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Effects of heat and burning on testing methods for blood and semen

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EFFECTS OF HEAT AND BURNING ON TESTING METHODS FOR BLOOD AND SEMEN

By

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EFFECTS OF HEAT AND BURNING ON TESTING METHODS FOR BLOOD AND SEMEN

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ABSTRACT

Perpetrators of crime may sometimes attempt to destroy links that connect them to the crime scene. Limited information is known about the appearance and behavior of blood or semen stains collected from fire scenes. The purpose of this study is to observe the effects of high temperatures and flame exposure on blood and semen stains using current traditional presumptive (phenolphthalein, AP Spot test and ALS fluorescence) and confirmatory (ABAcard® Hematrace®, microscopic identification of spermatozoa and RSID™-Semen) testing methods.

Liquid blood and semen was applied to swatches of denim, synthetic leather and carpet, and allowed to dry. In Part I of this study, the stains were exposed for various amounts of time to 130°C, 150°C, 170°C and 190°C using a laboratory oven. When bloodstains were exposed to high temperatures,

reaction times using phenolphthalein increased and only bloodstains on denim were undetected within one minute at the most extreme conditions. Scraping was determined to be a better collection method than swabbing to test samples exposed to higher temperatures, and some samples required longer than the typical 10-15 second time frame used by most forensic labs to yield a positive reaction. Detection of human hemoglobin using ABAcard® Hematrace® was not possible after 1 hour at 130°C, making it a less sensitive test for bloodstains exposed to high temperatures than phenolphthalein. Semen stains exhibited fluorescence using an alternate light source after limited exposure to all temperatures, but more successfully on denim and synthetic leather than on carpet. AP Spot test was successful on all semen stains on synthetic leather, and on a number of samples on denim and carpet. Microscopic identification of sperm was most successful on denim but became impossible at higher temperatures and exposure times. Semenogelin testing was most successful on carpet and could be detected even under the most extreme conditions. Additionally, as temperature increased, semen stains darkened in color and appeared visually similar to bloodstains.

In Part II of this study, blood and semen stains were exposed to direct flame contact for 5 and 10 seconds using a propane torch. Phenolphthalein testing was positive for all three materials at both burn times, and bloodstains could also be detected on the back of the porous substrates that allowed the blood to seep through. ABAcard® Hematrace® yielded consistently positive

results for denim and carpet, while only one bloodstain on synthetic leather tested positive for human hemoglobin. For analysis of semen stains, AP Spot test, microscopic identification of spermatozoa, ALS fluorescence and RSID™-Semen were most successful on carpet, suggesting this material provides a better protection of the seminal constituents. An evaluation of all the results shows that current presumptive and confirmatory tests for blood and semen can be used to successfully test evidence samples exposed to high heat and direct flame contact in some cases.

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List of Abbreviations

AP	acid phosphatase
ALS	alternate light source
ASTM	American Society for Testing and Materials
°C	degrees Celsius
CA	California
cm	centimeter
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Fab	fragment antigen-binding
FBI	Federal Bureau of Investigation
Hb	hemoglobin
IL	Illinois
Inc	incorporation
KPIC	Kernechtrot Picroindigocarmine
MA	Massachusetts

MI	Minnesota
mm	millimeter
μL	microliter
mL	milliliter
NEG	negative
NFPA	National Fire Protection Association
NFSTC	National Forensic Science Technology Center
NFR	nuclear fast red
NY	New York
PA	Pennsylvania
PSA	prostate specific antigen
P/TMB	phenolphthalein/tetramethylbenzidine
®	registered trademark
RBC	red blood cell
RBS	red-brown stain
RPM	revolutions per minute

RSID	Rapid Stain Identification
SAP	seminal acid phosphatase
SERI	Serology Research Institute
Sg	Semenogelin
™	trade mark
UCR	Uniform Crime Reporting
USA	United States of America
VA	Virginia
VAP	vaginal acid phosphatase

1. Introduction

Crime scene investigators frequently analyze fire crimes, including those determined to be arson. Arson is a criminal act and is defined according to the FBI's Uniform Crime Reporting (UCR) Program 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" (1). According to the National Fire Protection Association (NFPA), approximately 27,500 intentional fires occurred in the USA in 2010, leading to approximately $585,000,000 in damages (2). Although most research on evidence recovered from fire scenes has focused on the extraction of ignitable liquids, little has been reported about the effects of high temperatures on body fluids, like blood or semen, which are commonly found at crime scenes (3).

