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Evaluating the recovery of DNA after heated passive headspace concentration

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EVALUATING THE RECOVERY OF DNA AFTER HEATED PASSIVE HEADSPACE CONCENTRATION

by

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EVALUATING THE RECOVERY OF DNA AFTER HEATED PASSIVE HEADSPACE CONCENTRATION

CASSIDY MCGANN

ABSTRACT

It is not uncommon for an individual to commit arson as a method of concealing a crime, whether the goal be to destroy a body, DNA evidence, or any other information that may link the suspect to the scene. Fortunately, for investigators, setting a fire to the crime scene does not always destroy all evidence. Some pieces of evidence are more resilient than others. For example, evidence such as ignitable liquids and other accelerants can often be detected after the fire. In the event that an item of evidence like clothing is not completely incinerated, the presence of biological fluids may also be detected through presumptive testing and eventually lead to the identification of an individual through DNA analysis.

The purpose of this study was to determine whether or not DNA analysis can be performed effectively after heated passive headspace concentration, without causing irreparable degradation to DNA evidence. Heated passive headspace concentration is a common procedure for extracting ignitable liquids from a substrate to identify and confirm the presence of that substance. This process requires long incubations at fairly high temperatures within a tightly sealed vessel to prevent evaporation. If practitioners can delay DNA analysis steps, move straight into heated passive headspace concentration, and lower the chances of losing a portion of the ignitable liquid to evaporation, it may assist in arson investigations. This study explores DNA quantity and

quality in saliva and semen stains after incubation times and temperatures based on the recommended upper and lower temperature and timeframe limits of ASTM Guidelines E1412-19 and E1388-17 entitled “Standard Practice for Separation of Ignitable Liquid Residues from Fire Debris Samples by Passive Headspace Concentration with Activated Charcoal” and “Standard Practice for Static Headspace Sampling of Vapors from Fire Debris Samples,” respectively. Possible DNA analysis inhibitors such as gasoline, open flame, and burnt substrate were also explored. It was determined that while open flame in direct contact with a biological stain caused significant damage in all saliva stains and some semen stains, the presence of gasoline and burnt substrate did not appear to inhibit DNA analysis. Additionally, heated passive headspace concentration conditions did not appear to cause significant degradation or inhibition that would result in an incomplete genetic profile. Further experimentation is necessary given the presence of extraneous factors. For example, the initial amount of DNA deposited onto substrates was unknown. However, it is reasonable to state that performing heated passive headspace concentration before DNA analysis may be a feasible option if desired in a forensic laboratory.

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LIST OF ABBREVIATIONS

%	percent
ASTM	American Society for Testing and Materials
CE	capillary electrophoresis
Ct	threshold cycle value
°C	degrees Celsius
DI	degradation index
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HID	human identification
hr	hour
IL	ignitable liquid
ILR	ignitable liquid residue
ISO	International Standards Organization
LPH	low peak height
MA	Massachusetts
µL	microliter
mL	milliliter
mm^2	millimeter squared
MPH	max peak height
N/A	not applicable

ND	no data
ng	nanogram
NTC	No Template Control
OH	Ohio
PA	Pennsylvania
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
R^2	coefficient of determination
RFU	relative fluorescence units
rpm	rotations per minute
STR	short tandem repeats
TE	Tris-EDTA
TX	Texas
VA	Virginia
VOC	volatile organic compound
vs.	versus

1. INTRODUCTION

1.1 Arson

The term “arson” can be generally defined as “a criminal act of deliberately setting fire to a property (1).” Similarly, the Federal Bureau of Investigation’s Uniform Crime Reporting Program defines arson as “any willful or malicious burning or attempting to burn, with or without intent to defraud, a dwelling house, public building, motor vehicle or aircraft, personal property of another, etc. (2).” In 2017, there were approximately 13.3 arson offenses per 100,000 inhabitants across the United States of America (2). It is not uncommon for arson to be utilized as a way to cover up or conceal other crimes (3–6). For example, an offender may commit a crime like burglary, sexual assault, or homicide and set a fire in the hopes of destroying evidence that could be used against him/her. Juvenile fire setting is also not unheard of, and is sometimes related to prior sexual assault or molestation with the goal of covering up evidence of those crimes, which likely left behind biological fluid evidence (7). In many cases, accelerants or ignitable liquids may be used to cause more damage or increase the spread of the fire. However, fire does not necessarily always conceal every piece of evidence. When investigating a possible arson, there are several methods that investigators can utilize to detect the presence of ignitable liquid residues (ILR).

1.2 Field Detection of Ignitable Liquids

When present at a fire scene, investigators will have tools that can provide presumptive identification of ignitable liquids. It is not until a sample can be analyzed by

gas chromatography-mass spectrometry or another confirmatory method that the presence of ignitable liquids can be confirmed. Three overarching methods for the presumptive detection of ignitable liquids include detection based on burn patterns, detection of volatile organic compounds (VOCs) in headspace, or detection via fluorescence (1).

In the case of burn patterns, sometimes pool-shaped patterns may indicate the presence of an ignitable liquid that was poured onto a surface and settled in a particular area (1). However, not all burn patterns are as straightforward and revealing as they may appear (1). Given that burn patterns are more subjective and can be left up to interpretation, some forms of presumptive chemical detection can be utilized. If detecting VOCs in headspace is desired, electronic sniffers/portable gas detectors like catalytic combustion detectors, flame ionization detectors, and photoionization detectors may be utilized to presumptively identify the presence of compounds found in ignitable liquids (1). If an ignitable liquid was used to initiate or spread a fire, volatile compounds can be present in the air within range of the debris. VOCs exist mainly in a gaseous phase at temperatures and humidity levels that are often encountered indoors (8). If not contained in a sealed vessel, the VOCs readily evaporate into the air, and eventually evaporate off completely (9). Another common method of testing the headspace on-scene is the use of accelerant detection canines due to their highly developed olfactory organ. Dogs' noses are considered to be 200 to one billion times more sensitive than the nose of a human, and as a result, are able to locate and, sometimes, differentiate accelerant residues (10). The third, and final, method for presumptive testing often includes the detection of ignitable liquids via ultraviolet light. Ultraviolet light can be utilized to locate and

visualize ignitable liquids and fire patterns and those patterns can then be documented with fluorescent photography as evidence (11). However, this method is not very common due to the complexity and price of equipment (1). While presumptive testing methods are useful for sample selection at a scene, confirmatory identification is necessary.

1.3 Extraction and Analysis of Ignitable Liquids

As previously mentioned, one of the main presumptive methods of detecting ignitable liquids is via the VOCs present in headspace. This concept is based on the fact that volatile compounds evaporate more readily and, eventually, will evaporate completely. Due to this characteristic of ignitable liquids, fire debris evidence that may contain these types of compounds must be packaged in a very particular manner. Generally, evidence that may contain ignitable liquids or accelerants are packaged in metal paint cans with a sufficient seal for preventing evaporation (1). Some agencies may package evidence in glass canning jars, which also have a sufficient seal (1). Common types of evidence may include substrates such as carpet or wood flooring, which are highly absorbent and, fortunately, retain ignitable liquids well (1). The substrate may be collected directly or with a swab or an ignitable liquid absorbent. Once the properly-packaged pieces of evidence make their way to the laboratory, there are several different methods of extraction and analysis that may be utilized to identify and confirm the presence of ignitable liquids.

While ILRs can be analyzed via gas chromatography-mass spectrometry or other separation techniques, the residues need to first be made suitable for analysis. In order to separate the residues from a piece of evidence, the residues must be extracted via one of the four main categories of extraction techniques: *distillation, solvent extraction, headspace*, and *adsorption techniques* (1). Distillation techniques require vaporizing, separating, and condensing the residues back to a liquid form for analysis. This technique is advantageous because it results in the ILR being converted to liquid form, which can be visibly seen by practitioners and a jury. However, this method is highly destructive (1). Meanwhile, solvent extraction involves soaking debris in a solvent to desorb the ILR and later filter and concentrate it (1). Like distillation, this method is highly destructive, as well.

Headspace and adsorption techniques are two techniques that are the most relevant to the goals of this study. Headspace techniques rely on vapor pressure and the volatility of ILRs. When placed in a closed container, a portion of the ILR on the debris moves into the headspace, or air above the debris. The air can then be sampled via syringe or another method and analyzed. According to ASTM Guideline E1388-17, this sampling of headspace vapors from fire debris evidence can occur at room temperature (12). However, it is common to elevate temperatures to somewhere between 60°C and 90°C by placing the paint can containing debris into an oven (12). The increase in temperature helps recover compounds with lower volatilities (12). Headspace methods are nondestructive, straightforward, and are therefore advantageous.

Meanwhile, adsorption techniques utilize similar concepts to headspace techniques and partially rely on the volatility of ILRs. However, in the case of adsorption techniques, instead of remaining in the headspace of the container and being sampled directly, the ILR adsorbs onto a substance for which it has an affinity (9). Some adsorbents include activated charcoal strips, charcoal powders, solid phase microextraction fibers and Tenax® (1,13–19). *Figure 1* shows a general diagram of the process of heated passive headspace concentration in the presence of an activated charcoal strip. After the completion of extraction, the ILR can be desorbed from the adsorbent with a solvent or with heat and then analyzed (14, 20). Generally, when performing passive headspace concentration with an adsorbent like activated charcoal, the container, a metal paint can, should be heated anywhere between 50°C to 80°C for 2 to 24 hours (13). The heated passive headspace concentration conditions used in this study were based on ASTM Guidelines E1412-19 and E1388-17 (12,13). ASTM Guideline E1412-19 is the "Standard Practice for Separation of Ignitable Liquid Residues from Fire Debris Samples by Passive Headspace Concentration with Activated Charcoal" and it outlines important procedural guidelines for passive headspace concentration and the rationale behind the process (13). ASTM Guideline E1388-17 is the "Standard Practice for Static Headspace Sampling of Vapors from Fire Debris," and it outlines rationale and proper procedural guidelines for static headspace sampling (12). The temperature conditions utilized were based on the low and high temperatures of each guideline (50ºC and 90ºC) and time frames were based on what would be easiest for moving the evidence into and out of the ovens (6 hours and 16 hours).

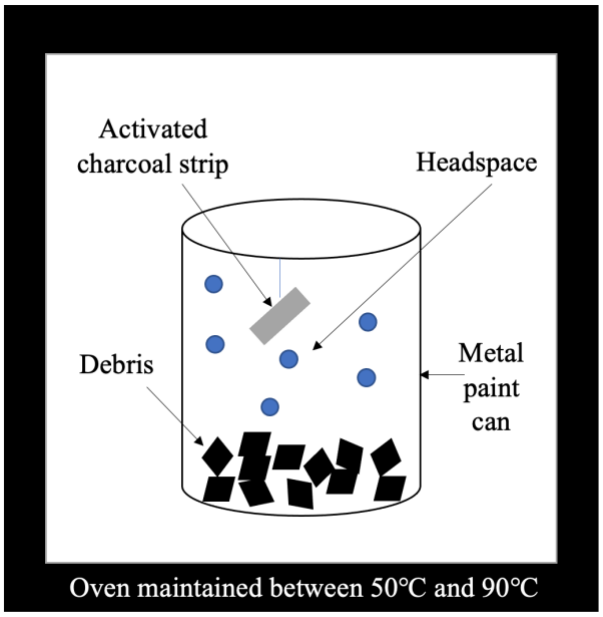

Figure 1. Heated passive headspace concentration. Fire debris from a scene is placed in a metal paint can with an activated charcoal strip suspended by a string. The can is sealed and incubated in an oven for some amount of time. This process was simulated during this study, with the exception of the addition of an activated charcoal strip.

1.4 Arson to Cover Up Crime

As previously mentioned, it is not uncommon for offenders to set fires in order to destroy evidence of crime or attempt to conceal the crime altogether. For example, this year, a wildfire was initiated in Vacaville, California by a man who murdered a woman he met online when he wanted to destroy evidence of the crime (21). That wildfire ended up killing two additional people that were innocent bystanders. Oftentimes, offenders believe that crime concealment can be achieved with fire, in the hopes of destroying all physical evidence that would link the offender to the crime. Offenders may attempt to destroy bodies to prevent identification, or to rid the scene of any potential DNA-

containing evidence, like biological fluids. While DNA is highly vulnerable to heat and degradation, it is not always impossible to obtain DNA evidence after a fire.

