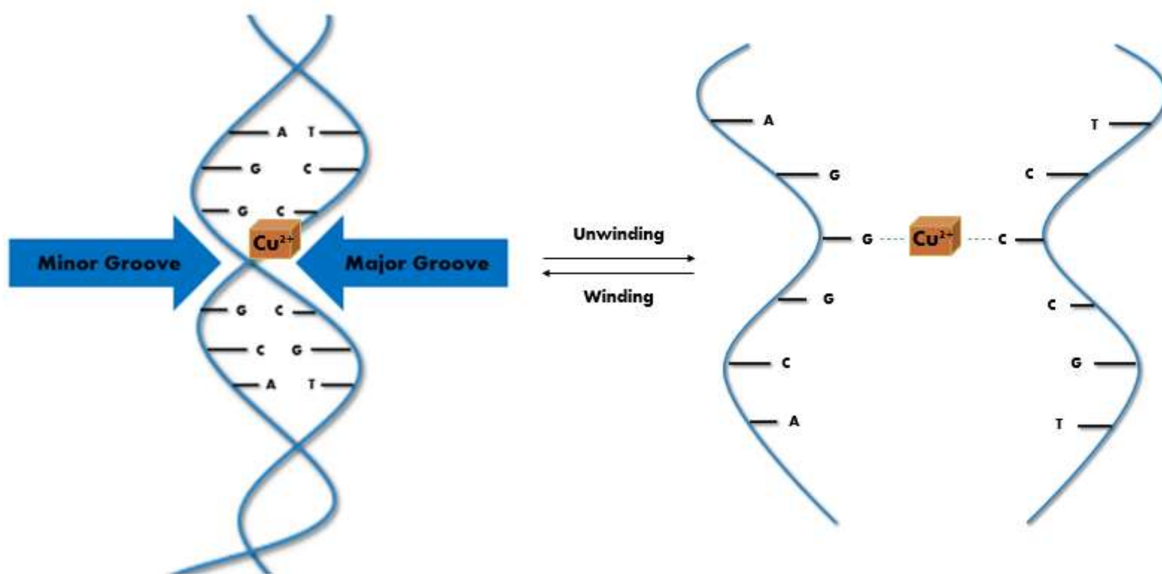

Figure 3. Illustration of site-specific binding of Cu2+ with nitrogenous bases in DNA, resulting in helical disruption (adapted from Shamsi and Kraatz, 2013 [56]).

3.3.2 M-DNA Formation through Outer and Inner-Sphere Binding

The first binding motif of metal to DNA is outer-sphere bonding. In this type of interaction, metal cations indirectly bind with the free oxygen anions on the phosphate backbone of the B-DNA helix via hydrogen atoms from surrounding water molecules [47]. By binding to the phosphate backbone of DNA, the helix is consequently lengthened, changing from its B-DNA conformation to M-DNA. Oftentimes, divalent metal cations, most clearly observed with copper,

will bind first to the anionic phosphate groups [10], disrupting the helical stability and allowing for easier access to the nucleobases.

Much stronger than its counterpart due to direct bonds formed between free electrons of the nitrogen and oxygen atoms on the nucleobases with metal cations is inner-sphere bonding [47]. When metal cations have access to the nucleobases of the DNA helix as a direct result of outer-sphere binding, they disrupt the interaction between bases, increase the winding angle of the helix, and subsequently weaken base stacking. As base stacking is weakened, the conformation of the guanine and adenine residues are altered, thereby allowing those metal cations (i.e., copper) to preferentially bind to the N7 position on guanine, adenine, and cytosine [10].

4. Instrumental Analysis of M-DNA

4.1 M-DNA Observed in Solution

M-DNA complexes have been the focus of much research as it pertains to cancer therapies and the development of novel biophysical models. While these studies (summarized in Table 2) do not explicitly correspond to forensically relevant topics, the information detailed therein lends itself as a useful tool in characterizing the nature of metal-DNA interactions.

Table 2. Brief selection of published research pertaining to M-DNA in an aqueous environment [10, 12, 57–61].

Objective	Findings	Comments and/or Conclusions	Author and Publication Year	Reference
Describe DNA conformation in which divalent metal ions play a major role	Dismutation does not involve strand separation; rate of formation increased with increasing temperature; lowering DNA concentration does not alter rate of transformation	Some features of the Watson-Crick base pairs are retained in M-DNA; imino protons replaced either by zinc or the formation of a complex; addition of EDTA rapidly restores B-DNA	Lee, Latimer, and Reid, 1993	[56]
Investigating if M-DNA behaves as an electron conductor via fluorescence-based assays, titration, and electrophoretic techniques	M-DNA contains a π stack as well as an interchelated metal ion for each base-pair; reformation of B-DNA using EDTA rapidly reversed fluorescent quenching	M-DNA behaves as a molecular wire; it's surrounded by a negatively charged organic sheath allowing for the manipulation of electric fields; M-DNA is readily interconverted with B-DNA	Aich et al., 1999	[57]
Evaluate the effects of divalent metal cations on DNA using CE	Extent of peak-broadening and mobility shifts dependent on the metal cation and its concentration, formamide exposure prior to injection, fragment size and sequence	The presence of metal ions decreased CE performance due to structural changes in the DNA; chelating agents or pH adjustments can reverse effects	Hartzell and McCord, 2005	[12]
Demonstrate conformational changes to DNA induced by copper using UV-vis, melting studies, CD, and fluorescence spectroscopy	Copper binds to nucleobases via altering the helix winding angle; copper binding damages the DNA and changes B-DNA conformation; binding is concentration dependent	Binding efficiency increased proportionally to the degree of helical unwinding; altered B-DNA may affect replication and transcription; copper-induced DNA damage in brain may cause neurodegeneration	Govindaraju et al., 2013	[10]
Use ATR-FTIR to analyze the interaction of platinum complexes with DNA in hydrated,	Platinum II anticancer drugs are disrupting the dsDNA backbone; the transition from B-DNA to A-DNA not	IR spectroscopy can be used to study interactions between some platinum anticancer drugs and DNA	Al-Jorani et al., 2018	[58]

dehydrated, and rehydrated states	reversible after Platinum II drug treatment			
Synthesized a benzimidazole-derived ligand and its copper and zinc complexes; interactions between complexes and DNA studied	Copper complex showed greater binding propensity than zinc complex; both complexes have the potential to cleave DNA	ROS responsible for cleavage activity; activity of complex 1 good on breast cancer cells as a chemotherapeutic intervention	AlAjmi et al., 2018	[59]
Binding interactions of zinc cationic porphyrin with B-DNA and Z-DNA examined using UV-vis and CD	Two coexisting binding modes identified for the zinc cationic porphyrin/Z-DNA complex; major groove binding by zinc coordination with N7 of guanine	Binding occurs only with Z-DNA via intercalation; intercalation results in a structural transition from Z- to B-DNA	Qin et al., 2018	[60]

4.2 Infrared Radiation (IR) Spectroscopy and M-DNA

To better characterize those M-DNA complexes and other structural changes which may be occurring, the infrared spectrum and associated techniques have provided pivotal information on a chemical or biological entity's vibrational modes, such as C-H stretching/bending, C=O stretching, etc. [61]. Infrared radiation (IR) spectroscopy is a universal principle based upon the mechanisms and foundations of light, energy, absorption, transmission, and reflection [62].

4.2.1 IR Spectroscopy

IR spectroscopy, in effect, uses light to measure the energy of bonds by exploiting the relationship between the wavelength of light and the frequency or relative energy of a bond [63]. For each sample tested, a portion of the light emitted is absorbed by the sample and the energy that is not absorbed is thus measured as transmission [62].

A standard output of an IR spectrophotometer provides information on the near- and mid-IR regions ranging from 12800-4000 cm^{-1} and 4000-200 cm^{-1}, respectively. Applications for these regions of IR include microscopic surface studies and attenuated total reflectance analysis of solids and surfaces [63].

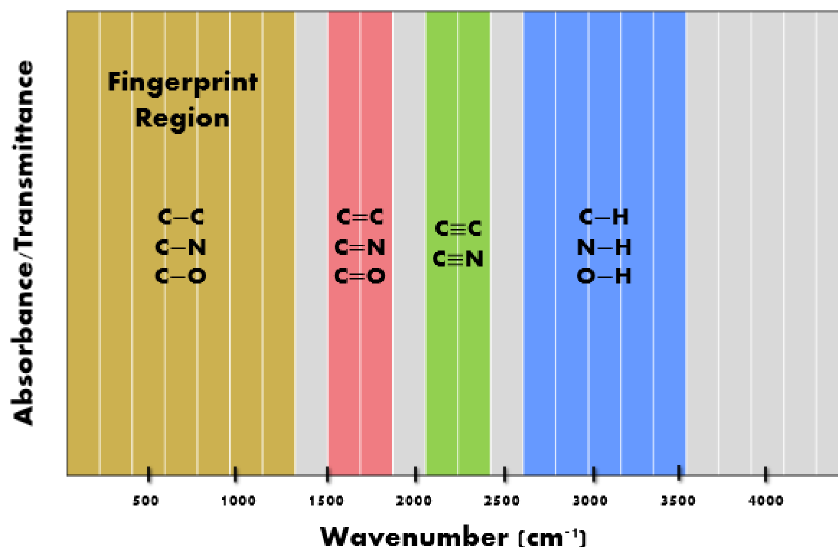

Figure 4. Typical ranges whereby IR functional groups behaviors (i.e.: stretching, bending, etc.) might be observed (adapted from Wade, 2016 [62]).

4.2.2 Attenuated Total Reflectance

Attenuated total reflectance (ATR) is a sampling technique that allows for the surface examination of highly absorbing samples [62]. Originally designed to measure the IR of polymers and films, it is now used for both solid and liquid sample types [62, 63]. A widely used technique that requires little to no sample preparation, ATR has two requirements for all samples tested: direct contact between the sample and ATR crystal, and that the crystal's refractive index is significantly greater than that of the sample [63].

The product of ATR is an IR spectrum. This occurs when an IR reference beam is directed into a highly refractive crystal, thus generating an evanescent wave that travels from the crystal to the sample. When the sample absorbs the energy from that wave, attenuation occurs [63]. The attenuation of the radiant reference beam is accomplished by removing a continuously variable portion of the reference beam in the IR region [63]. The attenuated energy then exits out of the crystal and into the IR detector [63].

4.2.3 Fourier Transform

Fourier transform (FT) spectrometers apply the same principles as other forms of IR spectroscopy with the addition of an interferometer [63]. In Fourier Transform Infrared Spectroscopy (FTIR), the IR beam passes through a Michelson interferometer whereby a beam is split and displaced by a series of fixed and moving mirrors (i.e.: modulation of optical radiation). Constructive and destructive interference is generated by the difference in mirror paths from the split beam for a single pass of the mirror. The displaced beam is recombined, and the intensity of interference light is measured and recorded in the form of an interferogram [63]. The interferogram records the signal from the laser in relation to the time of the mirror's path [63]. The light is then directed into a secondary detector whereby the time-domain signal is transformed into the

frequency-domain by means of Fourier Transform. In the frequency domain, the power of the IR beam is attenuated by the beam's capacity for absorption per sample [63].

FT spectrometers have several advantages as opposed to their counterpart IR instruments. These include a greater throughput, high resolving power, wavelength reproducibility, and simultaneous resolution element detection. Furthermore, the presence of the interferometer ensures that stray radiation does not unduly influence the measurements because each IR beam is split at a different frequency. However, there exist some drawbacks in using FTIR spectroscopy – with the higher throughput comes a lower sensitivity for interferometer measurements [63].

4.3 Spectral Imaging of M-DNA using ATR-FTIR

FTIR spectroscopy has in recent years become a powerful tool in analyzing the structure and characteristics of DNA, as it is an easy and rapid technique. Furthermore, it provides information regarding not only the standard B-DNA structure but has also been useful in demonstrating minute changes from the standard conformation by evaluating the height, width, and shape of the resulting absorption bands present in the spectra. As such, this method has been reported to provide information on DNA in a variety of physical states and quantities and has further been used to evaluate DNA damage (i.e.: degradation, denaturation, etc.) and complexation [64].