Perpetrators of crimes sometimes attempt to destroy links that connect them to the crime scene by setting fires (4-8). Biological fluids deposited on items such as clothing or bedding may be overlooked during fire scene processing due to the burnt physical state of the evidence. The careful collection of evidence items that might otherwise be sent directly for fire debris analysis could potentially be analyzed first by the forensic biologist.

1.1 Fire chemistry

When a fire starts, a combustion process takes place. Combustion is an exothermic reaction that releases energy in the way of heat and light (5,9). In order for ignition to occur it must follow the "fire triangle model" requirements (5,9). These requirements are: an ignition source, a fuel and an oxidizer. The ignition source, such as a flame or lit cigarette, starts the combustion reaction. A combustible is any material in the solid, liquid or gas state that when in contact with an ignition source, is able to combust, or burn. The oxidizer provides oxygen molecules to the reaction; air is the most common oxidizer because it has oxygen molecules in its composition. If one of the components of the fire triangle is removed, the fire is completely extinguished. When there is a continuous chain reaction between these three components, the chain reaction itself becomes the fourth component, transforming the "fire triangle model" into a "fire tetrahedron model" (5).

1.2 Blood composition

Human blood is composed of plasma and cellular components (10-13). The cellular portion is composed of red blood cells, white blood cells and platelets (10-12). Red blood cells (RBC), or erythrocytes, are produced in the bone marrow and have a biconcave shape due to the loss of their nuclei before they enter the circulatory system. There are approximately 4 to 6.5 million RBC per mm^3 of whole blood (13) and each erythrocyte is composed of approximately

280 to 300 million molecules of hemoglobin (12,14). The plasma portion contains nutrients and proteins such as albumins and enzymes as well as antibodies, which protect the body from foreign materials (10,12).

1.2.1 Hemoglobin

Hemoglobin (Hb) is an allosteric protein that transports oxygen to all the tissues of the body, and is responsible for the red coloration of blood. Hemoglobin has a tetrameric structure composed of four polypeptide chains (13,14). The transportation of oxygen is accomplished by the cooperation of these polypeptides that are non-covalently bonded (12,14). On each polypeptide there is a heme group known as ferroprotoporphyrin, which is composed of protoporphyrin IX and a ferrous iron, to which oxygen can bind (11,14).

1.2.2 Antigens and Antibodies

An antigen is a foreign macromolecule that, when in contact with a corresponding antibody, triggers an immune response. An epitope is the binding site where an antigen is capable of binding to an antibody (15,16). An antigen with more than one epitope is described as being multivalent (15,16). An antibody is a glycoprotein that belongs to the immunoglobulin family (16). Its structure is a Y-shaped complex composed of four polypeptide chains, two light and two heavy (11). On the heavy and light chain, an area called the fragment antigen binding (Fab) is located; within this area there are three hypervariable regions that play an essential role in the specificity of antigen–antibody binding sites (11,15).

1.3 Presumptive testing for blood: Phenolphthalein

During evidence collection, stains exhibiting a color similar to that of blood, such as ketchup, tomato juice or even red paint may be observed; this is the reason why presumptive testing is important in the forensic field. Presumptive testing in laboratories is often a required step before subjecting an evidence sample to further confirmatory analysis or DNA typing. A variety of presumptive tests for blood are available including phenolphthalein, leucomalachite green, benzidine derivatives, luminol, etc. (3,10,11). A study by Cox (17) concluded that phenolphthalein was an ideal screening test because of its sensitivity and relative specificity for blood.

Phenolphthalein is the colored product that results from a common catalytic color test for the presence of hemoglobin. A drop of colorless reduced phenolphthalein (phenolphthalin), also known as Kastle–Meyer reagent, is added to a suspected bloodstain, followed by a drop of hydrogen peroxide. If blood is present, the heme will catalyze an oxidation-reduction reaction. The oxidation of phenolphthalin converts it to phenolphthalein, causing the observation of a pink coloration under basic conditions. The change to a pink color is indicative of the presence of hemoglobin within the sample. This catalytic test is very simple to use and gives rapid results, as a positive result is obtained within a few seconds.