1.5 DNA Analysis

DNA (deoxyribonucleic acid) analysis is one of the most useful tools in criminal investigation and forensic science. While DNA evidence can be utilized to connect a suspect to a crime scene, it can also be used to establish the innocence of individuals. Current forensic DNA analysis utilizes short tandem repeat (STR) typing to produce a genetic profile (22). There are five main steps that are often followed during the workflow of collection and analysis of DNA, which includes sample collection, extraction, quantitation, amplification, and separation/detection (23). Prior to analysis, the workflow begins simply by the collection and storage of evidence at a crime scene or other location. DNA is generally collected in the form of biological material, whether that is blood, saliva, semen, or even as trace DNA. Collection may be achieved via cutting, scraping, or more commonly, swabbing. No matter the collection method, the main goal is to obtain cellular material, if present, package that material properly, and ensure that no contamination occurs. Reference DNA samples may also be collected in order to successfully perform identification. Reference samples may include the DNA of potential suspects, victims, or even relatives. Once collected and packaged, the evidence that is thought to contain biological material may be characterized at the crime laboratory via testing for biological fluids. For example, the evidence may go through presumptive testing for blood, semen, saliva, urine, feces, or vaginal fluid and/or confirmatory testing

for blood, semen, and saliva (23). Once biological material is identified, the evidence may move on to DNA extraction.

DNA extraction is the process of lysing the cells within the biological material to help release the DNA within those cells and separate the DNA from the rest of the cellular material (23). The DNA that is extracted may then be isolated and made amenable for later steps, including quantitation and PCR (polymerase chain reaction) amplification. The process of extracting and isolating the DNA is important for removing inhibitors, which could interfere with downstream DNA analysis steps, as well as the final results. Extraction methods will also differ depending on the type of suspected cells that are present. For example, most DNA extraction methods that utilize proteinase K, nucleases, and other components are useful for somatic cells like the epithelial cells found within a saliva sample (24,25). However, extraction of DNA from sperm cells requires a different approach. The packaging of DNA within a sperm head is more resistant than that of DNA in an epithelial cell (26). Therefore, DNA extraction from sperm cells requires the presence of dithiothreitol (DTT) or another reducing agent or enzyme in order to compromise the sperm head and release the DNA (26). During this study, both epithelial cell and sperm cell extraction methods were employed. After extraction, DNA can then be quantitated.

DNA quantitation is the process of determining how much *human* DNA material is present in the evidentiary sample, given that animal, bacterial, plant, or fungal DNA could also be present (23). Quantitation ensures that an ideal amount of DNA is utilized during amplification for a successful resulting genetic profile. If too little DNA is

amplified, detection will be minimal or non-existent, and if too much DNA is amplified, the resulting profile may be "overblown" or have so much signal that the profile is uninterpretable (27). The amount of target DNA for amplification varies across studies, but in our laboratory, the target DNA amount generally lies between 0.50 ng and 1.0 ng. In this particular study, the protocols utilized were validated for a target DNA amount of 0.75 ng. DNA is generally quantitated using real-time quantitative PCR (qPCR). Quantitative PCR is the process of exponentially amplifying a sample of DNA while also being able to detect each fluorescently labeled amplicon of DNA that is produced (5). As a result, the original amount of DNA can be estimated based on the use of a standard curve (5). In this study, the Quantifiler™ Trio DNA Quantification Kit from Applied Biosystems was utilized. One of the advantages of this kit is its ability to determine both the quantity and quality of human DNA (28,29). The loci that are targeted by this kit include three human-specific target loci: Small Autosomal, Large Autosomal, and a Y-chromosome target sequences (28). By comparing the quantity of small and large autosomal DNA, the kit allows the analyst to estimate the level of degradation of our sample. The level of degradation is conveyed by a Degradation Index (DI). DI is equal to the concentration of the small autosomal DNA target divided by the concentration of the large autosomal DNA target (28). Ideally, your ratio of concentration of small to large DNA target should be 1:1 if the DNA is fully intact. However, any DI over 1 can suggest the presence of degradation and a DI of 1 to 10 may indicate slight to moderate degradation. Likewise, a DI less than 1 suggests the DNA is not degraded or inhibited. One study suggested that a DI less than 1 may be due to small differences in the detection

of fluorescent probes during quantitation (30). In the same study, a DI of 4 or less was considered “no to mild degradation” and resulted in full genetic profiles (30). The Quantifiler™ Trio DNA Quantification Kit also utilizes an Internal Positive Control (IPC) to demonstrate whether or not inhibition occurred during quantitation. The IPC is a sample of synthetic DNA that takes a set number of cycles to amplify (28). An increase in the number of cycles indicated by the results would suggest that the IPC was inhibited, therefore notifying the analyst that an inhibitor may be present within the sample (28).

DNA amplification, like quantitation, also utilizes PCR. In this case, PCR is utilized to make millions or billions of copies of a particular region within the DNA sequence. Once copied, the resulting DNA product is abundant enough to be measured. These amplicons, or DNA amplification products, are also now labeled with fluorescent dyes which can be detected by capillary electrophoresis instrumentation during the separation step of DNA analysis. While the Federal Bureau of Investigation’s Combined DNA Index System requires 20 core STR loci for uploading a genetic profile, commercial kits for amplification often explore additional loci (31,32). For example, amplification for this project was completed using the GlobalFiler™ PCR Amplification Kit from Applied Biosystems, which amplifies 24 STR loci to be visualized after DNA separation and detection (33).

Human DNA separation and detection occurs most commonly via the process of capillary electrophoresis. There are several different genetic analyzer instruments for performing capillary electrophoresis, but all of them involve the same overall process **(Figure 2).** The amplicons of interest are loaded into a sample tray with an internal size

standard and allelic ladder. The internal size standard provides DNA fragments of known length in base pairs that can be compared to a DNA sample and the allelic ladder provides known alleles present at each locus to help determine allele designations for a DNA sample. The amplicons move through polymer-filled capillaries from the negatively charged cathode towards the positively charged anode, given the negatively charged nature of DNA (23). The fragments are separated by size given that smaller fragments move through the polymer faster than larger fragments. The internal size standards move with the amplicons, allowing for the calculation of the lengths of labeled DNA amplicons in base pairs. As the amplicons get closer to the anode, they pass a window and are exposed to a laser, resulting in the emission of fluorescence of the dyes attached to the amplicons. The fluorescence is detected and recorded. The resulting data can then be analyzed using a software like GeneMapper® ID-X in order to compare and interpret the genetic profiles, called electropherograms.

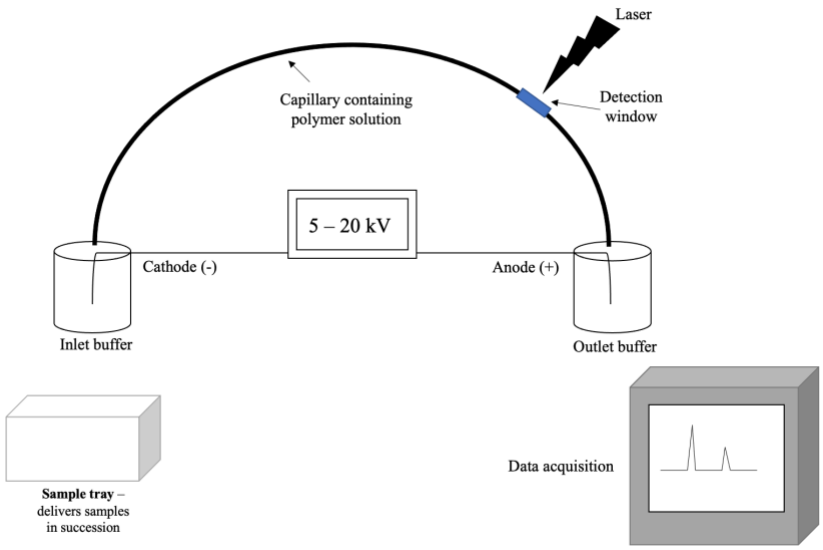

Figure 2. General schematic of capillary electrophoresis of DNA. DNA fragments move from the negatively charged cathode towards the positively charged anode and are detected at the detection window. The resulting data can be viewed as an electropherogram (23).

With an ideal piece of evidence, the resulting profile will be interpretable, complete, and suitable for comparison to reference DNA samples. However, not all genetic profiles are perfect. Profiles are subject to artifacts like stutter that may cause misinterpretation of a genetic profile (34). Some pieces of evidence are too degraded, mixtures of more than one DNA profile, or plagued with inhibitors, which can cause more issues during analysis and interpretation.

1.6 Degradation and Inhibitors of DNA Analysis

Degradation of genetic material may occur through a variety of processes, whether they be enzymatic or chemical (23). For example, cellular material is subject to enzymes like nucleases which cleave, or cut, the DNA. Organisms like bacteria, fungi, and insects

may have destructive effects on genetic material as well. If the cleavage of DNA occurs near or at the site of primer annealing, the efficiency of PCR amplification may be reduced or the region may not be amplified at all, resulting in the absence of allele peaks in the electropherogram. This cleavage is assisted by heat and humidity, which are important factors in fire debris analysis (35).

PCR is also inhibited by components of the evidence itself. For example, hemoglobin from blood and indigo dyes from denim clothing are commonly encountered inhibitors of PCR found in the field (23). While this study does explore the effect of weathered gasoline, or gasoline that has experienced evaporation and therefore slight changes in its chemical composition, a prior study suggested that accelerants and some suppressants do not affect the integrity of DNA (36,37). Additionally, outdoor crime scenes may include the presence of soil, sand, or wood, which can result in inhibition (23). Inhibitors are problematic due to their effects on extraction, PCR, and the resulting generation of a genetic profile. Some inhibitors may co-extract with DNA and interfere with polymerases used during DNA amplification. The resulting profiles may look similar to that of degraded DNA and produce a partial profile. Additionally, as previously mentioned, levels of degradation can be indicated through the DI established during quantitation. Throughout this project, heated passive headspace concentration, heat, ignitable liquids, and burning of a substrate are explored as possible inhibitors of DNA analysis.

2. MATERIALS AND METHODS

2.1 Proof-of-Concept Experiments

A series of proof-of-concept experiments were performed in order to establish optimal conditions for future steps. All of these experiments were performed using liquid saliva samples from Donor # R08-00587, a female individual who had not eaten one hour prior to collection. The donated sample contained approximately 3 mL of saliva, which was separated into two separate 2 mL microcentrifuge tubes containing approximately 1.5 mL of saliva in each tube. The tubes of saliva standard were labeled as "Tube 1" and "Tube 2." Each tube was centrifuged for 3 minutes at a maximum speed of 13,200 rpm. Approximately 1 mL of supernatant was removed and the remaining contents were resuspended in 500 μL of Tris-EDTA (TE) Buffer. Both tubes were stored at -20°C when not in use. These samples could now be more accurately referred to as suspensions of buccal cells, but will be referred to as saliva or saliva standards going forward.

The substrates utilized during these experiments were white, ISO Adjacent Cotton swatches (Testfabrics, Inc., West Pittston, PA). The swatches were approximately 10 cm long and 4 cm wide, which were cut in half lengthwise with clean scissors before use.

2.1.1 Saliva Stains After Heated Passive Headspace Concentration

All bench space and materials were cleaned with 10% bleach, followed by 70% ethanol unless sterile. Five swatches were laid out on clean Kimtech Science™ Kimwipes™ Delicate Task Wipes (Kimberly-Clark Corporation, Irving, TX) over clean bench paper. "Tube 1" of saliva standard was vortexed thoroughly and 20 μL of saliva

was spiked onto the approximate center of each of the five swatches using a manual single channel pipette. The swatches were left to dry in a biological safety cabinet for at least 24 hours on the original bench paper. Once the stains were dry, each swatch was packaged separately in glassine envelopes and then together in a coin envelope and tape-sealed. The swatches were stored in a drawer at room temperature for approximately five days.

Four separate experimental conditions were explored, along with positive and negative control samples. When it came time for incubation, the swatches were separated into different, labeled, pint-sized paint cans that were then incubated in an oven at the designated temperature for the designated amount of time. The temperature at the start and end of incubation was also checked and noted to ensure the oven was not set too high or too low. *Table 1* lists the sample numbers and descriptions. Sample 5 functioned as a positive control, which was a swatch spiked with 20 μL of saliva standard and stored at room temperature and processed without the application of heated passive headspace concentration. Sample 6, the negative control, was a clean swatch stored at room temperature, with no biological stain.

Table 1. Sample descriptions for unadulterated saliva stains. These four conditions at 50ºC and 90ºC for two different time periods were consistent across all experiments. While the timepoints do not completely encompass the full range for heated passive headspace, they are within the timeframe suggested by ASTM Guideline E1412.

Sample #	Description / Experimental Condition
1	50°C, 6 hours
2	50°C, 16 hours
3	90°C, 6 hours
4	90°C, 16 hours
5	Positive control
6	Negative control
7	Reagent blank

2.1.1.1 DNA Extraction

DNA extraction was performed on all seven samples using a *forensic*GEM Universal kit (MicroGEM, Charlottesville, VA) and following the manufacturer's recommended protocol. Sample 7, the reagent blank, tested the validity of the components of the extraction kit. A cutting of approximately 4 - 5 mm^2 was retrieved from the approximate center of the saliva stain using a rotary cutter and DNA was extracted utilizing 100µL of a master mix containing water, *forensic*GEM enzyme, and 10X BLUE buffer. The cutting and master mix were combined in 0.5 mL microcentrifuge tubes. The tubes were briefly vortexed and then briefly centrifuged. It was ensured that the cuttings were completely submerged in the liquid contents before incubation. The seven samples were incubated in the Veriti™ 60 Well Thermal Cycler (Applied Biosystems, Waltham, MA) using the "ZyGEM General Protocol" run method. This run method utilizes the cycle times and temperatures recommended by the manufacturer of

the *forensic*GEM Universal Kit. At the completion of the run, the samples were then taken directly into quantitation.