Because dsDNA has characteristic absorption peaks at 1690cm^{-1} (C=O of thymine residues), 1647cm^{-1} (C=O of overlapping thymine/cytosine residues), 1213cm^{-1} (phosphate backbone), and 1060cm^{-1} (C-O of deoxyribose), observable changes in the active vibrational modes associated with DNA-metal complexes have been observed in the mid and far-IR regions of normalized FTIR spectra [65]. When using FTIR with attenuated total reflectance (ATR-FTIR) to evaluate the effects of platinum (Pt^{II}) intercalation, disruptions in the conformation of B-DNA have been observed by shifts in the spectrum at 1711cm^{-1}, 1088cm^{-1}, 1051cm^{-1}, and 966cm^{-1} for both hydrated and dehydrated samples [58]. This study demonstrates the potential of ATR-FTIR in the identification of structural changes to DNA as a result of metal interactions, and as such, information regarding the nature of the bonds formed between the DNA and metal could lay the groundwork for collection techniques that might break these bonds without adverse effect on the integrity of the DNA sample.

5. Trace/'Touch' DNA Collection and Extraction from Various Substrates

5.1 Collection Techniques for 'Touch' DNA

Throughout the literature, a variety of collection techniques have been tested to determine the most advantageous recovery method for investigators to employ. These collection approaches have been developed to accumulate the maximum amount of sample from a variety of substrates and include traditional and novel swabbing techniques, tape-lifting, and more recently, vacuum collection [4].

5.1.1 Swabbing

Traditional swabbing techniques employ the use of a moistened cotton swab which is then applied to the entirety of the sample area, whilst continually rotating the swab [15]. However, this

technique has been demonstrated throughout the literature to be sub-optimal in collecting 'touch' DNA in that it oftentimes is only capable of picking up half of the sample present [66]. From this challenge came the double-swab method, whereby a swab is moistened and applied to the sample, followed by a second dry swab, or in some cases, a second wet swab. In the instances of double swabbing, the swab heads are typically co-extracted to ensure as much of the DNA is captured as possible [66, 67].

Oftentimes, DNA-free water is used to moisten the swab, however, in recent years, laboratories have been moving from water to alternative reagents (i.e., ethylenediaminetetraacetic acid, phosphate-buffered saline, etc.) to increase sample yield [67]. In 2017, researchers tested the use of lysis buffer (e.g., containinga detergent such as sodium dodecyl sulfate) as a moistening agent for the collection of 'touch' DNA. It was found that in using lysis buffer, not only did the ability to recover trace DNA from plastic, aluminum, and glass improve, but the sample integrity prior to DNA purification was enhanced [68]. Additional research has proved successful sample recovery using a wet:wet double swab method with sodium dodecyl sulfate (SDS) as the wetting agent [39]. Moreover, it has been reported that when recovering DNA from metal substrates, 0.5% EDTA resulted in significantly greater yields from more challenging metals including copper, brass, and nickel [13].

Improvements to the swab head sampling matrix have been presented as well. Traditional cotton-tipped swabs have dominated the market for years, but more recently, newer swabs have been developed that increase collection efficacy as well as the ability to extract cellular material from the sampling matrix. Nylon flocked and rayon swabs have demonstrated an ability to produce a greater DNA yield and stabilize the DNA for longer periods of time, as the fiber construction more readily releases collected cellular material [45]. However, while these alternate swab types have been examined to improve sampling of cellular material from fingerprint evidence, little has been studied regarding the utility of these swabs for cfDNA.

5.1.2 Tape-lifting

Tape-lifting has become an established recovery method for 'touch' DNA, as it allows for sampling of larger surface areas when compared to swabbing. This technique was developed first in the field of trace evidence and has since been applied to a variety of evidentiary types now including trace DNA evidence. The process of tape-lifting involves, as the name implies, applying tape to a surface to lift a sample by means of the adhesive properties of the tape for subsequent forensic analysis. The idea is that the adhesive on the tape will mechanically retrieve more DNA from surfaces such as wood and clothing by having a stronger attraction to the cellular material, as well as not dilute the sample from a wetting agent. However, considerations regarding the adhesive composition, adhesive strength, manufacture (i.e., sterile or non-sterile), etc. need to be made when selecting tape as the collection technique of choice [38].

Several studies have been performed regarding how the adhesive strength and number of applications increase the yield of DNA recovery, noting that the strength of adhesion varies depending on the type of tape as well as the substrate. Additionally, increasing the number of applications has been reported to increase the quantity of recovered DNA [38, 69]. In a study published in 2020, researchers found that when comparing the efficacy of fourteen different collection tapes, more DNA was recovered using store-bought tapes (specifically Packmate™ brown packing tape) rather than traditional crime scene sampling tapes. Their research also showed that increasing the number of applications of tape onto 'touch' deposits up to ten applications, the

recovery of DNA increased by almost 10% from initial application, whereas after a total of ten applications, the tape became oversaturated with cells (to include both anucleate corneocytes and nucleated epithelial cells) [69]. This is likely due to the chemical properties of the adhesive; however, in using store-bought tapes instead of sterile crime scene collection tapes, there remains the potential issue not only of manufacturing contamination *(refer to the Phantom of Heilbronn* [67]*)* but also the propensity for certain adhesives to increase PCR inhibition. As such, recommendations for sterilizing tapes prior to sample collection have been suggested [69]. However, the recovery efficacy of trace DNA from sterilized tapes, as well as a generalized selection of tapes with the greatest recovery yields have been presented in the literature [69].

5.1.3 Vacuum Filtration and the Bardole Modification

DNA collection by vacuum filtration is a method that has emerged in recent years in which a proprietary sterile rinse solution (SRS) is sprayed on the substrate, dislodging the cellular material from the sample, followed by vacuum suction and filtration of the collected buffer-cell solution. During the filtration process, the collected cellular material is trapped on a polyether sulfone (PES) filter which can then be processed much like a swab head in order to extract DNA for subsequent analysis. This technology has demonstrated itself as a way to overcome limitations in DNA collection from touch samples from a variety of substrates including wood, tape, and fabrics [70, 71]; yet, it has been reported that cfDNA in touch samples might not be collected on the filter [70].

The only current vacuum filtration system on the market that has been used for forensic casework thus far is the M-VAC® (Fig. 4) which has set itself apart from other collection approaches as it collects a greater amount of DNA from those samples in which traditional techniques, such as swabbing, produced an inconclusive result. While swabbing and tape-lifting have not been able to collect a sufficient quantity of DNA from large objects with low levels of DNA, as with trace samples, the M-VAC® has been internally validated by labs for casework and been demonstrated to increase the yield of DNA [70, 71].

Figure 5. Microbial wet-vacuum DNA collection system, alternatively referred to as the M-VAC® [72].

Another approach for collecting DNA from more challenging samples, including cartridge casings, keys, and bomb fragments is the Bardole method. This method, named after Francine Bardole from the West Utah Police Department, was designed by Ms. Bardole in an attempt to collect DNA from small evidentiary items. In this technique, the M-VAC® SRS is added to a sterile plastic collection bottle along with the evidentiary item and vortexed. A concentration PES filter is attached to the bottle and a vacuum suction is then applied to collect and bind the cells. The filter-bound cells can then be used for traditional DNA extraction and processing. While this method has been utilized for smaller items, it is restricted to the size of the collection bottle. However, while there are limitations in size, this method has been successful in collecting sufficient quantities of 'touch' DNA from cartridge casings and keys [71].

5.2 Extraction Methods for Cellular and cfDNA in 'Touch' Samples

DNA extraction using solid phase extraction allows for the isolation of nDNA from cellular deposits in touch samples [15], as well as the separation and purification of cfDNA from these same samples [7, 8]. Existing methods for the recovery of cfDNA have been developed and optimized for plasma; however, less is understood about those kits' potential application for trace DNA evidence. In 2021, researchers evaluated the capability of three cfDNA purification kits, originally designed for plasma, on the acellular component of 'touch' samples [8]. To do so, they presented a centrifugation technique for the separation of cfDNA from cellular components prior to cellular nDNA extraction. Once separated from the cellular fraction, the cfDNA was purified. While the separation via centrifugation was reported successful, the purification of the cfDNA fraction demonstrated that bead-based purification was the most effective, as it resulted in greater DNA yields than other approaches [8]. It was concluded that further study into the recovery of cfDNA was essential for future analysis of 'touch' DNA samples.

6. Theoretical Considerations

In achieving the project objectives (see Section 1) and given consideration of the theoretical framework (see Sections 2-5), there are listed below the foundational assumptions and limitations which must be acknowledged.

6.1 Foundational Assumptions

1. This project was developed assuming that the majority of DNA deposited in a touch sample was done so upon initial contact with the surface/object and does not include considerations for transfer or repeated depositions.

2. This project was designed under the premise that 'touch' DNA is not only shed nucleated epithelial cells and anucleate corneocytes, but also a majority of cfDNA. Therefore, a separation method for both cellular and cfDNA fractions of a sample was evaluated.

3. If, the majority of DNA deposited in a 'touch' sample is acellular, and the acellular DNA is not protected by any nuclear envelope or cellular membrane, then it is assumed that that cfDNA will more readily bind to those transition metals, forming M-DNA complexes.

6.2 Project Limitations

1. While shedder variability has been documented throughout the literature, there remain gaps in our understanding of how to classify one's shedder status. the objective of this thesis is to evaluate the nature of cfDNA's interactions with metal substrates and optimize collection and extraction of touch DNA using mock evidentiary samples. Consequently, the natural variability between donors is accepted and not considered in extensive detail due to the lack of research characterizing the variability in cfDNA deposition.

2. Despite research demonstrating the potential of DNA recovery from keratinocytes using lysis methods traditionally applied to hair samples, the standard processing for cellular portions of touch samples does not consider highly keratinized samples. Furthermore, there has yet to be research evaluating the effect of such lysis methods on the integrity of cfDNA. This thesis seeks to optimize DNA recovery from touch samples, and as such methods to extract DNA which may be present in keratinocytes was excluded.

Part II

A four-part study was conducted to qualitatively examine the binding effects of copper with cfDNA, as well as evaluate the efficacy of swabbing, tape-lifting, and the M-Vac® in recovering cellular and cfDNA from touch deposits from metal substrates. Additionally, a centrifugal separation technique for cellular and acellular fractions of touch samples was attempted to characterize the influence of each fraction on overall sample quantity and profile quality. The methods and findings therein will be presented in this part of the thesis, with the aim to optimize collection and extraction parameters for touch DNA samples on those more challenging metal substrates.

Prior to the start of this research study, ethics approval was obtained from the West Virginia University Institutional Review Board (Protocol I.D.: 2205577144). Informed consent was obtained from all volunteers who provided saliva and "touch" samples in accordance with the guidelines as detailed in the project's HIPPA IC Authorization Consent (OHRP-38) forms.

7. Evaluation of Trace DNA Recovery Techniques *(Preliminary Study)*

7.1 Materials and Methods

7.1.1. Sample and Substrate Preparation

Prior to sample deposition and recovery, a participant's saliva sample was acquired. The individual was instructed to abstain from eating, drinking, and/or use of any dental hygiene products for at least one hour prior to sample collection. A total of 150 mL of saliva was collected, and the extracts of DNA in ten, 50 µL aliquots acquired by processing each using Qiagen QIAamp® DNA Investigator Kit according to the manufacturer's "Isolation of Total DNA from Small Volumes of Blood and Saliva" protocol. The concentration of DNA in each extract was evaluated using the Quantifiler™ Trio kit with the Applied Biosystems™ 7500 Real-Time PCR System using half-reaction volumes. The average concentration was calculated across all ten replicates and used to determine approximate quantity of DNA deposited and subsequent recovery efficacy.

One of each copper, brass, and stainless-steel sheet metal (Hillman™ Metals, Hayward, CA) was cut into 4x5 inch pieces. Each sheet was sterilized using a 70% ethanol wash followed by UV irradiation for 30 min. Following decontamination, 10 µL of saliva was deposited onto the metal and allowed to dry. This process was repeated for the TaqMan Human Control DNA (ThermoFisher Scientific©, Waltham, Massachusetts).