It is important to note that a positive result can also be obtained by animal blood and certain vegetable peroxidases (11,17). Hence, this test is not specific for human blood.

1.4 Confirmatory testing for blood: Immunochromatographic assays

If a presumptive blood test is positive, further testing to confirm the presence of human blood is required (3,11,18-20). Currently, confirmatory testing for human hemoglobin is commonly performed utilizing antigen specific immunochromatographic assays cards that have proven to be a robust tool for the identification of human blood (11,18-21). These immunochromatographic assay cards provide rapid, sensitive and reproducible results for the presence of human blood. ABAcard® Hematrace® (Abacus Diagnostics, West Hills, CA), like several other commercially available assays, tests for the presence of human Hb. This test is highly specific due to the antigen-antibody (Ag-Ab) complex formed when a sample containing human Hb is loaded into the sample well (18,21,22).

When human Hb antigen in the extracted bloodstain sample binds to the mobile dye labeled anti-human Hb antibody present in the sample well region, an Ag-Ab complex is formed (18,21,22). This complex diffuses along the membrane of the card to the test zone, where the labeled Ag-Ab complex attaches to the polyclonal anti-human Hb antibody immobilized on the membrane. An Ab-Ag-Ab sandwich is formed, triggering the appearance of a pink band due to the accumulation of the dyes. Further up the membrane at the control zone, remaining dye labeled anti-human Hb antibodies bind to an immobilized anti-immunoglobulin (Ig) and a pink band is formed, acting as an internal procedural control. For a test to be considered positive, both the test zone and the control zone must have a visible pink band. It must be mentioned that ABAcard® Hematrace® immunochromatographic assays can cross react with blood from higher primates and ferrets, however, the tests are still widely utilized because the likelihood of having evidence samples from these species in forensic cases is typically very low. According to the manufacturer, ABAcard® Hematrace® is reported to have a detection limit of 0.05µg/mL (22).

1.5 Semen composition

Another body fluid of interest in forensic science is semen, which is composed of sperm cells and seminal fluid. Semen contains approximately 10 to 100 million sperm cells per milliliter (mL) and ejaculatory volume is approximately 2 to 5 ml (11,23). Spermatogenesis is the process in which the male germ cells proliferate and transform to spermatozoa (23). This process takes approximately 2 to 3 months and occurs in the seminiferous tubules of the testes (11,23). The maturation and storage of spermatozoa takes place in the epididymis. Emission and ejaculation occur when spermatozoa mixes with secretions from the seminal vesicle, prostate gland and bulbourethral grand to form semen, and is expelled from the body (11,23).

1.5.1 Sperm cells

An individual sperm cell measures approximately 50–70 μm (24,25). The morphology of a sperm cell is divided into three basic parts: the head containing the nuclear material and the acrosomal cap (4-5μm), a mid-piece which contains mitochondria, and a tail that provides the motility of the sperm (11,23,26). Several factors can cause sperm to be absent or difficult to detect in a semen stain deposited on an item of evidence, including azoospermia (no sperm) and oligospermia (low sperm count) (11,27).

1.5.2 Seminal fluid

Seminal fluid is mostly composed of seminal vesicle (60%) and prostatic fluid gland secretions (30%) (11,28). Prostate-Specific Antigen (PSA) and Semenogelin (Sg) are the major markers for seminal fluid (11,25,27-33). PSA, or p30, is a glycoprotein that is secreted from the prostate gland and its major function is liquefying seminal fluid (34-36). PSA is also found in lower concentrations in male serum, male/female urine, breast milk, sweat and fecal material (11,33,34,37,38). Semenogelin is a protein produced in the seminal vesicle and is composed of two parts: Semenogelin I and II (Sg I and Sg II) (39-41). During ejaculation, Sg I and II are non-covalently bonded, keeping semen coagulated for around 5 to 20 minutes. Later, PSA fragments Sg, enabling the motility of spermatozoa (28,40,41). Sg is also found in tissues that have high levels of Zn (II) ions including skeletal muscle, kidney, colon, and trachea tissue, but not in other biological fluids (11,40,41).