2.1.1.2 Quantitation

The DNA extracts were quantitated using the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, Waltham, MA) on the 7500 Real-Time PCR system (Applied Biosystems, Waltham, MA). The protocol that was followed was recommended by the manufacturer. A set of standards with known concentrations were prepared and plated twice along with the experimental samples and two No Template Control (NTC) samples. The standard concentrations utilized to develop a standard curve were 50 ng/μL, 5.0 ng/μL, 0.50 ng/μL, 0.050 ng/μL, and 0.0050 ng/μL of Quantifiler™ Trio DNA Standard diluted in Quantifiler™ Trio DNA Dilution Buffer.

2.1.1.3 Amplification

The samples were amplified using the GlobalFiler™ PCR Amplification Kit (Applied Biosystems, Waltham, MA) following the manufacturer's recommended protocol. The target DNA concentration for each sample was 0.75 ng of DNA and the total volume in each tube was 25 μL. Amplification positive and negative controls were also created. The positive control utilized the DNA Control 007 from the kit. The negative control contained 15 μL of TE Buffer and 10 μL of amplification master mix. After all of the samples were plated, the strip tubes were capped with non-optical cap strips and the tubes were vortexed and centrifuged briefly. The tubes were then moved to

the GeneAmp™ PCR System 9700 (Applied Biosystems, Waltham, MA) and the program "Glob" was selected. The cycling parameters were as follows:

Hold
95ºC for 1 minute, *then,*

94ºC for 10 seconds
59 ºC for 90 seconds
For 28 cycles, *then*

Time Delay File
60ºC for 10 minutes, *then,*

Soak at 4 ºC

At the completion of the 28 cycles, the strip tubes containing the samples were moved to a 96-well box and stored at -30ºC until capillary electrophoresis.

2.1.2. Saliva Stains After Heated Passive Headspace Concentration in the Presence of Gasoline

All bench space and materials were cleaned using 10% bleach, followed by 70% ethanol. Nine cotton swatches were laid out upon Kimtech Science™ Kimwipes™ Delicate Task Wipes on clean bench paper and spiked with 20 μL of recently thawed and vortexed saliva standard from "Tube 1." The stains were left to dry under the biological safety cabinet for at least 24 hours and then packaged separately in glassine envelopes, then in coin envelopes and tape-sealed. The samples were stored at room temperature for approximately one week.

After a week, four of the nine swatches were spiked with 20 μL of 50% weathered gasoline directly onto the approximate location of the saliva stain from the previous week. Four of the remaining five swatches were spiked with 20 μL of 50% gasoline on one corner of the swatch, away from the saliva stain. One of each type of swatch was designated to each of the four experimental conditions. *Table 2* lists the sample numbers and descriptions. One swatch was stored at room temperature to serve as a positive control. A clean swatch was designated as a negative control. A reagent blank sample was also created at the time of extraction.

Table 2. Sample descriptions for saliva stains spiked with 50% weathered gasoline. Samples denoted as "direct gasoline" received 20 μL of 50% weathered gasoline onto the approximate location of the saliva stain. Samples denoted as "indirect gasoline" received 20 μL of 50% weathered gasoline onto a corner of the cotton swatch, as far away from the saliva stain as possible. The goal of these two locations was to determine the effect of gasoline in direct contact with the biological fluid and the effect of gasoline presiding simply in the headspace during heated passive headspace concentration.

Sample #	**Description / Experimental Condition**
1	50°C, 6 hours, direct gasoline
2	50°C, 6 hours, indirect gasoline
3	90°C, 6 hours, direct gasoline
4	90°C, 6 hours, indirect gasoline
5	50°C, 16 hours, direct gasoline
6	50°C, 16 hours, indirect gasoline
7	90°C, 16 hours, direct gasoline
8	90°C, 16 hours, indirect gasoline
9	Negative Control
10	Positive Control
11	Reagent Blank

2.1.2.1 DNA Extraction

DNA extraction was performed on all eleven samples using the *forensic*GEM Universal kit and following the manufacturer's recommended protocol. A cutting of approximately 4 – 5 mm^2 was retrieved from the approximate center of the saliva stain using a rotary cutter and DNA was extracted utilizing 100 μL of a master mix containing water, *forensic*GEM enzyme, and 10X Blue Buffer. The cutting and master mix were combined in 0.5 mL microcentrifuge tubes. The samples were briefly vortexed and then briefly centrifuged. It was ensured that the cuttings were completely submerged in the liquid contents. The eleven samples were placed into the Veriti™ 60-Well Thermal Cycler using the "ZyGEM General Protocol" run method. This run method utilizes the cycle times and temperatures recommended by the manufacturer of the *forensic*GEM Universal Kit. The samples were then taken directly into quantitation.

2.1.2.2 Quantitation

The DNA extracts were quantitated using the Quantifiler™ Trio DNA Quantification Kit on the 7500 Real-Time PCR system. A set of standards with known concentrations were prepared and plated twice along with the experimental samples and two No Template Control (NTC) samples. The standard concentrations utilized to develop a standard curve were 50 ng/μL, 5.0 ng/μL, 0.50 ng/μL, 0.050 ng/μL, and 0.0050 ng/μL of Quantifiler™ Trio DNA Standard diluted in Quantifiler™ Trio DNA Dilution Buffer. Quantitation was performed using the manufacturer's recommended protocol.

2.1.2.3 Amplification

The samples were amplified using a GlobalFiler™ PCR Amplification Kit using the manufacturer's recommended protocol. The target DNA concentration for each sample was 0.75 ng of DNA and the total volume in each tube was 25 μL. Amplification positive and negative controls were also created. The positive control utilized the DNA Control 007 from the kit. The negative control contained 15 μL of TE Buffer and 10 μL of amplification master mix. After all of the samples were plated, the strip tubes were capped with non-optical cap strips and the tubes were vortexed and centrifuged briefly. The tubes were then moved to the GeneAmp™ PCR System 9700 and the program "Glob" was selected. At the completion of the 28 cycles, the strip tubes containing the samples were moved to a 96-well box and stored at -30°C until capillary electrophoresis.

2.1.3 Saliva Stains After Heated Passive Headspace Concentration in the Presence of Burnt Substrate and After Exposure to Open Flame (*forensic*GEM)

All bench space and materials were cleaned using 10% bleach, followed by 70% ethanol. Five cotton swatches were spiked with 20 μL of recently thawed and vortexed saliva standard from "Tube 1." The five swatches were left to dry under a biological safety cabinet for approximately 24 hours. One of the five swatches served as a positive control during this experiment, while a clean swatch served as a negative control. The other four spiked swatches were burned using an open-flame Worthington Pro Grade propane torch (Worthington Industries, Columbus, OH) from approximately six inches away for approximately nine seconds. All swatches that experienced burning were taped

to a clean piece of heat resistant tile (to prevent the swatch from moving) and then burned with the propane torch. Four new swatches were burned using the same burning methods and then spiked with 20 μL of saliva standard from "Tube 1" using a single channel manual pipette and left to dry for approximately 24 hours. *Table 3* lists the sample numbers and descriptions. *Figure 3* shows one of the ideal "spiked-then-burned" saliva stains from this experiment.

Table 3. Sample descriptions for burnt saliva stains extracted with *forensic*GEM Universal.

Sample #	Description / Experimental Condition
1	50°C, 6 hours, burned-then-spiked
2	50°C, 6 hours, spiked-then-burned
3	90°C, 6 hours, burned-then-spiked
4	90°C, 6 hours, spiked-then-burned
5	50°C, 16 hours, spiked-then-burned
6	50°C, 16 hours, burned-then-spiked
7	90°C, 16 hours, spiked-then-burned
8	90°C, 16 hours, burned-then-spiked
9	Negative Control
10	Positive Control
11	Reagent Blank

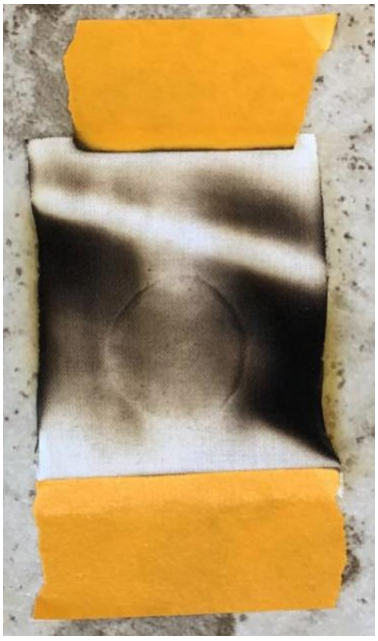

Figure 3. "Spiked-then-burned" saliva stain. This is an example of one of the "spiked-then-burned" saliva stains. The coloring of this swatch was ideal in terms of charring, but results varied across swatches. While all swatches were burned for approximately nine seconds, the rate at which the cotton swatch burned varied across samples.

2.1.3.1 DNA Extraction

DNA extraction was performed on all eleven samples using a *forensic*GEM Universal kit and following the manufacturer's recommended protocol. A cutting of approximately 4 – 5 mm^2 was retrieved from the approximate center of the saliva stain using a rotary cutter. If the area of saliva was excessively charred, the lightest portion of the stain (generally light brown in color, as opposed to a heavily charred black color) was cut and extracted. DNA was extracted utilizing 100 μL of a master mix containing water, *forensic*GEM, and 10X Blue Buffer from the kit. The cutting and master mix were combined in 0.5 mL microcentrifuge tubes. The samples were briefly vortexed and then briefly centrifuged. It was ensured that the cuttings were completely submerged in the

liquid contents. The samples were placed into the Veriti™ 60 Well Thermal Cycler using the "ZyGEM General Protocol" run method. This run method utilizes the cycle times and temperatures recommended by the manufacturer of the *forensic*GEM Universal Kit. The samples were then taken directly into quantitation.

2.1.3.2 Quantitation

The DNA extracts were quantitated using the Quantifiler™ Trio DNA Quantification Kit on the 7500 Real-Time PCR system. A set of standards with known concentrations were prepared and plated twice along with the experimental samples and two No Template Control (NTC) samples. The standard concentrations utilized to generate the standard curve were 50 ng/μL, 5.0 ng/μL, 0.50 ng/μL, 0.050 ng/μL, and 0.0050 ng/μL of Quantifiler™ Trio DNA Standard diluted in Quantifiler™ Trio DNA Dilution Buffer. Quantitation was performed using the manufacturer's recommended protocol.

2.1.3.3 DNA Concentration using Microcon® DNA Fast Flow Centrifugal Filters

Samples that did not contain enough DNA to add 1μL or more of sample to the amplification reaction were concentrated using Microcon® DNA Fast Flow Centrifugal Filters (MilliporeSigma, Burlington, MA). This included any samples that contained an "Undetermined" amount of DNA in the hopes of cleaning the sample up and making it viable. The samples that were concentrated included all four of the "spiked-then-burned"

samples (Sample 2, 4, 5, 7), as well as the "burned-then-spiked" sample held at 90ºC for 16 hours (Sample 8) and a portion of the reagent blank for this set of extracts. Concentration was performed using the manufacturer's recommended protocol. The extracts were stored overnight at -20°C and quantitated again.

2.1.3.4 Amplification

The samples were amplified using a GlobalFiler™ PCR Amplification Kit using the manufacturer's recommended protocol. The target DNA concentration for each sample was 0.75 ng of DNA and the total volume in each tube was 25 μL. Amplification positive and negative controls were also created. The positive control contained the DNA Control 007 from the kit. The negative control contained 15 μL of TE Buffer and 10 μL of amplification master mix. After all of the samples were plated, the strip tubes were capped with non-optical cap strips and the tubes were vortexed and centrifuged briefly. The tubes were then moved to the GeneAmp™ PCR System 9700 and the program "Glob" was selected. At the completion of the 28 cycles, the strip tubes containing the samples were moved to a 96 well box and stored at -30°C until capillary electrophoresis.

2.1.4 Saliva Stains After Heated Passive Headspace Concentration in the Presence of Burnt Substrate and After Exposure to Open Flame (QIAmp)

All bench space and materials were cleaned using 10% bleach, followed by 70% ethanol. Five cotton swatches were spiked with 20 μL of recently thawed and vortexed saliva standard from "Tube 2." The five swatches were left to dry under the biological

safety cabinet for approximately 24 hours. One of the five swatches served as a positive control during this experiment, while a clean swatch served as a negative control. The other four spiked swatches were burned using an open-flame Worthington Pro Grade propane torch from approximately six inches away for approximately nine seconds. Four new swatches were burned using the same burning methods and then spiked with 20 μL of saliva standard from "Tube 2" and left to dry for approximately 24 hours.