7.1.2. Sample Collection

Once the stock saliva and TaqMan Human Control DNA had dried onto the substrate, samples were collected in triplicate (N=72) using the following methods: double-swab, tape-lifting, and the M-Vac® (M-Vac® Systems Inc., Sandy, Utah).

7.1.2.1 Wet:dry double-swab.

Sterile cotton tipped wood swabs were obtained in sets of two. For all samples, the first swab from a set was moistened using 100 µL of nuclease-free water. The sample area was swabbed with the moistened cotton swab, and the swab head broken off into a sterile 1.5 mL microcentrifuge tube. The second swab within the set was then used dry to swab the entire sample area again, then broken off into the same 1.5 mL microcentrifuge tube. This procedure was repeated for another sample set using 0.5M EDTA as the moistening agent. All swabs were stored at -20 °C until extractions were performed.

7.1.2.2 Tape-lifting

Using a sterile pair of surgical scissors for each sample, a 1x1 in piece of Scotch™ heavy duty packing tape (3M©, Saint Paul, Minnesota) was cut. Holding the corner of each tape strip with a sterile pair of tweezers, the tape was firmly pressed onto the sample area, lifting for a total of ten (10) lifts per sample. The tape lifts were carefully rolled with the tweezers, placed into a sterile 1.5 mL microcentrifuge tube, and stored at -20 °C until extractions were performed.

7.1.2.3 Vacuum filtration

For those samples collected using the M-Vac® wet-vacuum DNA collection system, a new nozzle (M-Vac® Systems Inc., Sandy, Utah) and sterile Nalgene™ Rapid-Flow™ disposable PES filter (ThermoFisher Scientific©, Waltham, Massachusetts) were attached to the instrument for each sample set and sample, respectively. Unidirectional sampling, in accordance with manufacturer's guidelines, was employed to dislodge the sample from the substrate, using a total of 50 mL of sterile rinse solution (M-Vac® Systems Inc., Sandy, Utah) for each sample. The resulting flow-through from those M-Vac® collected samples was filtered a total of three (3) times to improve recovery of cellular material from the solution. Using sterile scalpel and tweezers, the PES filter containing the collected sample was cut from the filter unit (Nalgene™ Rapid-Flow™, ThermoFisher Scientific©, Waltham, Massachusetts), placed into a sterile 1.5 mL microcentrifuge tube, and stored at -20 °C until extractions were performed.

7.1.3. DNA Extraction and Quantitation

For all collected swab and M-Vac® samples, manual extractions were performed using the Qiagen QIAamp® DNA Investigator Kit according to the manufacturer's "Isolation of Total DNA from Surface and Buccal Swabs" protocol using Qiagen DNA Mini Spin Columns. All tape-lifted samples were processed similarly according to the manufacturer's "Isolation of Total DNA from Chewing Gum" protocol. All manual extractions were performed with the addition of 1 µL of carrier RNA to improve extraction efficiency and eluted to a final volume of 25 µL. Quantitation was performed as detailed previously using the QuantifilerTM Trio kit.

7.1.4 Data Analysis

DNA quantity was determined as detailed previously with consideration to manufacturer thresholds. For all preliminary (saliva and TaqMan Human Control DNA) data, the amounts of DNA recovered using each of the three recovery techniques were expressed as percentages of the initial amount deposited. This was also done across substrate types.

All raw data was compiled in JMP® Statistical Software (SAS® Institute, Cary, NC) for statistical analysis. A quantile-quantile plot was used to test the residuals for normal distribution of the data. Due to the low numbers evaluated in these data, to include both preliminary and primary datasets, a logarithmic transformation was applied to all data and a two-way fixed ANOVA with a balanced design performed. A Student's *t*-test was applied for all pairwise comparisons.

7.2 Results and Discussion

7.2.1 Recovery Rates

To assess general trends in both the efficacy of swabbing, tape-lifting, and the M-Vac® wet-vacuum DNA collection system on DNA recovery from metal substrates, as well as to consider the substrates' interaction effects on recovery, both mixed cellular and cfDNA samples (saliva) and exclusively cfDNA samples (TaqMan Human Control DNA) were deposited onto copper, brass, and stainless-steel substrates. The total DNA recovered for all sample types was only a fraction of the initial amount of DNA deposited for each saliva (~ 6 ng) and TaqMan Human Control DNA (100 ng).

For saliva samples deposited across all metal types, the maximum expected yield was estimated at 6 ng, with observed yields measuring 3.0% of expected for copper, 6.1% of expected for brass, and 4.7% of expected for stainless steel. While low, these data are comparable with the literature in that research has shown that significant quantities of DNA (up to 76%) can be lost during the collection and extraction procedures [54]. These data illustrate that despite the metal composition and when considering mechanical means of recovery, DNA yields initially present in low quantities will remain low after collection and extraction processes. This can be likely attributed to the lack of porosity and texture seen in metal substrates as opposed to wood or fabrics, which have been demonstrated to collect greater quantities of cellular material by sloughing and retaining more cellular material than a smooth, non-porous surface [14, 37].

Our collection and extraction efficiencies were lower than those ~15-30% previously reported for recovery of trace DNA from non-porous surfaces [14]. The reduction in recovery for our data could be due to differences in the DNA source as well as the respective collection protocol employed (i.e.: quantity of repeated tape lifts, M-Vac® sampling technique, etc.). Furthermore, the swab type evaluated in this study was cotton whereas other studies have evaluated the collection of trace DNA from non-porous surfaces using nylon-flocked swabs which have been reported to have a greater propensity to release DNA and increase yields [14].

The maximum expected yield for the TaqMan Human Control DNA was estimated at 100 ng. For all TaqMan Human Control DNA deposits, observed recovery yields of that DNA from the total elution for copper and brass were <1% of expected, whereas recovery was improved for stainless steel with resulting yields measuring 1.1% of expected. These findings are congruous to that reported in the literature whereby recovery of trace DNA from metal cables was <2% [14].

Lower recovery rates were predicted for all cfDNA samples as the DNA is unprotected from the cellular membrane and nuclear envelope and is, therefore, more susceptible to environmental agents, degradation, and transfer [4]. As illustrated by these data, the unrecovered DNA is likely resultant from the effects of metal composition on the unprotected, cfDNA, as it has been demonstrated both here (see Section 9.1) and *in vitro* that select transition metals, particularly

copper, have a propensity to chemically bind to the DNA backbone and nucleobases, forming metal-DNA interactions (M-DNA) [11].

7.2.2 Treatment and Substrate Effects

For mixed cellular and cfDNA samples (saliva deposits), results of a two-way ANOVA (Fig. 6) demonstrate a significant difference ($p < 0.05$) in trace DNA recovery between treatments (i.e.: recovery methods) irrespective of substrate, with tape-lifting resulting in overall greater mean recovery, followed by swabbing using nuclease-free water as a wetting agent. A statistically significant difference can also be observed ($p < 0.05$) in trace DNA recovery across materials irrespective of recovery approach, with copper having the lowest propensity to release DNA

Student's *t*-tests pairwise comparisons between sampling methods showed that for mixed cellular and cfDNA sample types, tape-lifting resulted in significantly greater ($p < 0.05$) DNA yields (0.82 ng) than either the wet:dry double-swab using 0.5M EDTA (0.02 ng) or the M-Vac® vacuum collection and filtration system (0.07 ng). Statistically significant differences ($p < 0.05$) were also observed between both wet:dry double-swabs and the M-Vac sample collection method, as well as for both swabbing approaches across all metal types. However, no significant differences ($p > 0.05$) were found between tape-lifting (0.82 ng) and the wet:dry double-swab using nuclease-free water (0.62 ng) as the wetting agent.

It is presumed here that for all mixed sample types across all substrates, what is being collected is not both cellular and cfDNA, but is primarily, if not all, cellular. Our findings are congruous with those of Prasad et al. [72], in that tape-lifting resulted in a higher DNA yield than swabbing when evaluating mechanical techniques for recovering cellular material, thus increasing intracellular DNA yields. Furthermore, it may be that the cotton matrix is more tightly trapping the collected cellular material, whereas those cells are exposed on the adhesive surface of the tape and thus more easily lysed. The insignificant differences ($p > 0.05$) observed between tape-lifting and the wet:dry double-swab with nuclease-free water further support this discrepancy in recovery efficacy.

For all mixed cellular and cfDNA sample types, no statistically significant differences ($p > 0.05$) were observed between brass and stainless steel, whereas copper was found to be different from the other substrate materials. However, this is believed to be caused by variability in the composition of sample deposited. It should be noted that while efforts were made to ensure equitable deposition of cellular and cfDNA from homogenized saliva deposits, the actual concentration of DNA deposited on the substrates was unknown. As a series of saliva samples were quantitated and their DNA concentrations averaged, without making a cell-suspension of a known quantity of cells, the ground truth can only be estimated.

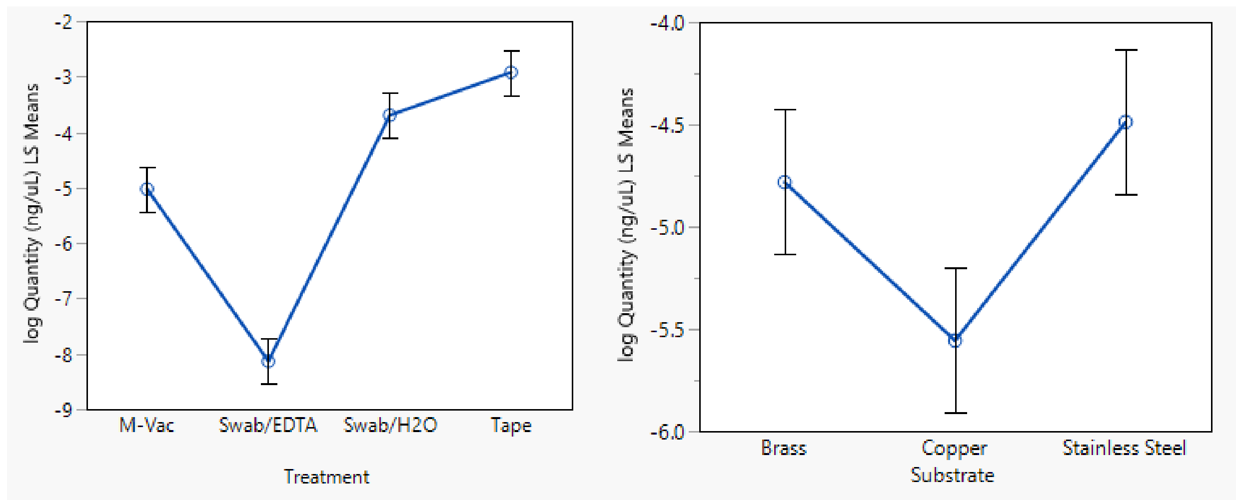

Figure 6. Least squares mean plots of average quantitated trace DNA yields as recovered from brass, copper, and stainless steel. For all samples (N = 45), ~ 6 ng of mixed cellular and cfDNA was deposited in the form of saliva.

For exclusively cfDNA samples (TaqMan Human Control DNA deposits), results of a two-way ANOVA (Fig. 7) demonstrate a significant difference ($p < 0.05$) in trace DNA recovery between treatments (i.e.: recovery methods) irrespective of substrate type, with swabbing using nuclease-free water resulting in overall greatest mean recovery, followed by tape-lifting. A statistically significant difference can also be observed ($p < 0.05$) in trace DNA recovery across materials irrespective of recovery approach, with stainless steel releasing the greatest amount of cfDNA.

The extremely low cfDNA yields observed for all collection techniques (Fig. 7) was found to be directly influenced by the substrate composition. In Figure 7, copper-containing materials (i.e.: copper and brass) were observed to release statistically significantly ($p < 0.05$) lower amounts of DNA from the substrate when compared to stainless-steel. Where copper has been demonstrated to inhibit sample recovery due to formation of M-DNA complexes [11], the lack of sample displacement by means of a chelating agent to preferentially bind to the divalent copper ions versus the DNA would mean that that cfDNA is still chemically fixed to the substrate. These data pertaining to the collection of cfDNA using 0.5M EDTA as a wetting agent do not correspond to that reported in the literature by Holland et al. whereby samples were swabbed [13]; however, this could be, in-part, explained by the type of DNA collected in their study as opposed to here (mitochondrial versus cfDNA), as well as the lack of interaction time for sample displacement (see also Section 9.2).

Substrates presented here that were copper containing (i.e.: copper and brass), and therefore presumed to form M-DNA, cannot be differentiated ($p > 0.05$) based on quantity of recovered DNA (Fig. 7). These results were congruous with expectations, as previous research under controlled conditions has demonstrated it is more difficult to retrieve trace DNA from copper containing alloys, as observed in brass cartridge casings versus nickel-plated cartridge casings [14, 39, 43]. Furthermore, the copper composition of brass has been reported to make up 70% of the alloy [51], as observed by the similarities in recovery from both the copper and brass materials tested here.

This data reinforces the findings that DNA recovery from copper and brass substrates remains problematic for analysts irrespective of collection technique, especially when considering

‘touch’ DNA samples which are comprised of majority cfDNA [7]. Not only is cfDNA unprotected, due to its mode of generation, it is initially more fragmented than intracellular DNA [73], potentially providing more M-DNA binding sites. Furthermore, the statistically significantly higher yields in cfDNA recovery ($p < 0.05$) from tested materials lacking copper ions (i.e.: stainless steel) serve to demonstrate that without those critical divalent copper ions present, metal surfaces are likely no more challenging for sample recovery than other non-porous substrates (i.e.: glass) [37, 42].

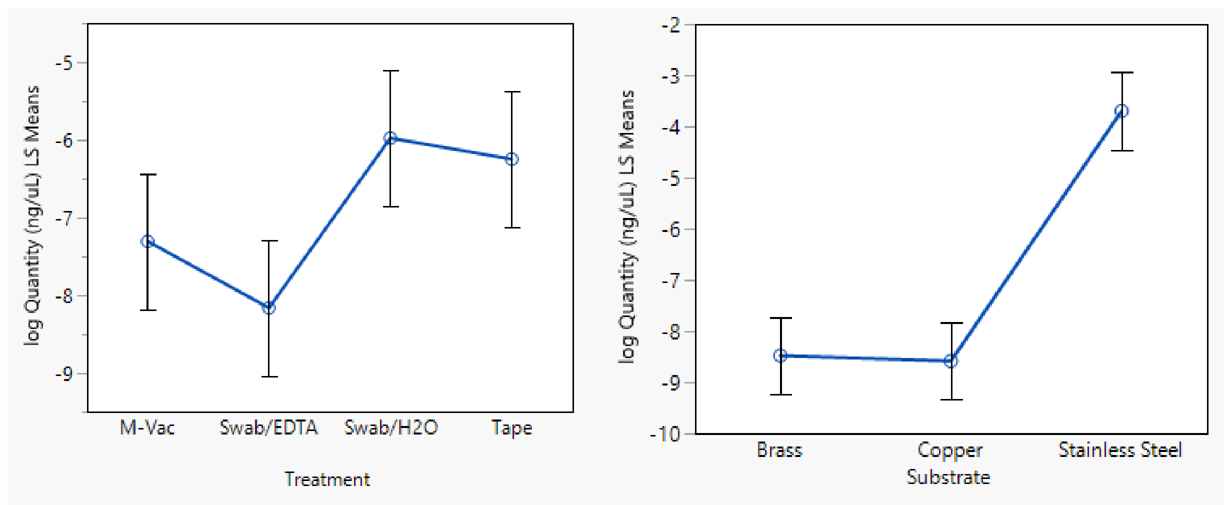

Figure 7. Least squares mean plot of average quantitated cfDNA yields as recovered brass, copper, and stainless steel. For all samples (N = 45), ~ 100 ng of cfDNA was deposited in the form of TaqMan Human Control DNA.

8. Evaluation of Trace DNA Recovery Techniques *(Primary Study)*

8.1 Materials and Methods

8.1.1. Participant Selection

Upon approval by the West Virginia University Internal Review Board, informed consent was obtained by five (5) donors. Each donor was aged 20-30 years old, to include both male and female participants.

8.1.2. Sample and Substrate Preparation

Glass microscope slides and one of each copper and brass sheet metal was cut into 4x5 inch pieces. Each slide and/or sheet was sterilized using a 70% ethanol wash followed by UV irradiation for 30 min.

Each participant was instructed to self-groom for approximately thirty (30) seconds upon arrival. No handwashing was permitted for one (1) hour prior to grooming or sample deposition to mimic real-life transfer of DNA from touching one’s face and arms to different surfaces. Once the grooming period concluded, participants were instructed to firmly place their thumb, pointer

finger, and middle finger on one (1) of the DNA-free substrate types for thirty (30) seconds per finger using their dominant hand, totaling one and a half (1.5) minutes deposition time per sample. This process was repeated for the remaining items at approximately the same time each day the following weeks to ensure consistency in deposition circumstances. After sample deposition, items were immediately transferred to sample collection.

8.1.3. Sample Collection

All touch samples were collected in triplicate from five (5) participants (N=135) as detailed in Section 7.1.2, excluding that wet:dry double-swab approach using 0.5M EDTA as a wetting agent.

8.1.4. DNA Extraction and Quantitation

For all collected swab and M-Vac® samples, manual extractions were performed using the Qiagen QIAamp® DNA Investigator Kit according to the manufacturer's "Isolation of Total DNA from Surface and Buccal Swabs" protocol using Qiagen DNA Mini Spin Columns. All tape-lifted samples were processed similarly according to the manufacturer's "Isolation of Total DNA from Chewing Gum" protocol. All manual extractions were performed with the addition of 1 µL of carrier RNA to improve extraction efficiency and eluted to a final volume of 25 µL. Quantitation was performed as detailed previously using the Quantifiler™ Trio kit.

8.1.5 Data Analysis

DNA quantity was determined as detailed previously with consideration to manufacturer thresholds. For all preliminary (saliva and 007 human control DNA) data, the amounts of DNA recovered using each of the three recovery techniques were expressed as percentages of the initial amount deposited. This was also done across substrate types. For all primary (touch DNA) data, participant deposition variability across substrate types and recovery approaches was additionally evaluated.

All raw data was compiled in JMP® Statistical Software (SAS® Institute, Cary, NC) for statistical analysis. A quantile-quantile plot was used to test the residuals for normal distribution of the data. Due to the low numbers evaluated in these data, to include both preliminary and primary datasets, a logarithmic transformation was applied to all data and a two-way fixed ANOVA with a balanced design performed. A Student's *t*-test was applied for all pairwise comparisons.

8.2 Results and Discussion

8.2.1 Treatment and Substrate Effects

For all touch samples, results of a two-way ANOVA (Fig. 8) demonstrate a significant difference ($p < 0.05$) in touch DNA recovery between treatments (i.e.: recovery methods) irrespective of substrate composition, with tape-lifting resulting in overall greater mean recovery. Glass also demonstrated a greater propensity to release DNA as opposed to those copper-

containing materials for all recovery methods. These findings are congruous with previously demonstrated results both here (Figs. 6 and 7) and throughout the literature [18].

The variability in recovered 'touch' DNA yields as observed here ranges from 0 to ~ 1 ng DNA after elution to 25 µL. If, assuming the deposited sample is primarily cfDNA, then results are lower than those of Miller et al. [74], where variability in cfDNA recovery from trace DNA samples ranged between 0 and > 10 ng DNA. If, however, we consider the contribution of intracellular DNA documented by Miller et al. [74] to be between 0 and ~230 pg DNA, then these data here are still within expectations, as the recovery observed, excluding tape-lifting from copper samples, reaches a maximum of ~130 pg DNA. Given the recovery approaches (tape lifting) are mechanical in nature, then it would be expected that the recovered DNA results from collected cellular material.

Despite the present variability across all collection techniques as seen with copper (Fig. 12), tape-lifting consistently produced statistically significantly ($p < 0.05$) greater yields of DNA (0.37 ng) as opposed to the wet:dry double swab (0.03 ng) or the M-Vac® wet-vacuum DNA collection system (0.04 ng). This is consistent with those findings recorded by Prasad et al. [41, 72] in that, for trace and 'touch' DNA deposited onto brass cartridge casings, tape-lifting produced the highest yields as compared to swabbing, vacuum collection, and soaking. As such, it was recommended as the most suitable approach, given the parameters tested therein, for DNA recovery from cartridge casings. Based off findings presented both here and, in the literature, [41, 72], it is presumed that what is primarily being collected is the cellular component of DNA, as each of the tested methods are mechanical in nature, with the adhesive of the tape having a greater propensity to collect a greater number of shed, and subsequently deposited, epithelial cells.

It should also be noted that while efforts were made to ensure equitable deposition of 'touch' DNA, the actual quantity of DNA deposited on the substrates was unknown and likely variable [75] between participants across collection days. Nevertheless, these trends observed both here with 'touch' deposits as well in preliminary testing reinforce the need for additional research concerning 'touch' DNA recovery from metal substrates, particularly focused on the interactions between cfDNA and copper, as well as the contribution of cfDNA to a 'touch' deposit.

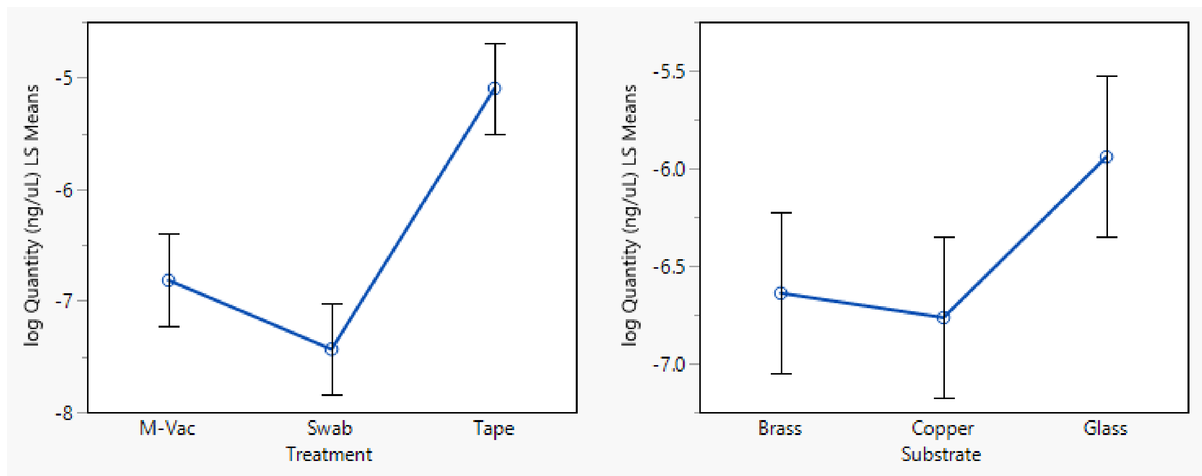

Figure 8. Least squares mean plot of average quantitated DNA yields as recovered using the M-Vac®, swabbing, and tape-lifting. For all samples (N = 135), participants firmly placed their thumb, index, and middle fingers onto the substrate for a total of 90 seconds.

8.2.2 Interaction Effects Observed when Recovering 'Touch' DNA

The results from the two-way ANOVA (Fig. 9) reveal effects between recovery approaches and substrate material were found to be insignificant ($p > 0.05$); however, pairwise comparisons using the Student's *t*-test reveal that few interactions were significant. For all 'touch' DNA deposits, samples collected via tape-lifting were not statistically significantly different across substrate types ($p > 0.05$). Additionally, for all substrate materials, all samples collected using the M-Vac® and wet:dry double-swab approach, with the exception of M-Vac® touch DNA recovery from glass, were not found to be statistically significantly different ($p > 0.05$).