One of each type of swatch was designated to each of the four experimental conditions. During these times, the positive control sample was stored at room temperature. *Table 4* lists the sample numbers and descriptions.

Table 4. Sample descriptions for burnt saliva stains extracted with QIAmp DNA Investigator Kit. These samples were extracted using a different kit with the goal of obtaining DNA from the "spiked-then-burned" samples that were not successful with *forensic*GEM Universal extraction. Even though only the "spiked-then-burned" samples were of interest, all conditions were repeated for the sake of comparison.

Sample #	Description / Experimental Condition
1	50ºC, 6 hours, spiked-then-burned
2	50ºC, 16 hours, spiked-then-burned
3	90ºC, 6 hours, spiked-then-burned
4	90ºC, 16 hours, spiked-then-burned
5	50ºC, 6 hours, burned-then-spiked
6	50ºC, 16hours, burned-then-spiked
7	90ºC, 6 hours, burned-then-spiked
8	90ºC, 16 hours, burned-then-spiked
9	Negative Control
10	Positive Control
11	Reagent Blank

2.1.4.1 DNA Extraction

DNA extraction was performed on a cutting of each swatch that measured approximately 4 – 5 mm^2. The cutting was retrieved from the approximate center of the saliva stain using a rotary cutter, or from the lightest portion of the stain if the swatch was heavily charred. The protocol used for extraction was recommended by the manufacturer of the QIAmp DNA Investigator Kit (QIAGEN, Hilden, Germany). After extraction, the extracts were taken directly into quantitation.

2.1.4.2 Quantitation

The DNA extracts were quantitated using the Quantifiler™ Trio DNA Quantification Kit on the 7500 Real-Time PCR system. A set of standards with known concentrations were prepared and plated twice along with the experimental samples and two No Template Control (NTC) samples. The standard concentrations utilized to generate a standard curve were 50 ng/μL, 5.0 ng/μL, 0.50 ng/μL, 0.050 ng/μL, and 0.0050 ng/μL of Quantifiler™ Trio DNA Standard diluted in Quantifiler™ Trio DNA Dilution Buffer. Quantitation was performed using the manufacturer's recommended protocol.

2.1.4.3 Amplification

The samples were amplified using a GlobalFiler™ PCR Amplification Kit using the manufacturer's recommended protocol. The target DNA concentration for each sample was 0.75 ng of DNA and the total volume in each tube was 25 μL. Amplification

positive and negative controls were also created. The positive control contained the DNA Control 007 from the kit. The negative control contained 15 μL of TE Buffer and 10 μL of amplification master mix. After all of the samples were plated, the strip tubes were capped with non-optical cap strips and the tubes were vortexed and centrifuged briefly. The tubes were then moved to GeneAmp™ PCR System 9700 and the program "Glob" was selected. At the completion of the 28 cycles, the strip tubes containing the samples were moved to a 96-well box and stored at -30°C until capillary electrophoresis.

2.2. Applying Proof-Of-Concept Experimental Conditions to Semen Stains

All of the following experiments were performed using a donated sample of neat semen from Donor # R08-00635. The semen was removed from a donation cup and transferred to a 2 mL microcentrifuge tube that was stored at -20°C for approximately one day before use. The sample remained a neat semen sample for all of the experiments. The substrates utilized during these experiments were the same white, ISO Adjacent Cotton swatches that were used in previous experiments.

2.2.1 Semen Stains After Heated Passive Headspace Concentration

All bench spaces, instruments, and tools were cleaned with 10% bleach and then 70% ethanol before preparing samples. Seventeen clean cotton swatches were laid out on bench paper on top of clean Kimtech Science™ Kimwipes™ Delicate Task Wipes. Each swatch was spiked with 20 μL of neat semen from the donor previously mentioned. Every swatch was spiked at the approximate center of the swatch. All swatches were

moved to the biological safety cabinet and allowed to dry overnight for approximately 24 hours. Four of these 17 samples remained unadulterated and were utilized to evaluate the recovery of DNA after enduring heated passive headspace concentration conditions. A fifth sample remained unadulterated and did not undergo heated passive headspace concentration conditions in order to serve as a positive control. This sample remained at room temperature and was processed in the same manner as the other samples. A clean cotton swatch with no semen served as a negative control.

2.2.2 Semen Stains After Heated Passive Headspace Concentration in the Presence of Gasoline

Four of the previously mentioned cotton swatches spiked with neat semen were spiked with 20 μL of 50% weathered gasoline directly onto the approximate location of the semen stain once the stain was dry. Four additional spiked swatches were also spiked with 20 μL of 50% weathered gasoline, but were spiked indirectly, on a corner of the cotton swatch, away from the semen stain.

2.2.3 Semen Stains After Heated Passive Headspace Concentration in the Presence of Burnt Substrate and After Exposure to Open Flame

Four clean cotton swatches (in addition to the previously mentioned seventeen swatches) were burned with an open-flame Worthington Pro Grade propane torch from approximately 6 inches away until visibly brown in color and were then spiked with 20 μL of semen and allowed to dry for 24 hours. These four swatches were considered the

"burned-then-spiked" samples and were spiked at the same time as the seventeen previously mentioned cotton swatches. The remaining four dried, spiked swatches were also burned with an open-flame Worthington Pro Grade propane torch until visibly brown in color. These four swatches were considered the "spiked-then-burned" samples. *Table 5* lists the sample numbers and descriptions. *Figure 4* shows one of the ideal "spiked-then-burned" semen stains.

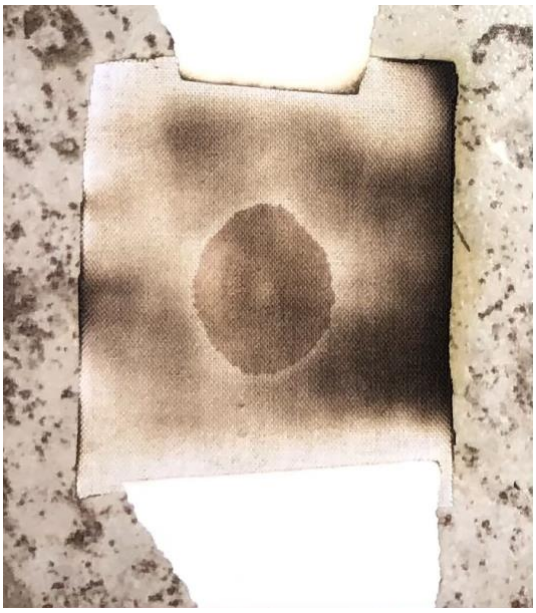

Figure 4. "Spiked-then-burned" semen stain. After some saliva stains experienced different rates of burning when exposed to the flame, it was decided that swatches should be burned until the desired level of charring was reached. This was done to prevent swatches from becoming too burnt and therefore, almost completely incinerated. This is an example of one of the ideal "spiked-then-burned" semen stains.

Table 5. Semen stain sample numbers and descriptions. Unlike with the saliva stains, all of the semen stain sample preparation, quantitation, and amplification were carried out within the same week due to time constraints. All conditions were virtually identical to those used on saliva stains, however rather than burning the swatches for 9 seconds, the amount of burning was based off of the amount of charring visualized to avoid extreme charring. All of these swatches were also incubated in new, clean paint cans or paint cans that were decontaminated with 10% bleach and 70% ethanol.

Sample #	Description / Experimental Condition
1	50°C, 6 hours
2	50°C, 16 hours
3	90°C, 6 hours
4	90°C, 16 hours
5	50°C, 6 hours, direct gasoline
6	50°C, 16 hours, direct gasoline
7	90°C, 6 hours, direct gasoline
8	90°C, 16 hours, direct gasoline
9	50°C, 6 hours, indirect gasoline
10	50°C, 16 hours, indirect gasoline
11	90°C, 6 hours, indirect gasoline
12	90°C, 16 hours, indirect gasoline
13	50°C, 6 hours, burned-then-spiked
14	50°C, 16 hours, burned-then-spiked
15	90°C, 6 hours, burned-then-spiked
16	90°C, 16 hours, burned-then-spiked
17	50°C. 6 hours, spiked-then-burned
18	50°C, 16 hours, spiked-then-burned
19	90°C, 6 hours, spiked-then-burned
20	90°C, 16 hours, spiked-then-burned
21	Positive Control
22	Negative Control
23	Reagent Blank

2.2.4 DNA Extraction

Extraction was performed on cuttings from all 22 samples using a *forensic*GEM Sperm Lysis Kit (MicroGEM, Charlottesville, VA)**.** The cuttings taken were approximately 4 – 5 mm^2 and were taken from the approximate center of the stain. In the

case of burnt samples, extremely charred locations on the swatch were avoided if possible. The cuttings were separated into labeled 0.5 mL microcentrifuge tubes. A master mix containing water, 10X Red Buffer, Acrosolv®, and *forensic*GEM enzyme was prepared. All steps taken were retrieved from the manufacturer's recommended protocol, unless otherwise noted. The protocol calls for the use of 10X Orange Buffer, but the use of 10X Red Buffer instead is common in the laboratory. Once samples were combined with master mix and vortexed, tubes were moved to the Veriti™ 60-Well Thermal Cycler. The samples were incubated for the recommended time and temperature from the manufacturer of the kit. After incubation, the cuttings were removed and moved to a spin basket in a 1.5 mL microcentrifuge tube and centrifuged for 3 minutes at 15,000 rpm. The extract remaining in the 1.5 mL microcentrifuge tube after centrifugation was combined with rest of the extract contained in the 0.5 mL tubes. The tubes were moved to -20° storage and quantitated the next day.

2.2.5 Quantitation

The DNA extracts were quantitated using the Quantifiler™ Trio DNA Quantification Kit on the 7500 Real-Time PCR system. A set of standards with known concentrations were prepared and plated twice along with the experimental samples and two No Template Control (NTC) samples. The standard concentrations used to generate a standard curve were 50 ng/µL, 5.0 ng/µL, 0.50 ng/µL, 0.050 ng/µL, and 0.0050 ng/µL of Quantifiler™ Trio DNA Standard diluted in Quantifiler™ Trio DNA Dilution Buffer. Quantitation was performed using the manufacturer's recommended protocol. Unlike the

saliva stain extract samples, all of the semen stain extract samples were plated in triplicate for the purpose of increasing the accuracy of the quantitation value. When utilizing the quantitation values for amplification, an average of the three values was used for calculating the necessary amount of sample needed to reach the target of 0.75 ng of DNA.

2.2.6 DNA Concentration using Microcon® DNA Fast Flow Centrifugal Filters

Samples that did not contain enough DNA to add 1μL or more of sample to the reaction were concentrated using Microcon® DNA Fast Flow Centrifugal Filters. This included any samples that contained an "Undetermined" amount of DNA in the hopes of cleaning the sample up and making it viable. The samples that were concentrated included Sample 18, 19, and 20, as well as a portion of the reagent blank (Sample 23) for this set of extracts. Microcon® concentration was performed using the manufacturer's recommended protocol. The extracts were stored overnight in a -20ºC freezer.

2.2.7 Amplification

The samples were amplified using a GlobalFiler™ PCR Amplification Kit using the manufacturer's recommended protocol. The target DNA concentration for each sample was 0.75 ng of DNA and the total volume in each tube was 25 μL. Amplification positive and negative controls were also created. The positive control utilized the DNA Control 007 from the kit. The negative control contained 15 μL of TE Buffer and 10 μL of amplification master mix. Samples that did not contain enough DNA to achieve 0.75

ng in were not combined with TE buffer (Sample 18-M and 20-M). In order to obtain the closest value to 0.75 ng, 15 μL of only sample was added to the reaction with the amplification master mix. This is the maximum volume of sample that could be added to the reaction. After all of the samples were plated, the strip tubes were capped with non-optical cap strips and the tubes were vortexed and centrifuged briefly. The tubes were then moved to GeneAmp™ PCR System 9700 and the program "Glob" was selected. At the completion of the 28 cycles, the strip tubes containing the samples were moved to a 96-well box and stored in a -30°C freezer until capillary electrophoresis.

2.3 Capillary Electrophoresis of Amplification Products from Saliva and Semen Stains

Amplified saliva and semen DNA extracts were prepared for capillary electrophoresis using the GlobalFiler™ PCR Amplification Kit, the GlobalFiler™ Allelic Ladder, and the GeneScan™ 600 LIZ™ Size Standard v2.0 (Applied Biosystems, Waltham, MA). Sample preparation was performed based on the manufacturer's protocol for amplification. Human Identification and amplicon separation/detection was performed using the SeqStudio Genetic Analyzer Instrument (Applied Biosystems, Waltham, MA) and the protocol was based on the manufacturer's recommendations in the *Software User Manual.* The injection of samples occurred at 1200 volts for 10 seconds with a capillary temperature of 60°C.