In Figure 9, these interaction effects can be observed by the overlap in trendlines whereby the mean concentration of recovered 'touch' DNA for copper and brass substrates was quantitatively similar, with the exception of M-Vac® collected samples. The distance observed between glass samples as compared to brass and copper substrates is also consistent with expectations, as it was not expected to hinder 'touch' DNA recovery but to simply serve as a control. For all averaged yields, interactions were similar for brass and glass, whereas copper diverged from the status quo for both M-Vac® and tape-lifting samples. This is consistent with previously documented touch DNA recovery from non-porous substrates [14, 37, 39, 41–44, 72] whereby DNA-metal interactions resulting in low DNA yields make it a challenging surface for sample recovery.

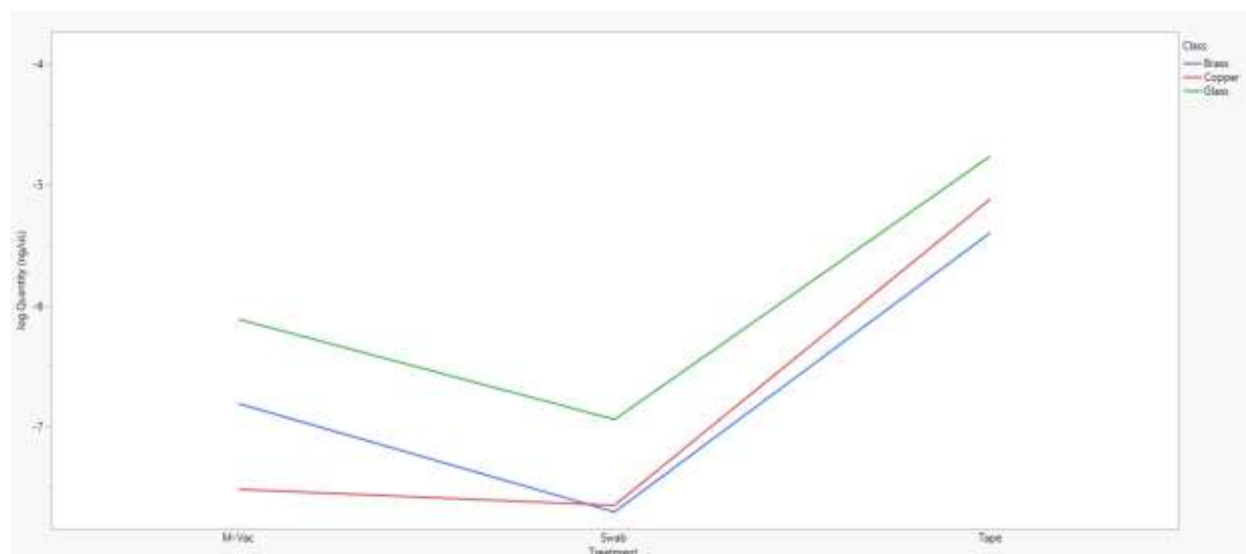

Figure 9. Interactions effects between treatment and substrate material for all 'touch' deposits. For all samples (N=135), interactions were found to be insignificant ($p > 0.05$); however, similar trends can still be observed for all metal-types with swabbing and tape-lifting, as well as between brass and glass for the M-Vac® and swabbing approaches.

Given these findings, it appears that when attempting to collect 'touch' DNA from non-porous materials, including those traditionally challenging metal substrates, tape-lifting should be considered a primary approach, as cellular DNA yields were consistently greater for all treatments and substrate types, provided that latent fingermark evidence need not be collected afterwards. Not only does this approach provide more cellular material for analysts to work with in a lab, something

of critical importance, but it additionally is an inexpensive alternative to other techniques such as the M-Vac® which necessitates either the use of large volumes of reagents or requires several non-reusable attachments to collect a single sample. It should be noted that the tape examined here in this study was packing tape, and as such, a sterile alternative should be tested in future work to ensure there is no contamination present from manufacturing *(consider the Phantom of Heilbronn case)*. Nevertheless, this data, when considering the interaction effects as present in 'touch' samples between recovery approach and substrate material, provide useful information that should be considered by practitioners as 'touch' DNA evidence becomes more prevalent in crime laboratories.

8.2.3 Participant Variation Observed in 'Touch' Deposits

For all recovery approaches and substrate types, it should be noted that there were significant differences observed across participants in the quantity of DNA recovered consistently throughout the collection period. On average, Participant #3 deposited significantly greater amounts of DNA, as indicated in recovery means. While it is widely accepted that shedder variation exists and is not well characterized, this increase in sample deposition was found to significantly ($p < 0.05$) elevate recovery yields when compared to all other participants (Fig. 10).

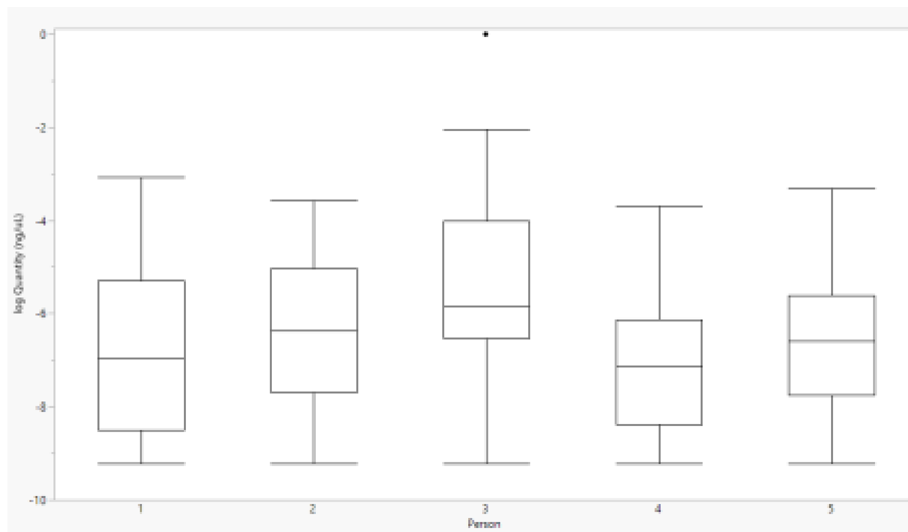

Figure 10. Participants effects for all 'touch' deposits (N = 135). For all samples, effects were found to be statistically significantly different using a Student's t-test ($p < 0.05$) for Participant #3, as observed by the outlying sample in the upper third of the figure; however, similar interactions can still be observed for all other participants involved in the study.

While not the primary focus of this study, it is interesting to note that variation that exists in touch deposits collected on subsequent days for the same participant, so much so that yields were found to also be significantly greater ($p < 0.05$) for the outlying sample which was 'touch' DNA recovered from copper by tape-lifting. This is important to note because it illustrates the necessity for further characterization in 'touch' DNA depositions, be it shedding of cellular material or improved recovery and processing of cfDNA yields given their relative contribution of DNA within a 'touch' sample.

9. DNA-Metal Interactions

9.1 Materials and Methods

9.1.1 Substrate Preparation

A total of two materials were considered in this study: glass microscope slides (AmScope™, Irvine, CA) and copper sheet metal (Hillman™ Metals, Hayward, CA). All substrates were decontaminated using a 70% ethanol wash, irradiated under UV light for 30 mins, and allowed to dry under a fume hood.

9.1.2 IR Instrumental Parameters

Fourier Transform Infrared Spectroscopy (FTIR) with Universal Attenuated Total Reflectance (U-ATR) was used to evaluate the bonds formed between cfDNA with copper. A PerkinElmer® Spectrum Two™ FTIR (PerkinElmer® Inc., Waltham, MA) with an ATR diamond crystal attachment collected spectra from 4000 cm^{-1} to 400 cm^{-1} with resolution set to 4 cm^{-1}. All samples were compressed against the crystal at a Force Gauge of approximately 95, and spectra collected across 64 scans.

9.1.3 IR Spectra and Sample Rinsing

To evaluate cfDNA interactions with copper, spectra were collected of the DNA sample on the glass and copper surfaces using ATR-FTIR spectroscopy (see Section 9.1.2 for instrumental parameters) both before and after rinsing with nuclease-free water and 0.1X TE buffer.

On the glass microscope slides, 10 µL of extracted DNA (~ 120 ng), eluted in nuclease-free water, was aliquoted and allowed to dry. Once dried on the surface, the sample area was measured using ATR-FTIR spectroscopy. Following initial measurement, 200 µL of nuclease-free water was deposited onto the sample area, allowed to remain for approximately two minutes, and the rinse collected with a pipette in a sterile 1.5 mL microcentrifuge tube. The sample area, after rinsing, was allowed to dry and re-measured. Following measurement, 0.1X TE buffer was used to rinse the sample area again, the rinse collected, and the sample area re-measured. This process was also repeated for all copper substrates.

9.1.4 Evaluation of Wetting Agents

To evaluate the efficacy of nuclease-free water and 0.1X TE buffer in the collection of cfDNA (i.e., extracted DNA eluted in water) from copper and glass, 10 µL of extracted DNA (~130 ng), eluted in nuclease-free water, was aliquoted onto the two substrates and allowed to dry.

Once dried on the surface, sterile cotton tipped wood swabs (Uline©, Pleasant Prairie, Wisconsin) were used for sample collection. For all samples, a swab was moistened using 100 µL of nuclease-free water, the sample area was swabbed for approximately 30 seconds with the moistened cotton swab, and the swab head broken off into a sterile 1.5 mL microcentrifuge tube. This procedure was repeated for another sample set using 0.1X TE buffer as the moistening agent.

To recover DNA from the swab matrix, manual extractions were performed using the Qiagen QIAamp® DNA Investigator Kit (Qiagen©, Hilden, Germany) according to the

manufacturer's "Isolation of Total DNA from Surface and Buccal Swabs" protocol using Qiagen DNA Mini Spin Columns (Qiagen©, Hilden, Germany). All manual extractions were performed with the addition of 1 µL of carrier RNA to improve extraction efficiency and eluted to a final volume of 25 µL. All extracts were quantified as previously described.

9.1.5 Data Analysis

The resulting.csv files containing the raw spectral data from all samples were processed using RStudio® (RStudio®, Boston, MA). All spectra were plotted and qualitatively evaluated to determine if there were any noticeable peak shifts, significant alterations in transmittance, or to identify and characterize the presence or loss of any peaks.

Using the quantitation data from Quantifiler Trio, for both copper and glass substrate data, the amounts of DNA recovered using each of nuclease-free water and 0.1X TE buffer wetting agents were expressed as percentages of the initial amount of DNA deposited.

9.2 Results and Discussion

There have been numerous studies using spectroscopic techniques to illustrate the basis of DNA-metal interactions, specifically with reactive oxygen species (ROS)., however, these studies are often conducted under optimal binding conditions by spiking DNA samples with copper nanoparticles or by using commercially pooled DNA [45]. Neither scenario provides a realistic model for determination of DNA-metal binding in a forensic casework scenario. The present study is considering only cfDNA-metal interactions since pure, extracted DNA is used and recent studies have documented that the majority of 'touch' DNA is comprised of cfDNA [7, 8].

9.2.1 Spectral Characterization

Infrared spectroscopy can help in understanding the affinity of copper with DNA, and the ways in which that copper complexes with DNA, forming M-DNA [64]. Various papers (Table 2) have reported using NMR and micro-FTIR, among other approaches, to examine this phenomenon *in vitro* [10, 12, 56–60, 64]. To evaluate copper-DNA complexation from a surface-level interaction, extracted DNA (eluted in water) was aliquoted onto copper and glass surfaces, and the signal measured. While it is known that DNA produces peaks with weak infrared intensity [64], in Figures 11 and 12, it can be observed that when DNA is fixed to the surface of non-porous materials, it is still able to be detected using ATR-FTIR in transmittance mode at a very low relative intensity.

Across all samples, there were several strong spectral bands identified at approximately 3000 cm^{-1}, 1600 cm^{-1}, 1500 cm^{-1}, and 1000 cm^{-1}. Those strong peaks located in the region between 1800 cm^{-1} and 1500 cm^{-1} have been documented to correspond with the nucleobases' purine and pyrimidine rings [64]. Additionally, those bands present between 1500 cm^{-1} to 1250 cm^{-1} and 1200 cm^{-1} to 800 cm^{-1} have been associated with sugar-nucleobase vibrations and phosphate groups located on the backbone, respectively [64].