2.4 Profile Generation Using GeneMapper ID-X

At the completion of capillary electrophoresis, the data was exported from the SeqStudio instrument and imported into a computer with GeneMapper ID-X Forensic Data Analysis and Expert System Software Version 1.6 (Applied Biosystems, Waltham, MA). The Analytical Method selected was "Globalfiler SeqStudio CJM Thesis." *Table 6* lists a portion of the settings utilized for initial profile generation using GeneMapper ID-X.

Table 6. GeneMapper ID-X settings for profile generation. The left column lists titles of GeneMapper ID-X settings sections and the right column lists settings within those sections.

General	Analysis Type: HID
Allele	Bin Set: SequStudio_Bins_v6.1X Use marker-specific stutter ratio and distance if available
Peak Detector	Peak Amplitude Thresholds: 100 RFU for all dye colors Smoothing: Light Analysis: Full Range Sizing: All Sizes
Peak Quality	Homozygous min peak height (LPH): 50.0 Heterozygous min peak height (LPH): 50.0 Max Peak Height (MPH): 5000.0
Panel	SeqStudio_GlobalFiler_Panel_v2X
Size Standard	GS600_LIZ(60-460)

3. RESULTS AND DISCUSSION

3.1 Quantitation and Degradation of DNA in Saliva

Quantitation was performed on the saliva stain cuttings for each experiment using the Quantifiler™ Trio DNA Quantification Kit.

3.1.1 Unadulterated Saliva Stains

The resulting standard curve produced from the two sets of five standards yielded an R^2 value of over 0.998 for all three loci targets and efficiency percentages of over 99 percent for each target as well. All IPC Ct values appeared normal. *Table 7* lists the resulting quantitation values truncated to two significant figures for the small autosomal target for each sample from this experiment.

Table 7. Quantitation results of DNA extracted from unadulterated saliva stains. As expected, samples that were incubated at higher temperatures resulted in higher degradation indices. The degradation indices for the negative control and reagent blank were considered to have no data (ND), given that no DNA was present to begin with. While the reagent blank did yield a value, it is a value that is extremely low and expected. If reagent blank values were closer to sample quantities, this would suggest contamination. However, a value of 0.00028 merely suggests that the reagents themselves may cause a variation in quantitation.

Sample #	Description	Quantity (ng/μL)	Degradation Index
1	50°C, 6 hours	0.35	0.85
2	50°C ,16 hours	0.25	0.85
3	90°C, 6 hours	0.21	1.1
4	90°C, 16 hours	0.35	1.6
5	Positive Control	0.70	0.93
6	Negative Control	Undetermined	ND
7	Reagent Blank	0.00028	ND

While this sample set was small, it can be theorized that the increase in temperature and time spent in an oven could be the cause for increased degradation index. While the degradation index was higher in samples that experienced higher temperatures, the value is still on the low-end of the spectrum, suggesting that the degradation that is present is minimal. This trend of higher degradation indices

accompanying higher oven temperatures is maintained consistently through most of the quantitation performed.

3.1.2 Saliva Stains in the Presence of Gasoline

The resulting standard curve produced from the two sets of five standards yielded an R^2 value of over 0.998 for all three loci targets and efficiency percentages of over 100 percent for each target as well. All IPC Ct (threshold cycle) values appeared normal. *Table 8* lists the resulting quantitation values truncated to two significant figures for the small autosomal target for each sample from this experiment.

Table 8. Quantitation results of DNA extracted from saliva stains in the presence of gasoline. These samples maintained a common trend of increased DI with increased time and temperature. While some experimental samples did yield higher concentrations of DNA, it is not possible to say whether that was due to the experimental conditions or if the DNA spiked onto the swatch was higher from the beginning. Sample numbers are out of order so that any possible trends amongst "direct gasoline" and "indirect gasoline" samples could be easily noticed.

Sample #	Description	Quantity (ng/μL)	Degradation Index
1	50°C, 6 hours, direct gasoline	0.25	0.74
5	50°C, 16 hours, direct gasoline	0.22	0.74
3	90°C, 6 hours, direct gasoline	0.36	0.84
7	90°C, 16 hours, direct gasoline	0.49	1.2
2	50°C 6 hours, indirect gasoline	0.19	0.67
6	50°C, 16 hours, indirect gasoline	0.14	0.71
4	90°C, 6 hours, indirect gasoline	0.31	0.91
8	90°C, 16 hours, indirect gasoline	0.47	1.0
9	Negative Control	Undetermined	ND
10	Positive Control	0.26	0.60
11	Reagent Blank	0.00013	ND

Once again, while the samples held at higher temperatures and longer timepoints have higher degradation indices, these values are still on the low-end of the spectrum for low to moderate degradation, suggesting that there is still likely minimal degradation occurring. There is also no extreme degradation difference between the samples that received direct spiking of gasoline versus those that merely had gasoline on the edge of the swatch, away from the biological stain, suggesting that direct contact with gasoline is no more harmful than the presence of gasoline in the headspace.

3.1.3 Burnt Saliva Stains

The rationale behind exploring both burned and then spiked swatches and spiked and then burned swatches was to help distinguish the cause of inhibition/degradation, if there was any. If "burned-then-spiked" samples experienced more inhibition, then the presence of burnt substrate was likely the cause. If "spiked-then-burned" samples experienced more inhibition, the exposure to open flame and the high heat of that flame was likely the cause. The resulting standard curve for the *forensic*GEM Universal sample set produced from the two sets of five standards yielded an R^2 value of over 0.996 for all three loci targets and efficiency percentages of over 100 percent for each target as well. All IPC Ct values appeared normal. *Table 9* lists the resulting quantitation values truncated to two significant figures for the small autosomal target for each sample from this experiment. Given that Samples 2, 4, 5, 7, and 8 were low or "undetermined" in DNA concentration, these samples were concentrated using Microcon® DNA Fast Flow Centrifugal Filters. Samples 2, 4, 5, and 7 had unknown expected concentrations after

Microcon® concentration due to their initial concentrations being around zero or "undetermined." Microcon® was performed on these samples in the hopes that if there were some inhibitors preventing quantitation, they could be cleaned out. Sample 8, now referred to as 8-M after concentration, had an expected concentration of 0.11 ng/μL.

Table 9. Quantitation results of DNA extracted from burned saliva stains using *forensic*GEM Universal. None of the "spiked-then-burned" samples were successful, likely due to the presence of direct contact with a flame. Sample numbers are out of order so that any trends in quantity or quality, if present, can be easily noticed. Sample numbers are out of numerical order to allow for grouping certain samples together to compare their quantities and DI values.

Sample #	Description	Quantity (ng/μL)	Degradation Index
1	50°C, 6 hours, burned-then-spiked	0.33	0.90
6	50°C, 16 hours, burned-then-spiked	0.37	0.81
3	90°C, 6 hours, burned-then-spiked	0.091	1.0
8	90°C, 16 hours, burned-then-spiked	0.033	1.3
2	50°C, 6 hours, spiked-then-burned	Undetermined	ND
5	50°C, 16 hours, spiked-then-burned	0.00017	1.2
4	90°C, 6 hours, spiked-then-burned	Undetermined	ND
7	90°C, 16 hours, spiked-then-burned	8.2×10^{-5}	ND
9	Negative Control	Undetermined	ND
10	Positive Control	0.42	0.88
11	Reagent Blank	Undetermined	ND

While Sample 8-M was viable after performing concentration (0.12 ng/μL), Samples 2, 4, 5, and 7 had little to no DNA after being quantitated again and were considered failed samples. Moving forward, these samples were excluded from future steps. If our focus is turned to the "burned-then-spiked" samples, the typical trend of increased DI with increased temperature remains. Additionally, samples that were incubated at 90°C, rather than 50°C, were anywhere from three-fold to ten-fold less in DNA concentration. It is unknown as to whether this could be attributed to the incubation temperature, quantitation inhibition from the charred substrate, or a mixture of both. Given that the IPCs were normal after quantitation, it is more likely that this phenomenon can be attributed to the incubation conditions.

Given the unsuccessful nature of the "spiked-then-burned" samples of the previous experiment using *forensic*GEM Universal extraction method, the same procedure was performed after extracting with QIAmp DNA Investigator Kit to determine if a different extraction method could improve results. However, like with the previous extraction method, the "spiked-then-burned" samples were unsuccessful, and were therefore abandoned for future steps. The "burned-then-spiked" samples using this extraction method also experienced increased degradation indices with increased temperature and time as shown in *Table 10.*

The resulting standard curve for the QIAmp DNA Investigator Kit sample set produced for the two sets of five standards yielded an R^2 value of over 0.998 for all three loci targets and efficiency percentages of over 99 percent for each target as well. All IPC Ct values appeared normal. *Table 10* lists the resulting quantitation values truncated to

two significant figures for the small autosomal target for each sample from this experiment.

Table 10. Quantitation results of DNA extracted from burnt saliva stains using QIAmp DNA Investigator Kit. A different extraction method was used to determine if "spiked-then-burned" samples could be successful. However, these samples contained essentially zero DNA and were abandoned for future testing. Sample numbers are out of order so that any trends in quantity or quality, if present, could be easily noticed.

Sample #	Description	Quantity (ng/μL)	Degradation Index
5	50°C, 6 hours, burned-then-spiked	0.31	0.72
6	50°C, 16 hours, burned-then-spiked	0.87	0.77
7	90°C, 6 hours, burned-then-spiked	0.84	1.0
8	90°C, 16 hours, burned-then-spiked	0.23	1.2
1	50°C, 6 hours, spiked-then-burned	Undetermined	ND
2	50°C, 16 hours, spiked-then-burned	Undetermined	ND
3	90°C, 6 hours, spiked-then-burned	0.00025	ND
4	90°C, 16 hours, spiked-then-burned	Undetermined	ND
9	Negative Control	Undetermined	ND
10	Positive Control	1.42	0.87
11	Reagent Blank	Undetermined	ND

3.2 Capillary Electrophoresis of DNA from Saliva

After quantitation and amplification with a target of 0.75 ng of DNA, capillary electrophoresis was performed on all samples, with the exception of only performing CE on one of the several positive control samples. All samples generated the expected LIZ Size Standard profile and all reagent blanks and negative control samples were free of contamination. The DNA Control 007 positive control amplification sample and the negative control for amplification both yielded expected results. Additionally, all allelic ladder samples were successful. CE was not performed on any of the "spiked-then-

burned" saliva stain extracts, for both *forensic*GEM Universal and QIAmp DNA Investigator Kit extraction methods, given that there was little to no DNA detected during quantitation. Full genetic profiles were generated for all saliva stain extracts.

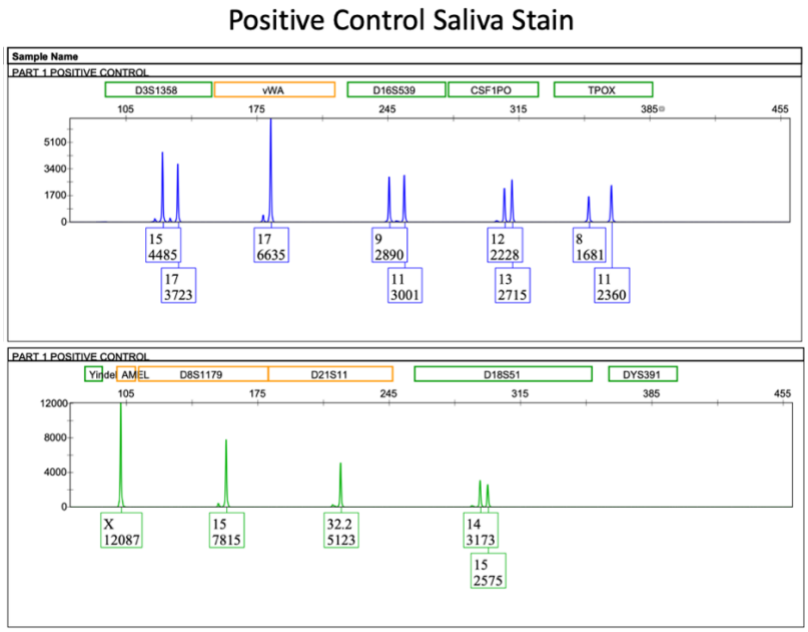

Figure 5. Electropherogram of saliva stain positive control. The saliva stain positive control sample resulted in a full profile that was suitable for interpretation and comparison to the rest of the saliva stain electropherograms.

Figure 5 shows the electropherogram panels for the blue and green dye-labeled loci for the untreated saliva stain that served as a positive control. This electropherogram served as a reference for the expected alleles for each locus for experimental samples, as well as displaying what the genetic profile would look like if the saliva sample did not endure the conditions of heated passive headspace concentration or the other variables explored. The average peak height in relative fluorescence units (RFU) for this profile was approximately 3179 RFU.

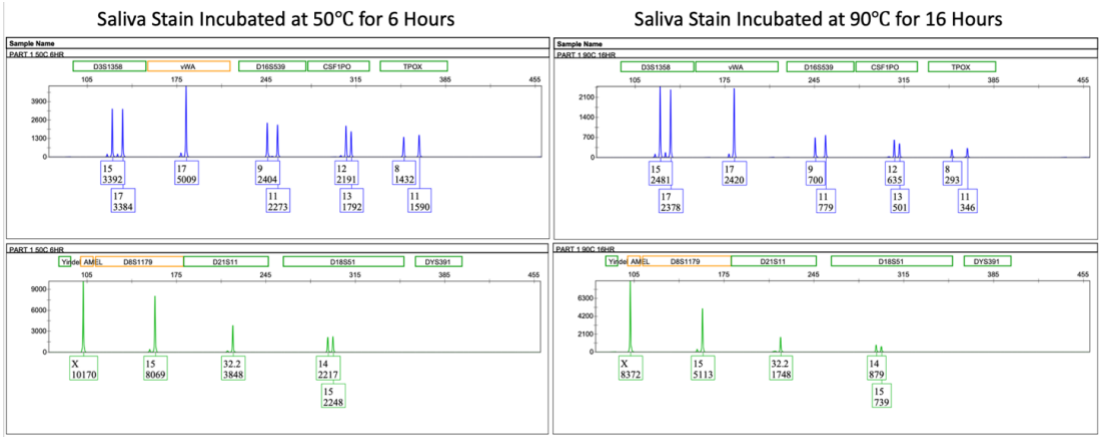

Figure 6. Electropherograms of unadulterated saliva stains incubated in the least and most extreme heated passive headspace concentration conditions. The saliva stain incubated at 50ºC for 6 hours (left) resulted in a full profile with substantial peak heights. The saliva stain incubated at 90ºC for 16 hours (right) also resulted in a full profile that had slightly lower peak heights. However, these peak heights were substantially higher than the analytical threshold of 100 RFU.

Figure 6 shows the resulting electropherograms for the saliva stains that were incubated at 50°C for 6 hours and 90°C for 16 hours. These two profiles were chosen from this set of samples in order to compare the least extreme and most extreme of the experimental conditions for this experiment. The RFU values for the sample incubated at 90°C for 16 hours appeared substantially less than those for the sample incubated at 50°C for 6 hours. In fact, the average peak height in RFU for the sample at 50°C was approximately 2690 RFU, while the average peak height in RFU for the sample at 90°C was approximately 1377 RFU. When viewing the entirety of the profile for the saliva stain at 90°C for 16 hours (right side of Figure 6), a “ski slope” pattern, a classic sign of degradation, was visible. This pattern of moving from taller peaks to shorter peaks from left to right is due to the fact that fragments of DNA that are larger in base pairs (towards

the right side of the panel) tend to break down first when degradation occurs. This phenomenon is what increases the resulting DI values.

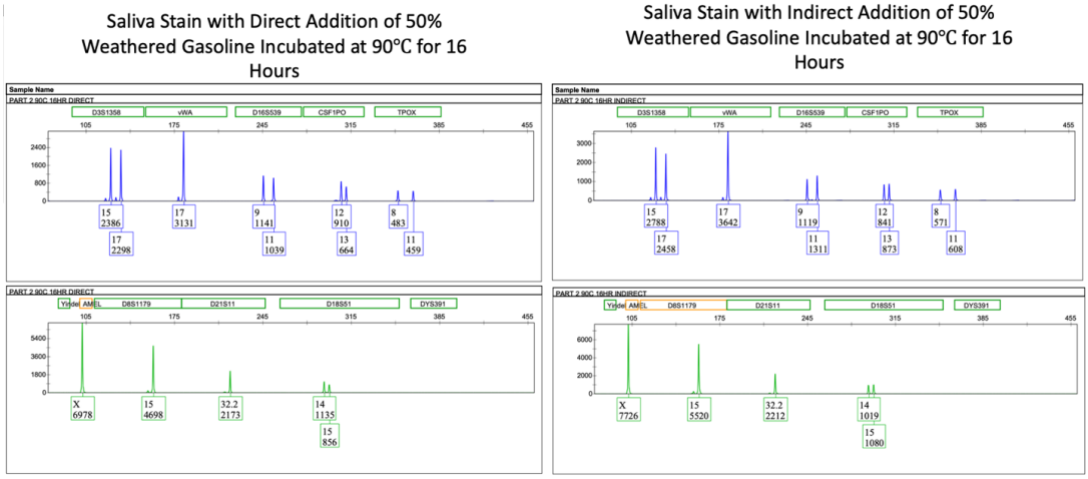

Figure 7. Electropherograms of saliva stains in contact with or in the presence of 50% weathered gasoline. The electropherograms displayed here show results from samples that endured the highest incubation temperature and time in order to observe the worst-case scenario. Both samples resulted in full genetic profiles with peak heights above the analytical threshold.

Figure 7 shows the electropherograms of the saliva stain samples for direct and indirect addition of 50% weathered gasoline in the most extreme heated passive headspace concentration condition explored – 90°C for 16 hours. Even after enduring this high temperature for 16 hours, both samples yielded full profiles, with a slight "ski slope" pattern when viewing the entirety of both profiles. Additionally, even though one stain was in direct contact with the ignitable liquid and the other was more so in the presence of the liquid, both electropherograms appear similar in peak heights. The stain experiencing direct addition of gasoline had an average peak height of approximately 1525 RFU across the whole profile, while the stain indirectly experiencing gasoline had an average peak height of 1731 RFU. This, and the fact that both stains yielded full

profiles, suggest that gasoline minimally inhibited analysis, whether in direct contact with the sample or if present in the headspace. The lack of interference from gasoline and minimal degradation from heated passive headspace concentration conditions is important for the handling of arson evidence with the possible presence of a bodily fluid. While DNA evidence is important, the volatility of ignitable liquids may warrant performing extraction of ILR prior to DNA analysis, given the results shown in this portion of the study.

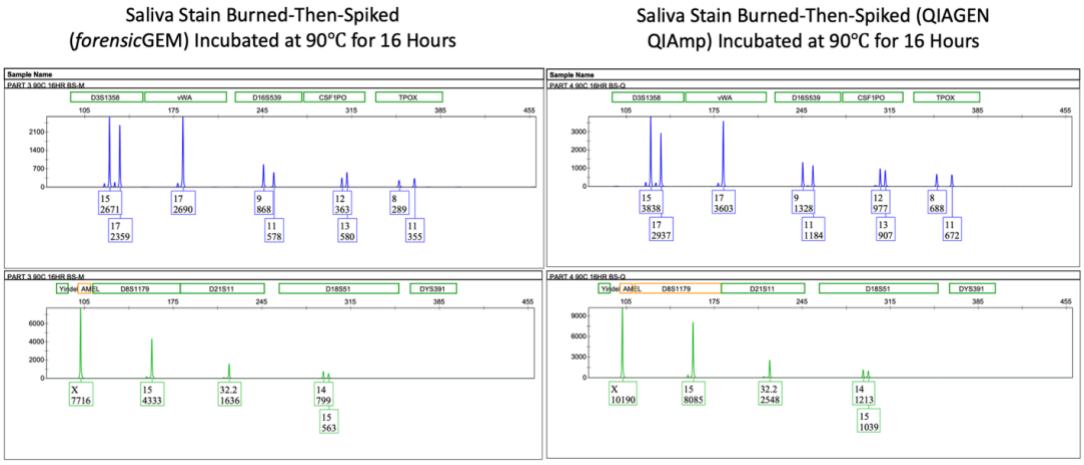

Figure 8. Electropherograms of burnt saliva stains extracted with *forensic*GEM Universal vs. QIAmp DNA Investigator Kit. The two profiles pictured show the difference, if any, between the two "burned-then-spiked" samples at the most extreme conditions and extracted using two different extraction methods. While the sample extracted with QIAmp resulted in a higher average peak height across the whole profile, both samples yielded full genetic profiles with peak heights well above the analytical threshold.

Figure 8 shows the electropherograms for the two "burned-then-spiked" saliva stains extracted with two different methods after enduring 90°C for 16 hours. While both stains yielded full genetic profiles, the peaks resulting from extraction with the QIAmp DNA Investigator Kit within the blue and green dye-labeled loci are substantially taller

than those resulting from extracting with the *forensic*GEM Universal kit. When looking at the entirety of the two profiles, the average peak height after *forensic*GEM Universal extraction was approximately 1446 RFU while the average peak height after QIAmp extraction was approximately 2154 RFU. This may suggest that while QIAmp could not overcome the issue of a biological stain in contact with a flame, it may have allowed for a cleaner extraction. Additionally, both profiles appeared to contain the "ski slope" pattern mentioned with previous profiles.

3.3 Quantitation and Degradation of DNA in Semen

DNA quantitation was performed using the Quantifiler™ Trio DNA Quantification Kit. All semen stain samples were quantitated at the same time and were quantitated in triplicate. Samples were quantitated in triplicate simply to improve the accuracy of quantitation results. The resulting standard curve produced for the two sets of five standards yielded an R^2 value of over 0.999 for all three loci targets and efficiency percentages of over 97 percent for each target as well. All IPC Ct values appeared normal. *Tables 11-13* are organized based on experiment type (i.e. unadulterated stains, direct/indirect contact with gasoline, etc.). Therefore, each table contains the positive control, negative control, reagent blank, and only sample numbers that pertain to that experiment type.

3.3.1 Unadulterated Semen Stains

Table 11. Quantitation results of DNA extracted from unadulterated semen stains. These stains were considered unadulterated due to the absence of ignitable liquid or burning/charring. All samples were deemed successful and similar to that of the group of saliva stains.

Sample #	Description	Average Quantity (ng/μL)	Average Degradation Index
1	50ºC, 6 hours	10	1.1
2	50ºC, 16 hours	8.2	1.0
3	90ºC, 6 hours	13	1.1
4	90ºC, 16 hours	11	0.99
21	Positive Control	9.0	1.1
22	Negative Control	Undetermined	ND
23	Reagent Blank	Undetermined	ND

The same heated passive headspace concentration conditions that were applied to saliva stains were also applied to neat semen stains. However, in this first set of samples with no additional adulterants besides the heating conditions, the degradation indices were similar across all samples and the average quantity of DNA was much higher in comparison to the quantity found in the saliva stains. Given that the highest degradation index was 1.1, which is also that of the positive control, it can be safe to assume that the heated passive headspace concentration conditions did not have a significant effect on the integrity of the DNA in the semen stains.

3.3.2 Semen Stains in the Presence of Gasoline

Table 12. Quantitation results of DNA extracted from semen stains in the presence of gasoline. Direct contact with gasoline and the presence of gasoline in the headspace both appeared to have little, if any, negative effect on the DNA quantity or quality.

Sample #	Description	Average Quantity (ug/μL)	Average Degradation Index
5	50ºC, 6 hours, direct gasoline	11	1.1
6	50ºC, 16 hours, direct gasoline	9.6	1.0
7	90ºC, 6 hours, direct gasoline	10	1.0
8	90ºC, 16 hours, direct gasoline	15	1.0
9	50ºC, 6 hours, indirect gasoline	10	1.0
10	50ºC, 16 hours, indirect gasoline	9.8	0.96
11	90ºC, 6 hours, indirect gasoline	11	0.96
12	90ºC, 16 hours, indirect gasoline	10	0.85
21	Positive Control	9.0	1.1
22	Negative Control	Undetermined	ND
23	Reagent Blank	Undetermined	ND

Similarly, to the last set of conditions, the degradation indices of most samples was around the same as the positive control, with some experimental samples having lower indices than the control. Additionally, the DNA concentration of these samples is extremely high, with no noticeable trend stemming from the gasoline, time, or temperature.

3.3.3 Burnt Semen Stains

Table 13. Quantitation results of DNA extracted from "burned-then-spiked" and "spiked-then-burned" semen stains. Unlike the saliva stains, the "spiked-then-burned" semen stains were more successful and one of them did not need to be concentrated (Sample 17). Sample 18, 19, and 20 were concentrated using the Microcon® DNA Fast Flow Centrifugal Filters. Only Sample 20 was deemed to be affected by moderate degradation, likely due to exposure to open flame.

Sample #	Description	Average Quantity (ug/μL)	Average Degradation Index
13	50ºC, 6 hours, burned-then-spiked	9.1	0.80
14	50ºC, 16 hours, burned-then-spiked	12	0.93
15	90ºC, 6 hours, burned-then-spiked	11	0.93
16	90ºC, 16 hours, burned-then-spiked	11	1.0
17	50ºC, 6 hours, spiked-then-burned	2.7	1.1
18	50ºC, 16 hours, spiked-then-burned	0.0013	0.99
19	90ºC, 6 hours, spiked-then-burned	0.0089	1.0
20	90ºC, 16 hours, spiked-then-burned	0.0027	5.3
21	Positive Control	9.0	1.1
22	Negative Control	Undetermined	ND
23	Reagent Blank	Undetermined	ND

A slight trend in increased degradation index with increased time and temperature can be noticed with the samples that were burned and then spiked with semen. However, while this trend exists, there is still no extreme difference between the four samples and no indication of degradation. While the spiked and then burned saliva stain samples were unsuccessful, something different can be said of the spiked and then burned semen stain samples. Not only were none of them "undetermined" in DNA concentration, one of the "spiked-then-burned" samples had a high enough DNA concentration that it did not require concentration with Microcon® (Sample 17). One possible theory is that given the increased resilience of the sperm head and the packaging of DNA in sperm, that same

resilience may be responsible for the survival of the semen stains through exposure to open flame. However, this phenomenon may also be attributed to the level of charring on this particular substrate. While the stain did experience burning in a similar fashion to the other samples, it did not char as significantly as some of the other samples. Between the previous saliva stain experiments and this set of samples, it can be stated that the effects of heat and burning were far more degrading and harmful than that of the presence of an ignitable liquid or the heated passive headspace concentration conditions themselves.