For those samples fixed to glass (Fig. 11), the major peaks indicative of DNA can be easily visualized due to the contrast between the glass background spectrum and the sample with DNA introduced. No characteristic peaks were present when glass was scanned independently, and therefore, all peaks in the DNA-containing spectrum were determined to be that of the cfDNA

sample. Upon addition of DNA, there were detected strong sharp peaks at ~ 1600 cm^{-1} and 3000 cm^{-1}. Those peaks observed at ~ 1600 cm^{-1} have been reported to be characteristic of in-plane base vibrations in adenine, guanine, and cytosine, as well as C=N ring vibration in adenine and guanine residues [64]. At ~3000 cm^{-1}, C-H stretching can be observed, perhaps from alkanes and alkenes present in the nucleobases [61].

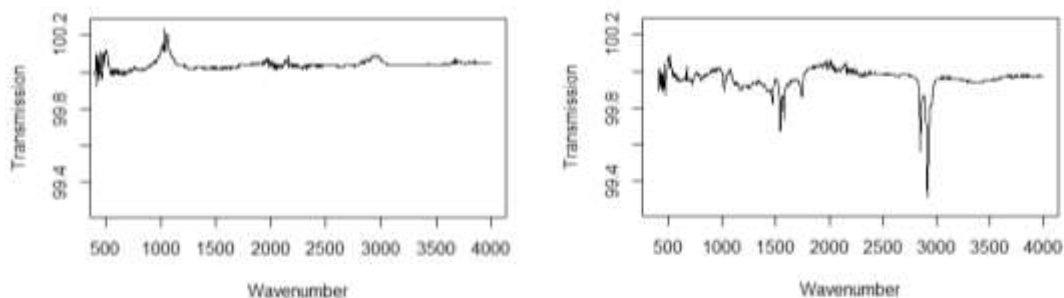

Figure 11. FTIR spectra of glass microscope slide both without (left) and with (right) extracted DNA (eluted in water) dried on its surface. The presence of the strong doublet in the spectra to the right at ~ 3000 cm-1 is indicative of C-H stretching, as well as those peaks may also be reflective of N-H stretching of the nitrogen residues of the nucleobases. Furthermore, those peaks in the spectra to the right appearing from ~ 1600 cm^{-1} and on into the fingerprint region provide further support that those peaks are originating from the DNA sample and not from a contaminant.

For samples dried on copper (Fig. 12), the major peaks indicative of DNA cannot be readily visualized due to elevated background noise created by the copper during measurement collection. However, while there is little contrast between the copper background spectrum and the spectrum associated with the DNA-containing sample, slight alterations between samples can be observed at ~ 1000 cm^{-1} (moderately strong peak characteristic of C-O stretching of the deoxyribose) and ~ 3000 cm^{-1} (weak broad peak potentially characteristic of C-H stretching). No characteristic peaks were present when copper was scanned independently.

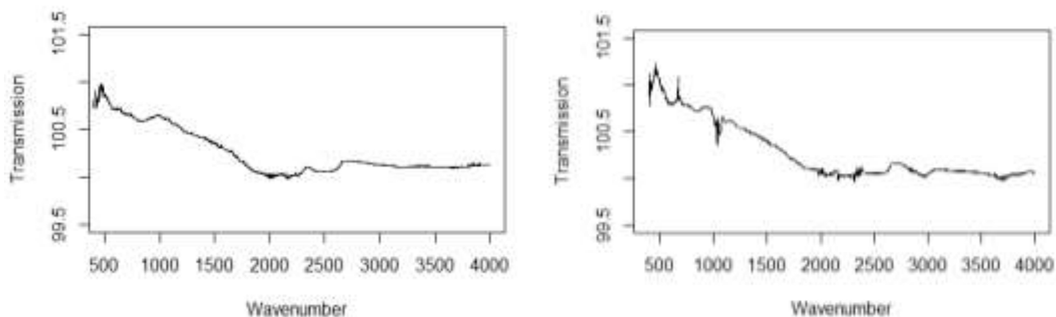

Figure 12. FTIR spectra of copper sheet metal both without (left) and with (right) extracted DNA (eluted in water) fixed to its surface. At ~ 3000 cm-1, a slight shift in the spectra can be observed when comparing the DNA containing sample (right) to the negative control (left). This may be indicative of either C-H stretching or N-H stretching of the nitrogen residues of the nucleobases, as observed in Figure 1; however, the relative intensity is such that no peaks can be reliably identified. Those peaks in the spectra to the right appearing at ~ 1000 cm^{-1} appear to be C-O stretching, indicating that those peaks originate from the DNA and not a contaminant.

This data illustrates that DNA can be visualized, albeit at very low relative intensities, on non-porous surfaces, and as such, the effects of rinsing agents in their ability to recover DNA from non-porous surfaces can be qualitatively evaluated based on the alteration and/or disappearance of those characteristic peaks as previously identified. The resulting spectra after rinsing, using first nuclease-free water followed by 0.1X TE buffer, can be seen in Figures 13 and 14.

Upon rinsing the sample area with nuclease-free water, a significant decrease in the relative intensity of peaks (specifically at ~3000 cm^{-1}) can be observed, indicating a majority of the sample was easily able to be recovered from the glass substrate (Fig. 13). This is consistent with expectations in that there have been no reports of complexation between glass and DNA, and therefore, the entire sample would be more easily recovered than from those metallic substrates. The spectra further reveal that with the secondary rinse with 0.1X TE buffer, not much more of the remaining sample was recovered, with the exception of the slight decrease in peak intensity at ~ 1000 cm^{-1}. This data shows that water is sufficient to collect DNA from non-porous glass surfaces. Further research would need to be conducted to confirm.

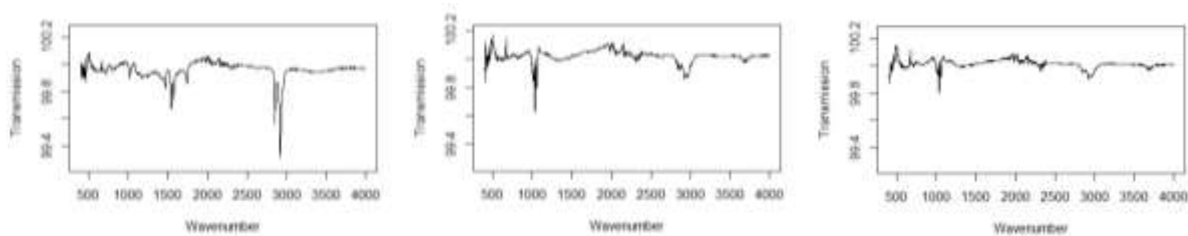

Figure 13. FTIR spectra of glass microscope slide with initial DNA deposit (left), after rinsing with 200 µL nuclease-free water (center), and after rinsing with 200 µL 0.1X TE buffer (right). At ~ 3000 cm-1, a significant decrease in relative intensity of the peaks can be observed between the initial DNA deposit and the nuclease-free water rinse, thus indicating that a majority of the DNA was recovered from glass simply using water. In the spectra from the 0.1X TE buffer rinse, there are minimal changes in peak intensity at ~ 3000 cm^{-1} from the nuclease-free water rinse and the TE rinse. A slight shift in the peaks and their relative intensities for the initial DNA deposit and the remining two samples can be observed from ~ 1500 cm^{-1} to ~ 1000 cm^{-1}.

In Figure 14, subtle spectral changes can be seen between the initial DNA-fixed spectrum on copper and those of the sample after rinsing with nuclease-free water and TE buffer. After rinsing with nuclease-free water, there is a slight decrease in intensity in those peaks observed at ~ 1000 cm^{-1} and ~3000 cm^{-1}. Only when examining the spectra of the sample after having been rinsed with 0.1X TE buffer are the peaks greatly reduced from the original DNA-containing sample, thus giving the final spectra a similar appearance to that of the copper control (Fig. 11). These findings are consistent with the literature in that compounds such as BSA with GGT and the chelating agent EDTA in TE, have been demonstrated to preferentially bind to transition metals (i.e.: copper), effectively maximizing DNA recovery [13, 48, 76]. However, in this study the relative intensities measured are within such a small range that no conclusive remarks concerning those potential DNA-copper interactions may be made without additional data.

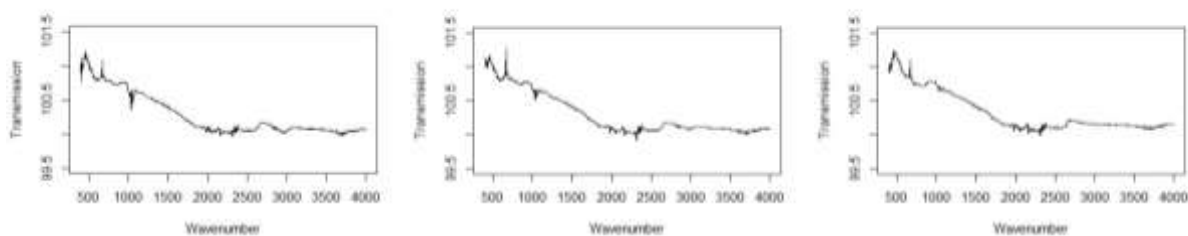

Figure 14. FTIR spectra of copper sheet metal with initial DNA deposit (left), after rinsing with 200 µL nuclease-free water (center), and after rinsing with 200 µL 0.1X TE buffer (right). At ~ 1000 cm-1, a slight decrease in relative intensity of the peaks can be observed between the initial DNA deposit and the nuclease-free water rinse; however, in the spectra from the 0.1X TE buffer rinse, the peak has almost entirely disappeared. This is indicative of a displacement reaction occurring between the buffer and the substrate, effectively removing the DNA.

ATR-FTIR has been shown to measure cfDNA on nonporous substrates (i.e.: glass and copper). All spectra collected (Figs. 11-14) in this study, with the exception of the negative controls, presented peaks that are characteristic of those vibrational and stretching modes previously reported for those chemical structures within B-form DNA (i.e.: nucleobases, deoxyribose, etc.). However, while these characteristic peaks can be observed to change after the rinse treatments (Figs. 11-14), it remains that this method is not currently sensitive enough to stand on its own to characterize those DNA-metal interactions.

9.2.2 Effects of Wetting Agents

The effects as observed spectroscopically were further characterized, as summarized in Table 3, by using each rinsing agent (i.e., nuclease-free water and 0.1X TE buffer) as wetting agents for the collection of cfDNA from copper using cotton-tipped swabs. On average, net yields from copper (Table 3) remained relatively similar between wetting agents, and it is speculated that the consistency within the dataset can be attributed to the lack of time provided for chemical recovery to occur, and therefore, the mode of sample recovery was primarily mechanical.

Both swabbing with nuclease-free water and swabbing with 0.1X TE buffer yielded similar DNA recovery percentages of approximately 3-5% of initial DNA deposited. While our recovery rates are lower than those in previous studies, which have reported greater yields when using chelating buffers for trace DNA collection from copper-containing substrates, the significance of these results is consistent with recently published findings. In 2019, Holland et al. [13] reported that DNA samples deposited onto copper cartridge casings experienced greater sample recovery at 13.0% when using 0.5M EDTA *versus* water which had a 3.9% recovery rate; however, the authors do note that p-values between wetting agents were not significant which is consistent with our present findings.

While our yields were lower than those reported when using 0.1X TE buffer (4.36% as opposed to 13.0%), they were virtually identical regarding swabbing with water (3.92% and 3.9%). It should be noted that Holland et al. [13] deposited extracted DNA eluted in low TE buffer for all samples, whereas in this study, extracted DNA was eluted in nuclease-free water. Any discrepancies between recovery results may be explained by the fact that their cfDNA was already suspended in TE buffer, and therefore, it is possible given the findings in this study, that the eluant upon deposition preferentially bound to the copper substrates, making sample recovery using

additional chelating agents (i.e.: 0.5M EDTA) easier and yield percentages greater than observed here (Table 3).