Sample 18, 19, 20, and a portion of 23 all underwent concentration with Microcon® DNA Fast Flow Centrifugal Filters in order to increase the DNA concentration prior to amplification. The final target volume was 15 µL of sample to be added to the amplification reaction. *Table 14* lists the expected concentrations of Samples 18, 19, 20, 23 after Microcon® concentration.

Table 14. Expected DNA concentrations of semen stain samples post-Microcon® concentration. Sample 18, 19, and 20 were concentrated in order to lessen the volume of sample needed for amplification. The sample volumes were decreased from 100µL to 15µL post-Microcon®. A portion of the reagent blank was concentrated as well to serve as a reagent blank for Microcon®.

Sample #	Concentration Before Microcon® Concentration (ng/µL)	Expected Concentration After Microcon® Concentration (ng/µL)
18-M	0.0013	0.008
19-M	0.0089	0.059
20-M	0.0027	0.018
23-M	Undetermined	Undetermined

After Microcon® concentration, samples 18-M and 20-M were not high enough in expected concentration to achieve 0.75 ng of DNA in 15 µL. Given that only 15 µL of

sample can be added to the reaction, the final amount of DNA within those two sample reactions was lower than 0.75 ng.

3.4 Capillary Electrophoresis of DNA from Semen

After quantitation and amplification with a target of 0.75 ng of DNA, capillary electrophoresis was performed on all samples. All samples generated the expected LIZ Size Standard profile and all reagent blanks and negative control samples were free of contamination. The DNA Control 007 positive control amplification sample and the negative control for amplification both yielded expected results. Additionally, all allelic ladder samples were successful. Full genetic profiles were produced for all semen stain samples with the exception of two samples, Sample 18-M (50ºC, 16 hours, spiked-then-burned) and Sample 20-M (90ºC, 16 hours, spiked-then-burned). These two samples yielded partial profiles.

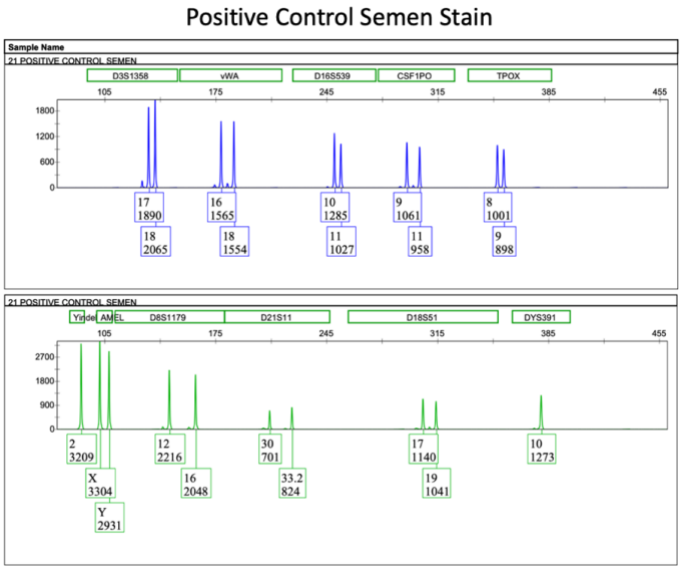

Figure 9. Electropherogram of positive control semen stain. The semen stain positive control sample resulted in a full profile that was suitable for interpretation and comparison to the rest of the semen stain electropherograms.

Figure 9 shows part of the resulting electropherogram for the positive control semen stain. This profile also served as a reference to be compared to other resulting profiles. The average peak height for this profile was approximately 1618 RFU.

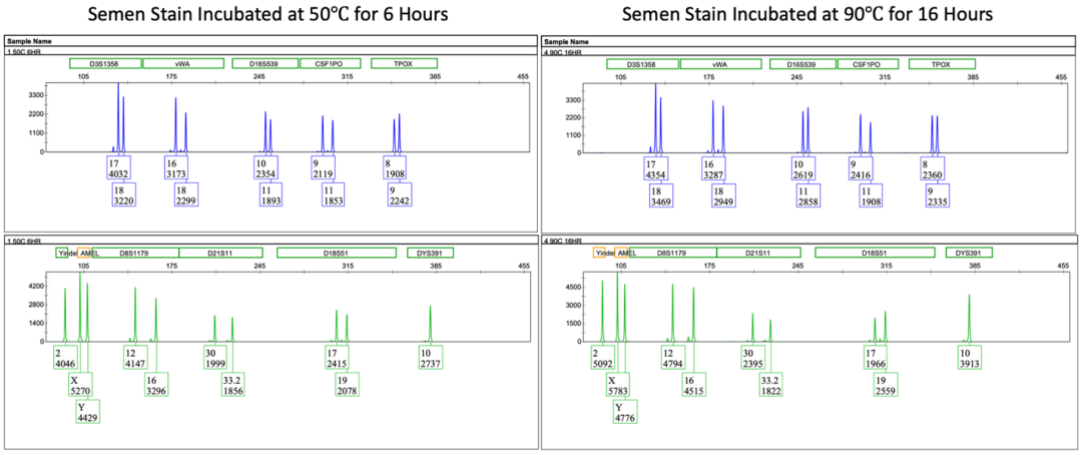

Figure 10. Electropherograms of semen stains incubated in the least and most extreme conditions. These semen stains were unadulterated and compared to show the difference between the lowest incubation temperature and time (left) and the highest incubation temperature and time (right). Both resulted in full profiles with peaks well above the analytical threshold.

Figure 10 shows Sample 1 and Sample 4, semen stains incubated at 50°C for 6 hours and 90°C for 16 hours, respectively. Both samples produced full profiles with similar peak heights at most loci, suggesting that the difference in time and temperature did not have an effect on the quality of DNA. Sample 1 had an average peak height of approximately 2880 RFU, while Sample 4 had an average peak height of approximately 3415 RFU.

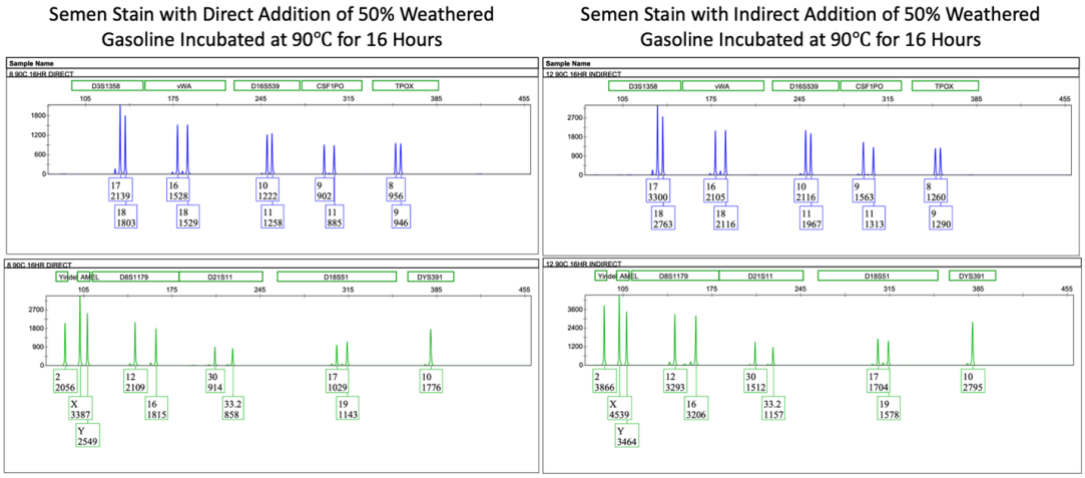

Figure 11. Electropherograms of semen stains in contact with or in the presence of 50% weathered gasoline. Neither sample displayed here seemed to be severely impacted by the direct or indirect addition of 50% weathered gasoline. Both samples resulted in full profiles.

Figure 11 compares the electropherograms of semen stains at the most extreme times and temperatures with direct (Sample 8) and indirect (Sample 12) addition of 50% weathered gasoline. Both samples yielded full profiles appropriate for interpretation with peak heights well over the analytical threshold. Sample 8 had an average peak height of 1437 RFU, while Sample 12 had an average peak height of 2218 RFU. While the sample with direct addition of the ignitable liquid had a lower average peak height across the whole profile, the ignitable liquid did not appear to significantly degrade or inhibit DNA analysis downstream.

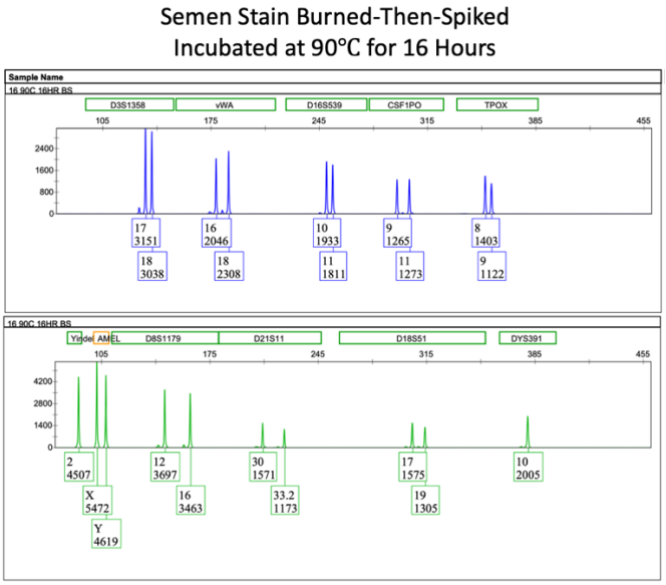

Figure 12. Electropherogram of "burned-then-spiked" semen stain. While this sample endured the most extreme heated passive headspace concentration plus the presence of substrate charring, it still resulted in a full profile with peak heights higher than that of the positive control.

Figure 12 shows an electropherogram of Sample 16, which was burned and then spiked with semen and incubated at 90°C for 16 hours. The approximate average peak height across this profile was 2222 RFU. A slight "ski slope" pattern was recognizable when viewing the whole profile, suggesting mild degradation.

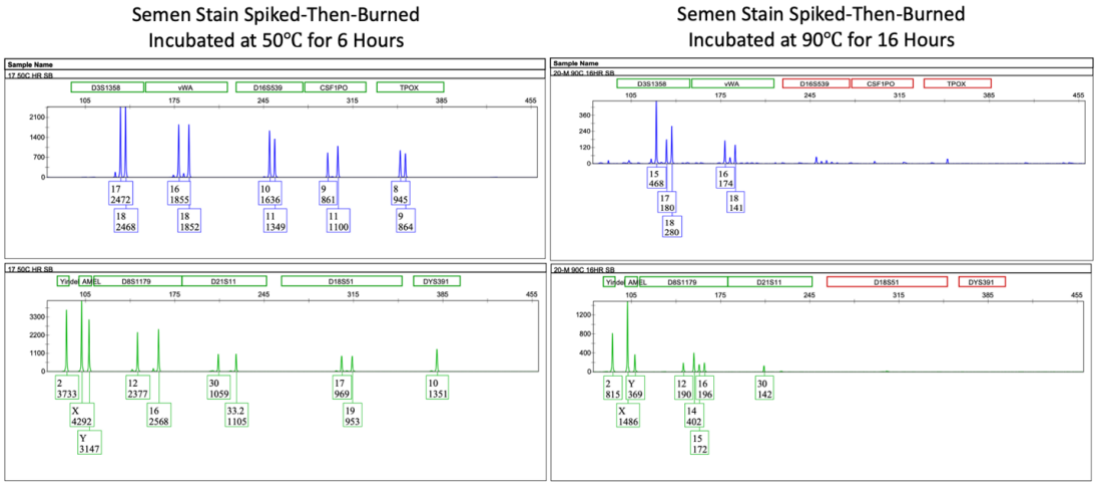

Figure 13. Electropherograms of "spiked-then-burned" semen stains incubated in least and most extreme conditions. While Sample 17 (left) was spiked with semen and then burned, it still resulted in a full DNA profile, while Sample 20-M (right), resulted in a partial profile. The difference between these samples could be the level of charring, given that the measure of burning was subjective, or the fact that Sample 20-M experienced more extreme heated passive headspace conditions. Sample 18-M also resulted in a partial profile, but only 3 loci were unsuccessful.