Table 3. Summary of cfDNA recovery (completed in triplicate) from copper substrates with nuclease-free water and 0.1X TE buffer to moisten sampling matrix. All samples (N=6) were extracted with the addition of 1 µL carrier RNA and eluted to a final volume of 25 µL.

Wetting Agent	DNA Deposited (ng)	Average Recovery (ng)	% Recovery	H_2O:TE
Nuclease-Free Water	128.56	5.04	3.92	0.90
0.1X TE	128.56	5.60	4.36	–

The lack of statistically significant results for cfDNA recovery from copper when swabbing could be due to several factors. Firstly, DNA upon introduction to a sampling matrix during collection may inadvertently become trapped in that matrix, resulting in a percent yield reduction. Second, when using water and TE as wetting agents, the only interaction each has with DNA is during the mechanical act of swabbing the sample. If given more time to react, it is presumed that the EDTA in TE would preferentially bind to the copper ions, thus freeing the DNA from the crystalline lattice and allowing it to be recovered for downstream processing. This idea is similar to those published soaking methods with few exceptions. Rather than fully submerging the sample, which requires a large volume of buffer for any item larger than a cartridge, bullet, or casing, the sampling area could be isolated/targeted, only necessitating a small volume of reagent (in this case 200 µL). Furthermore, many soaking methods inadvertently leach metal ions into the collected sample [45], resulting in further oxidative damage to the DNA and potentially inhibiting amplification. By only allowing the reaction to occur for a brief amount of time (two minutes as opposed to fifteen or twenty minutes), the proportion of leached ions may be greatly reduced, improving profile quality. Additional research is needed in order to determine if this would be the case.

Overall, the differences in DNA recovery based on the composition of the deposition substrate reveal that further research needs to be placed on characterizing cellular and cfDNA interactions with copper containing substrates. Based on the findings of Moreno and McCord [11], divalent metal ions replace the hydrogen atoms within base pairing, forming M-DNA, with copper ions strongly interacting with the DNA. For this reason, continued study of these substrate and sample types is vital.

10. Centrifugal Separation

10.1 Materials and Methods

To separate the cellular and cfDNA fractions of touch deposits, protocols from Burrill et al. [8] and Stanciu et al. [7] were adapted for this study.

10.1.1 Substrate Preparation and Touch Deposits

Glass microscope slides were sterilized using a 70% ethanol wash followed by UV irradiation for 30 min. A single participant was instructed to provide a touch deposit as detailed previously onto one of the DNA-free glass slides. This process was repeated for the remaining

items (N=20) at approximately the same time the following days. After sample deposition, items were immediately transferred to sample collection.

10.1.2 Sample Collection

The entire sample area for the first ten (10) deposits was swabbed using the wet:dry double swab approach with 100 µL of sterile water as the wetting agent. This was repeated for the second set of ten touch deposits, instead using 100 µL of PBS to moisten the swab. Swab heads were then placed in 800 µL of PBS and vortexed for fifteen (15) seconds to dislodge the cells from the sampling matrix. Afterwards, swab heads were transferred independently to sterile lyse and spin columns (Qiagen©, Hilden, Germany), centrifuged at maximum speed for three (3) minutes per swab head, and the flow through incorporated into the initial cell suspension. Swab heads were then set aside in sterile 1.5 mL microcentrifuge tubes for later processing.

10.1.3 Centrifugal Separation

To separate the cellular and acellular fractions for all samples, the cell suspension for all samples (to include those swabbed with nuclease-free water and PBS) was centrifuged for thirty (30) minutes at 12000 x g, the supernatant transferred to a clean microcentrifuge tube, and the cell pellet resuspended in 100 µL PBS and vortexed for fifteen (15) seconds. This process was repeated a total of three (3) times to ensure separation of the cellular and cfDNA fractions.

10.1.4 DNA Extraction/Purification

To the cellular fraction, the swab heads were combined with the pellet, and DNA extracted from the sampling matrix and pellet using the Qiagen QIAamp® DNA Investigator Kit according to the manufacturer's "Isolation of Total DNA from Surface and Buccal Swabs" protocol using Qiagen DNA Mini Spin Columns. The cfDNA fractions were manually purified using the Qiagen QIAamp® MinElute® ccf DNA Kit (Qiagen©, Hilden, Germany). All extractions and purifications were performed to a final elution volume of 25 µL, with extractions having 1 µL carrier RNA added to improve extraction efficiency.

10.1.5 Quantitation, Concentration and Amplification

For all samples, quantitation was performed as previously detailed using QuantifilerTM Trio. Prior to STR amplification, extracted and/or purified DNA from all samples was concentrated to 7 µL using Microcon® centrifugal filters (Millipore® Sigma®, Burlington, Massachusetts). STR amplification was performed in half-reaction volumes using the GlobalFiler™ PCR Amplification Kit (ThermoFisher Scientific©, Waltham, Massachusetts) on the VeritiPro™ PCR System (ThermoFisher Scientific©, Waltham, Massachusetts) according to manufacturer protocols.

10.1.6 Fragment Analysis

After amplification, products were prepared for separation and detection on the Applied Biosystems® 3500 Genetic Analyzer by addition of 1 µL of PCR product, 0.4 µL GeneScan™ 600 LIZ® Size Standard, and 9.6 µL Hi-Di formamide to a 96-well tray. All samples were run on the

ABI3500 using a 36 cm capillary with POP-7™ (ThermoFisher Scientific©, Waltham, Massachusetts).

10.1.7 Data Analysis

For all samples, profiles were analyzed via GeneMapper™ *ID-X* v1.4 (ThermoFisher Scientific©, Waltham, Massachusetts). Profile quality was compared between cellular and cfDNA fractions using proportion of called alleles to evaluate completeness to a known reference profile. Percentage of total alleles called was computed in Microsoft® Excel® for each fraction.

10.1.8 Cell Rupture/Lysis Evaluation

To evaluate if the process of centrifugal separation was causing the cells to lyse and artificially increase the total cfDNA yield, saliva samples were tested in triplicate (N=9) at the following relative centrifugal forces (RCF): 12000 x g, 7500 x g, and 3000 x g. For each sample, aliquots containing 250 µL of saliva and 750 µL of PBS were vortexed for fifteen (15) seconds and centrifuged for thirty (30) minutes at one of each RCF. After the initial centrifugation, the supernatant was transferred to a clean microcentrifuge tube, and the pellet re-suspended in 100 µL of PBS. This process was repeated a total of three (3) times as with the touch samples. DNA extraction/purification, quantitation, concentration, amplification, and fragment analysis were performed as detailed above. For this sample set, quantitation data was compared in Microsoft® Excel® between RCFs using a two-sample *t*-test.

10.2 Results and Discussion

10.2.1 Fraction Contribution

To investigate the correlation between the relative contributions of cellular and cfDNA on profile quality, multiple touch deposits on glass slides were collected from a single donor across several consecutive days using both dominant and non-dominant hands (N = 20). Half of these samples were collected via swabbing with nuclease-free water, and the other half with PBS.

For all touch deposits, consistently low proportions of cellular and cfDNA were quantitated with measured concentrations ranging from zero to 0.014 ng and zero to 0.056 ng, respectively (Table 4). This could be due to the fact that at the time of sample collection, the participant's hand were unwashed, and previous reports have indicated a greater transfer of cells from unwashed versus washed hands [8]. However, the proportions of cellular DNA observed here were found to be much lower than that recorded by others [7, 8]. This may be due to the deposition substrate. In the present study, all samples were deposited onto glass microscope slides, whereas in a report published by Stanciu et al., participants deposited samples onto plastic (i.e.: conical tubes), which has been demonstrated to be a less challenging substrate for touch DNA sample recovery [37, 42].

Table 4. Summary of average DNA recovery from glass substrates with nuclease-free water and PBS to moisten sampling matrix (N=20). Net yields for all samples remained lower than reported values after centrifugal separation. All samples quantitated in half-reaction volumes (10 µL) with quantification thresholds as low as 0.005 ng/µL [77].

Fraction	Wetting Agent	Average Recovery (ng)	Wetting Agent	Average Recovery (ng)
Cellular	Nuclease-free water	0.005	PBS	–
Cell-free	Nuclease-free water	0.02	PBS	0.01

It should also be noted that for PBS-collected samples, no DNA from the cellular fraction was able to be quantified despite subsequent CE results revealing a small number of called alleles in the profile (Fig. 15 and Table 5). It is possible that those cells collected in the pellet may have been comprised of a majority of shed, anucleate corneocytes, and thus, the very low levels of nDNA present may have been below the limit of quantitation while still present in sufficient quantities to be concentrated and amplified.

While this data may provide further support regarding the majority composition of DNA in a touch deposit being acellular, it is possible that between the several wash steps, as well as the high relative centrifugal force applied in this study versus other reported protocols, that the cells ruptured, thus artificially elevating the DNA content as measured in the cell-free fraction. This was explored in greater detail, and the findings presented in Section 10.2.2. In comparison to similar studies however, the distribution of cellular versus cfDNA within a touch sample observed here is generally consistent. Stanciu et al. [7] reported that in using a centrifugal separation protocol with multiple washing steps, anywhere between 84-100% of the DNA detected was comprised of cfDNA. Furthermore, the authors found that by increasing the number of wash steps, additional cDNA was recovered with only few cells being unintentionally transferred from the cellular to the cell-free fraction, as confirmed through microscopic surveys [7]. It is imperative to note, however, that in the previous report [7], the RCF was much lower than used in the present study (3220 x g and 12000 x g, respectively).

As suggested in the literature [7, 8] and given the distribution of cellular versus cfDNA present in touch samples as illustrated in this study, profiles from both cellular and cfDNA fractions were generated, and the quality of each assessed by percent contribution (Fig. 15). These proportions are not consistent with the author's expectations, as profiles are traditionally thought to be produced through higher quality DNA samples. It is well documented that cfDNA is highly fragmented, typically ranging between 140-200 bp in length [8]. Furthermore, the intracellular DNA as traditionally targeted in STR analysis was until recently presumed to contribute majority of the alleles called within a touch sample, and as such, efforts have focused on enhancing cellular DNA recovery. However, this data illustrates that not only does the cell-free fraction comprise a greater percentage of overall donor profile across all loci (Tables 4 and 5, Figure 15), but additionally, it was found to consistently produce a greater number of alleles within a single profile as observed in Table 5.

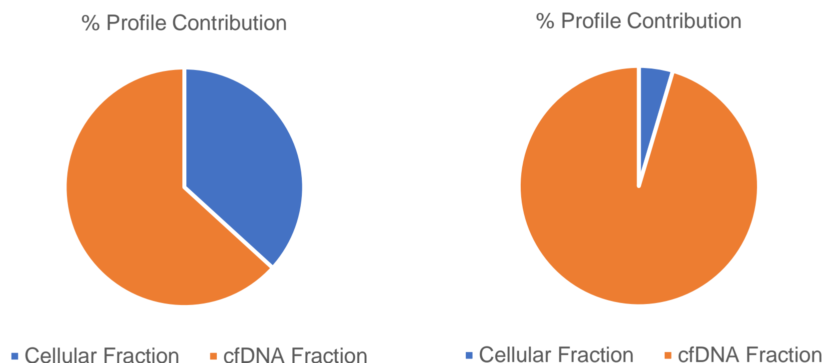

Figure 15. Total contribution of cellular and cfDNA to allelic profiles as observed in samples (N = 20) collected using nuclease-free water (left) and PBS (right).

Table 5. Summary of called allele for both cellular and cfDNA fractions of touch deposits (N=20). This data indicates that the cfDNA in touch samples, while understood to be more highly fragmented (140-200 bp [8]), contributes a greater proportion of genomic data to STR profiles than the more commonly target cellular fraction.