Figure 13 shows a comparison between the electropherograms of the semen stains experiencing the least extreme and most extreme conditions in addition to being burned after being spiked with semen. Sample 17 (left) generated a full profile even after burning with a slight "ski slope" pattern, while Sample 20-M (right), which had to be concentrated due to low DNA quantity, produced a partial profile after enduring the most extreme conditions. Sample 18-M, which was also spiked and then burned and was incubated for 16 hours at 50°C also resulted in a partial profile. However, Sample 19-M at 90°C for 6 hours resulted in a full profile. *Tables 15-18* were produced in order to compare the average degradation indices for each biological fluid within each experiment and the average degradation indices for each biological fluid at the various times and

temperatures. Additionally, a table was compiled to compare the average peak heights for the electropherograms from each experiment and for each biological fluid.

Table 15. Average degradation indices (DI) across different experimental conditions and biological fluids. Both biological fluids did not appear to be significantly degraded by any of the experimental conditions, with the exception of spiking and then burning with an open flame. The semen stains also appeared to have a higher initial DI for positive control and unadulterated samples.

	Unadulterated stains	**Direct Gasoline**	**Indirect Gasoline**	**Burned-then-Spiked (*forensic*GEM)**	**Burned-then-Spiked (QIAmp)**	**Spiked-then-Burned**
Saliva	1.1	0.90	0.82	1.02	0.95	N/A
Positive Control	0.93	0.68	0.68	0.88	0.87	N/A
Semen	1.07	1.07	0.95	0.93	N/A	2.1
Positive Control	1.1	1.1	1.1	1.1	N/A	1.1

In most cases, the experimental stain was slightly more degraded than the respective positive control sample, however even when the DI was above 1, it was only very slightly above 1. The cotton swatches that were spiked with semen and then burned under an open flame were the most degraded based on DI, but half of the "spiked-then-burned" semen stains still yielded full genetic profiles.

Table 16. Degradation indices across different incubation times and temperatures for different biological fluids. There appeared to be a trend for increased DI during increased incubation time and temperature for saliva stain samples. Semen stains experienced a similar trend to a lesser degree and a higher initial DI for the positive control and samples at 50ºC for 6 hours.

	50°C, 6hr	**50°C, 16hr**	**90°C, 6hr**	**90°C, 16hr**	**Positive Control**
Saliva	0.78	0.77	1.0	1.2	0.81
Semen	1.0	0.99	1.0	1.8	1.1

Given that the differences in DI across experiments were not overwhelming, *Table 16* was created in order to compare the average DI for each heated passive headspace concentration condition. In general, an increase in temperature accompanying an increase in incubation time appeared to cause an increase in DI, even though it was minimal.

Table 17. Average peak heights across different experimental conditions for saliva stains. Incubation time and temperatures appeared to have a larger effect on peak heights than those of the additional variables that were explored. *Only one positive control profile was taken to CE. Therefore, there is only one average peak height value.

	50ºC, 6hr	**50ºC, 16hr**	**90ºC, 6hr**	**90ºC, 16hr**
Unadulterated	2690.40	2725.73	2491.02	1377.5
Direct Gasoline	4697.47	2391.09	2429.78	1525.95
Indirect Gasoline	2894.23	2762.59	1969.71	1731.19
Burned-then-Spiked (*forensic*GEM)	2786.45	2415.42	1805.07	1446.07
Burned-then-Spiked (QIAmp)	3376.76	3878.07	1682.54	2154.33
Average	3289.06	2834.58	2075.62	1647.00
Positive Control*	3179.92	3179.92	3179.92	3179.92

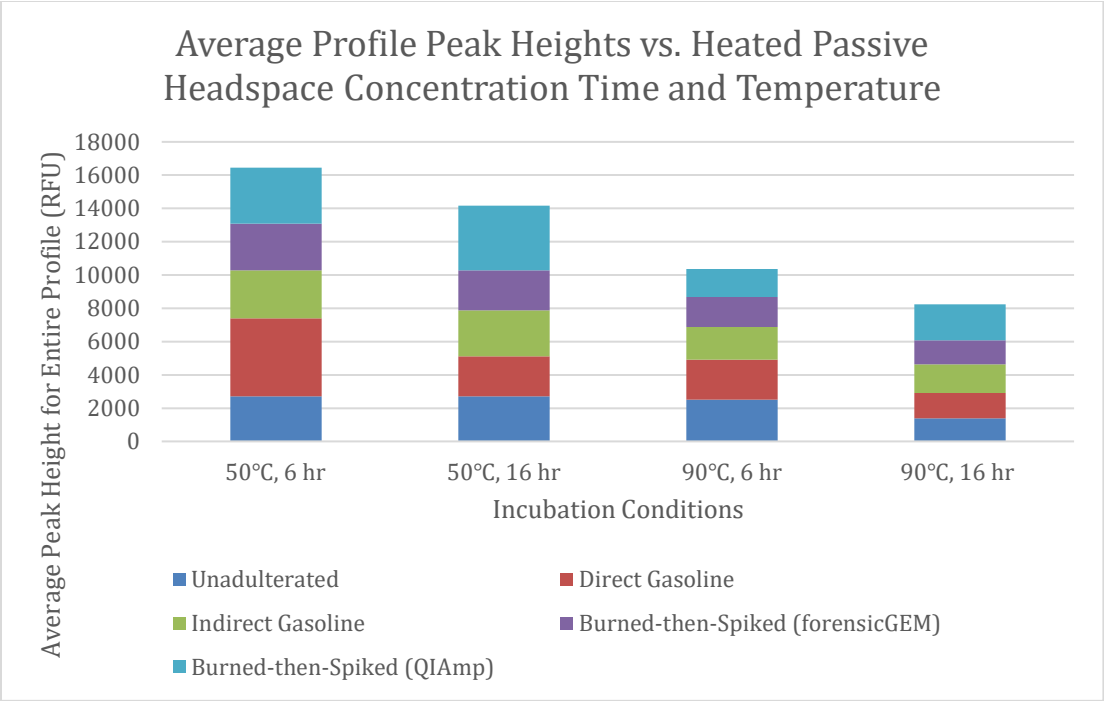

Figure 14. Average profile peak heights vs. heated passive headspace concentration time and temperature conditions for saliva samples. As the incubation conditions explored become more intense, there is an obvious decrease in average peak height when viewing all saliva stain DNA profiles.

While degradation indices are useful for predicting the quality of DNA, the proof is in the resulting electropherogram. The average peak heights of each experiment type at each heated passive headspace concentration condition were recorded and compared for saliva stain samples (**Table 17)**. It is obvious that the degradation caused by an increase in incubation time and temperature was enough to decrease peak heights sufficiently. For example, in most experimental groups, the average peak height for the resulting electropherograms decreased when moving from left to right on *Table 17*. This trend is more clearly visualized in *Figure 14.* The dramatic decrease in average peak heights from the left of the table to the right suggests that the increase in incubation time and

temperature had a much more noticeable effect on the resulting peak heights. If focusing on the "burned-then-spiked" stains extracted with *forensic*GEM, the average peak heights of overall profiles in that category decrease from approximately 2786 RFU to approximately 1446 RFU as time and temperature increased. This trend is maintained within all five experiment categories. However, when viewing *Table 17* from top to bottom, the average peak heights for the experiment with "Unadulterated" saliva stains were not always the highest in RFU, even though you would expect them to be. Additionally, the average peak heights of stains with "Indirect Gasoline" were not always higher than those with "Direct Gasoline." This inconsistency and lack of a trend can likely be attributed to extraneous factors like not knowing the concentration of DNA deposited onto the cotton swatch, performing these separate experiments at different times, and also the inability to produce cuttings of the exact same size across all cotton swatches. Additionally, there is inherent variability during DNA amplification and capillary electrophoresis.

Table 18. Average peak heights across different experimental conditions for semen stains. Heated passive headspace concentration incubation times and temperatures did not appear to have the same effect on semen stains as they did on saliva stains. In fact, there did not appear to be any noticeable trend for the resulting peak heights for semen stains. However, the resulting average peak heights were clearly affected most by spiking and then burning, given that Samples 18-M and 20-M were partial profiles. *Only one positive control profile was taken to CE. Therefore, there is only one average peak height value.

	50ºC, 6hr	**50ºC, 16hr**	**90ºC, 6hr**	**90ºC, 16hr**
Unadulterated	2880.73	2906.10	2200.86	3415.97
Direct Gasoline	2544.34	2206.56	2376.89	1437.50
Indirect Gasoline	2329.30	2017.45	2226.21	2218.54
Burned-then-Spiked	3074.39	2068.65	1714.82	2222.58
Spiked-then-Burned	2413.58	281.28	1494.93	201.93
Average	2648.46	1896.00	2002.74	1899.30
Positive Control*	1618.60	1618.60	1618.60	1618.60

Table 18 has a few less extraneous variables to consider given that preparation and extraction of semen stains for each experiment was performed all at once. Additionally, the trends that were more obvious for the average peak heights of saliva stain profiles are not as obvious or even present within the average peak heights for the semen stains. For example, if looking from left to right, the average peak height at each temperature/time condition does not always decrease consistently. In fact, for the semen stains that were not subject to adulteration, the average peak height at 50°C for 6 hours was approximately 2880 RFU while the average peak height at 90°C for 16 hours was approximately 3415 RFU. Once again, it can be said that it is unknown how much DNA was initially deposited onto these cotton swatches. It is also very clear that the "spiked-

then-burned" swatches resulted in the lowest quantity and quality DNA. Half of these profiles were partial. That being said, no profiles could be obtained from the saliva stains in similar conditions.

4. CONCLUSIONS

While the results of each aspect of the experiment displayed possible negative effects of an increased time and temperature within the oven during heated passive headspace concentration, it is evident that with the ideal piece of evidence (i.e. a piece of clothing with an undiluted biological fluid), heated passive headspace concentration could be performed before DNA analysis. This case is especially true for pieces of evidence that are not entirely charred. Even some samples, like Samples 17 and 19-M, which were burned after the deposition of semen, resulted in full genetic profiles that would be acceptable for interpretation. Semen stains especially appeared to be more resilient to the explored conditions (i.e., incubation in an oven, open flame, etc.), while saliva stains appeared more easily degraded.

The idea that ignitable liquid residue extraction could be performed before moving on to DNA analysis is significant for evidence that may contain highly volatile accelerants such as alcohols (38). The more time that a piece of evidence with VOCs spends outside of a sealed container, the more those vital VOCs evaporate and can no longer be detected. By holding off DNA analysis until after ILR extraction, the loss of VOCs can be limited. Additionally, given the recommended timeframe for passive headspace concentration in particular is 2 to 24 hours, the most time DNA analysis would

be delayed is likely around one to two days (13). This data is also significant given the fact that research on this topic is minimal and the parameters that were explored here can be expanded and studied more in-depth in the future. Resulting data may open the doors for altered workflow of testing evidentiary items.

4.1 Limitations and Future Directions

While this study explored various experimental variables, there were several limitations. For example, as previously mentioned, while the sample volume was the same, the concentration of DNA deposited onto each cotton swatch was unknown (i.e. the number of buccal or sperm cells may, and likely did, vary). While the saliva and semen standards were vortexed before each deposition, it is possible that varying numbers of epithelial and sperm cells were present on the swatch at the end. Additionally, while the cutting size for each sample was approximately the same, the sizes were estimated and not strictly measured and cut for extraction. Like the unknown starting concentration of DNA, this factor would result in varying quantities of DNA, which could result in misinterpretation of results. One may assume a lower quantity of DNA was due to high incubation temperature, when the reality is that the starting concentration of DNA was low to begin with. A third limitation to this study was the small sample size and limited incubation times explored. While it would have been beneficial to have multiple swatches undergoing the same conditions, the oven is only so large and it would have been difficult to explore many more incubation times. For example, only two timeframes, 6 and 16 hours, were explored and were chosen based off of ASTM Guideline E1412's

recommendation of 2 to 24 hours because they could be easily implemented in the laboratory. Ideally, all experiments should be repeated, with multiple swatches per condition and possibly quantitation and amplification performed in triplicate. While future studies are still required to ensure that it would be appropriate for DNA analysis to occur after heated passive headspace concentration in the field, this small study demonstrates that some DNA evidence can endure said conditions if the sample appears to contain a neat biological fluid without severe degradation (i.e., a stain that is not charred). This conclusion, if substantiated with future research, could alter the workflow in a forensic laboratory and aid in more successful ignitable liquid residue and DNA recovery from burnt materials containing biological materials of interest.

LIST OF JOURNAL ABBREVIATIONS

Anal Chem	Analytical Chemistry
J Forensic Sci	Journal of Forensic Science
Forensic Sci Int	Forensic Science International
Forensic Sci Int Genet	Forensic Science International: Genetics
FRCIJ	Forensic Research and Criminology International Journal

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