Wetting Agent	Cellular Fraction	cfDNA Fraction
H_2O	17.1	29.4
PBS	0.5	10.4

These results reveal the necessity for the reassessment of the contributing alleles to a profile from non-intracellular DNA samples (i.e.: cfDNA). As demonstrated, it appears that profiles may be generated from highly fragmented, circulating free DNA. When considered with those data presenting cfDNA as majority contributor to a 'touch' DNA sample, this could significantly alter the approach in recovery and processing of 'touch' DNA. Not only does this mean that improving 'touch' DNA recovery, to consider both cellular and cell-free fractions is of critical importance, but it is also imperative that downstream processing of these sample types be further examined.

However, if the contribution of cfDNA is artificially elevated due to cell rupture during centrifugal separation, and therefore the cell-free fraction is contaminated with higher quality intracellular DNA, then profiles would be predicted to appear as observed here. Therefore, the ability to form any conclusions as to the distribution of cellular versus cfDNA within a touch sample, as well as each fraction's contribution to an individual's profile rests upon the crux of efficient and reliable centrifugal separation.

10.2.2 Assessment of Artificial Lysis

To ensure the cfDNA component previously evaluated was from extracellular DNA and not the result of artificial lysis or cell rupture during centrifugal separation, the proportions of cellular and cfDNA fractions across three different relative centrifugation forces (RCFs) was measured in saliva samples (N = 18). The DNA concentration for separated fractions at each RCF was compared (Fig. 16), and two-sample *t*-test conducted.

While there is a slight decrease in the quantity of cellular DNA for those samples separated at 12000 x g when compared to 7500 x g and 3000 x g (Fig. 16), no significant changes can be observed between any RCFs for all samples (Table 6). These data suggest that contamination from artificial lysis/cell-rupture was not found to be an issue for the tested RCFs, and therefore, this

method of separation may present itself as another viable approach when seeking to characterize touch DNA components.

These findings are consistent with that reported by Burrill et al. [8], whereby the cfDNA content before and after centrifugation was measured, and percent change calculated. Their results show that unless heat was applied to a sample, few, if any, cells were found to rupture and those that did were still retained in the cellular pellet. Furthermore, the authors confirmed those findings by microscopic examination and cell counting for each sample in which there were no visible alterations in cells collected from hands, as well as those ruptured cells were primarily found to be associated with heat treatment [8]. However, it should be noted that in their protocol, samples were subjected to a single wash step, whereas here, additional wash steps were applied to all samples.

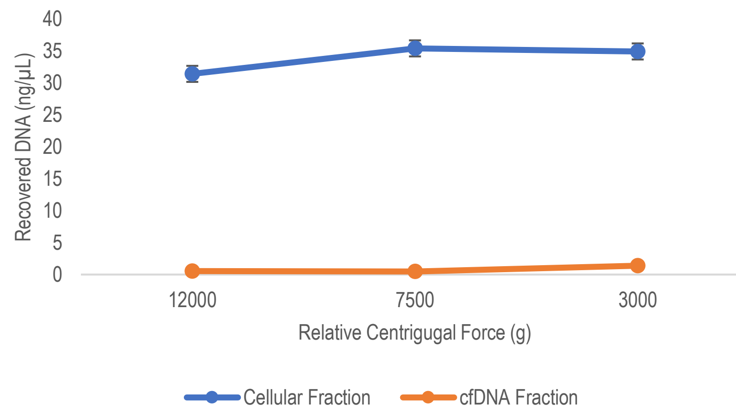

Figure 16. Concentration of recovered DNA from saliva samples (N=18) as measured using Quantifiler™ Trio Quantification Kit following 30 min centrifugation at increasing speeds. All samples were collected from a single donor. Centrifugal separation was conducted in triplicate and the averages used to evaluate trends. For both cellular and cfDNA fractions, no significant increase in cfDNA yields at higher centrifugal forces are observed, illustrating that few, if any, cells were artificially ruptured, and their contents transferred to the supernatant.

Table 6. Summary of p-values of pairwise comparisons for all sample types and RCFs. At $\alpha = 0.05$, no significant changes were observed for any RCF. These data indicate that elevating centrifugal force did not result in artificial cell lysis.

Sample	12000 v 7500 g	12000 v 3000 g	7500 v 3000 g
Cellular Fraction	0.269	0.592	0.939
cfDNA Fraction	0.777	0.196	0.175

Our results indicate that elevated centrifugal force was not found to mechanically rupture cells during centrifugation at 12000 x g for 30 min when tested against two slower RCFs. Therefore, this method of separation of the cellular and cell-free components may provide sufficient fraction separation without contamination by cellular DNA. However, there are limitations within this study that should be considered when reviewing the presented data.

Firstly, despite the statistically insignificant differences in DNA quantity for all samples measured across three different RCFs, we were unable to visually confirm this data either by microscopic examination or automated cell counting. Second, the currently published protocols purifying cfDNA are intended for cfDNA from plasma [7, 8], and therefore, observed yields were found to be lower both for these data when considering the acellular component of 'touch' DNA, as well as in other published studies evaluating several cfDNA purification kits on 'touch' DNA

[8]. Finally, and most importantly, each fraction is assumed to be either one of cellular or cfDNA. It is presumed, based on the extraction/purification kit and corresponding protocol used, that the "cellular" fraction contained only intracellular DNA. Similar assumptions were made concerning the "cfDNA" fraction in that the kit used to purify the cfDNA was presumed to remove any proteins or contaminants present in the supernatant. However, because a more detailed characterization of each fraction was not conducted, it is not conclusive as to whether there exists cross-contamination between fractions during the centrifugal separation step. Further investigation is needed to evaluate if these parameters continue to provide reliable separation.

Part III

11. Recommendations for Future Practice

Given the findings presented in Part II of this thesis, as well as those previously documented throughout the discipline, the following are the author's recommendations pertaining to the recovery of 'touch' DNA from non-porous substrates (Fig. 17). This is not intended to be an all-encompassing decision tree; however, it is to begin setting the foundation for others to build upon given additional and more comprehensive research, as well as it is intended to evolve as standards and guidelines are updated.

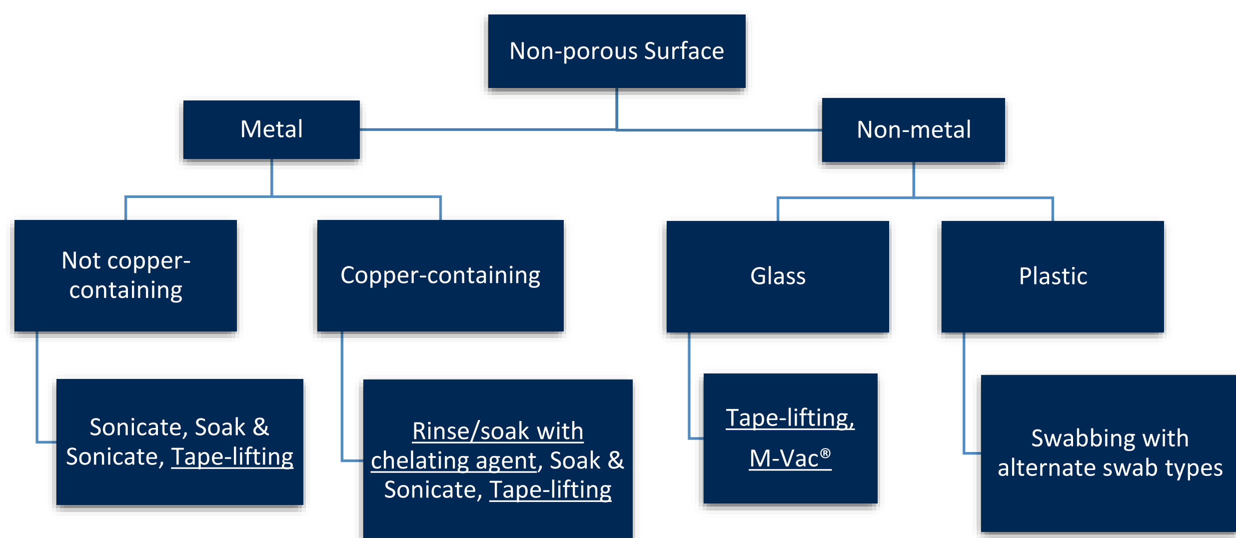

Figure 17. Proposed decision matrix for the collection of 'touch' DNA based on both the present findings as well as those previously published in the literature, to consider optimal collection of both cellular and cfDNA components, from non-porous substrates. Underlined recommendations are those which were evaluated in this thesis.

12. Concluding Remarks

While ATR-FTIR remains a key instrumental technique in many applications of trace evidence for its robustness and reliability, our data indicates it is currently not sensitive enough to reliably visualize cfDNA binding to copper surfaces as has been demonstrated *in vitro*. Despite the presence of some additional peaks in the spectra after the introduction of cfDNA to the substrate, these data only serve as proof of concept that cfDNA interacts with copper ions at the surface level similarly to what has been previously described in the literature. The supplemental quantitation data in this study further supports these findings by demonstrating a reduced net recovery of cfDNA from copper-containing materials as opposed to glass or stainless-steel surfaces in all cases. Further research would need to be performed if ATR-FTIR were to be used to characterize with any confidence those interactions observed here; however, there is sufficient evidence to suggest that practitioners familiarize themselves with the metal composition from which they are trying to collect biological evidence.

Across all collection methods and substrate types, recovery rates for acellular samples are significantly lower than those of cellular sample types deposited on copper-containing materials, indicating the possible formation of M-DNA complexes which make collection of the DNA much more taxing. However, in allowing chelating agents only a few minutes to react with the sample and substrate, recovery was greatly improved. Considerations may need to shift towards chelating approaches for the recovery of 'touch' DNA samples from metals to improve both cellular and cfDNA yields as opposed to the currently standardized mechanical techniques.

Regardless of metal composition, tape-lifting proved to have the greatest DNA yields as opposed to double-swabbing procedures and the M-Vac® wet-vacuum DNA collection system for cellular containing samples, however, no collection methods proved significantly different from one another for cfDNA collection. This is likely attributed to the tape-lifting's adhesive having a greater propensity to collect cellular material from which the DNA is later released from the adhesive during lysis steps, whereas cotton swabbing matrices and the PES filters appear to not retain as much cellular material for processing.

13. Key Take-Aways

- ATR-FTIR is not sensitive enough to stand alone in characterizing cfDNA-metal interactions.
- DNA is found to preferentially bind to copper-containing surfaces.
- Tape-lifting recovers the majority of cellular material present in 'touch' samples.
- Chelating collection methods need to be considered for touch DNA from copper-containing metals to optimize recovery.
- Further research is needed to optimize processing of cfDNA component of 'touch' samples.
- It is critical that investigators/practitioners familiarize themselves with substrate material.

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Appendix I – List of Abbreviations

ATR	Attenuated total reflectance
BCC	Body-centered cubic lattice
B-DNA	Right-handed DNA conformation
BSA	Bovine serum albumin
CD	Circular dichroism
CDC	Center for Disease Control and Prevention
CE	Capillary electrophoresis
cfDNA	Cell-free deoxyribonucleic acid
CODIS	Combined DNA Index System
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPG	Electropherogram
ETOH	Ethyl Alcohol/Ethanol
FBI	Federal Bureau of Investigation
FCC	Face-centered cubic lattice
FTIR	Fourier transform infrared spectroscopy
GGT	Gamma glutamyl transferase
HCP	Hexagonal close packing
LCN	Low copy number
LT-DNA	Low-template deoxyribonucleic acid
M-DNA	Metal-deoxyribonucleic acid complex
M-Vac®	Microbial vacuum
mtDNA	Mitochondrial deoxyribonucleic acid
nDNA	Nuclear deoxyribonucleic acid
NMR	Nuclear magnetic resonance

OH	Hydroxy group
OSAC	Organization of Scientific Area Committees for Forensic Science
PES	Polyethersulfone
PBS	Phosphate buffered saline
PCR	Polymerase chain reactions
QAS	Quality assurance standards
qPCR	Quantitative polymerase chain reactions
RCF	Relative centrifugal force
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SRS	Sterile rinse solution
ssDNA	Single-stranded DNA
STR	Short tandem repeat
SWGDAM	Scientific Working Group on DNA Analysis Methods
TE	Tris-EDTA *(see also Tris and EDTA)*
Tris	Tris(hydroxymethyl)aminomethane