1.6 Semen screening: Fluorescence

In cases where the presence of semen is suspected, the first step is to localize a stain by visual examination. Due to semen's colorless to white coloration, it can be difficult to find on light garments using the naked eye alone (18,25,42). Other characteristics like stiffness in a localized area or a yellowish coloration on older stains can also be indicative of semen. Unlike blood, semen stains may fluoresce with the use of an alternate light source (ALS), a non-destructive screening method. Fluorescence of a seminal stain is thought to be caused by the growth of the bacterium *Pseudomonas fluorescens* or by the transformation of non-protein components to compounds that are able to fluoresce when exposed to an ALS (25,42,43). The reflected or emitted fluorescence energy has a longer wavelength than the excitation light, thus an orange barrier filter (530nm) is used to allow only the longer wavelength to be visualized (42-44). Other bodily fluids like urine, saliva and vaginal secretions and some non-biological materials such as detergents, also fluoresce under ALS illumination (42). Other factors that might affect fluorescence screening include the type and the absorbency of the substrate on which the stain is deposited (11,42,43).

1.7 Presumptive testing for semen: Acid phosphatase

Semen contains an enzyme called acid phosphatase (AP), which is synthesized in the epithelium of the prostate gland (11,45-47). This enzyme is produced in high quantities in the prostate gland and its detection can be used as an indicator that semen may be present (11,27,45-48). AP is a glycoprotein made of two identical subunits that are bound together by non-covalent bonds. Under acidic conditions, AP hydrolyzes phosphate ester groups to inorganic phosphates (29,46,48). In 1945, Lundquist recommended that the detection of AP should be used as a way to detect semen stains due to the high levels encountered with this body fluid (27). There are several color tests that detect the presence of AP but they lack specificity because they can give positive results for lower AP levels found in other biological fluids (11). Another factor that affects the detection of AP activity is the variation of AP concentration between men. (23,25).

The AP Spot test (SERI, Richmond, CA) is a commercially available presumptive test for AP. It consists of a one-step reaction, where the dephosphorylating capabilities of AP remove an ester linked phosphate group from α-naphthyl phosphate, producing α-naphthol. The free α-naphthol couples with an insoluble azo base in the test reagent called Fast Blue B, resulting in the appearance of a characteristic purple color. Positive results are obtained within several seconds to 5 minutes (48-52).

1.8 Microscopic identification of sperm

The microscopic identification of spermatozoa is the most reliable confirmation for the presence of semen (3,11,25,53). In order to more easily identify spermatozoa in sexual assault samples, analysts utilize staining techniques to create contrast between cellular material and sperm cells. Kernechtrot Picroindigocarmine (KPIC) and Hematoxylin and Eosin (H&E) are two such staining methods.

KPIC or “Christmas Tree” staining provides a high level of contrast and is commonly used in forensic laboratories in the United States (54). Staining of the cells occurs due to the physicochemical action between the dye and the biological material (55). Two dyes are utilized with the KPIC method: nuclear fast red and picroindigocarmine. Nuclear fast red (NFR), or Kernechtrot, is water soluble and is a basic dye which is attracted to the negative charge of nuclear material, staining sperm heads and epithelial cell nuclei red (54,55). Picroindigocarmine is an acidic dye that gives a light green/bluish green coloration to the sperm tails and the cytoplasm of epithelial cells (11).

1.9 Analysis of seminal fluid

Currently, one immunochromatographic test cartridge is commercially available that detects the presence of Sg called RSID™-Semen (Independent Forensics, Lombard, IL). RSID™-Semen is specific to semen due to the anti-human Sg antibodies present on the cartridge membrane. For a test to be considered positive, both the test and the control area must show the presence of a red band (56). As previously mentioned, the advantages of using an immunochromatographic card are the rapid, reproducible and specific results for the targeted body fluid (31,33)

Other immunochromatographic assays that test for semen detect the presence of p30 (32,33,37,57). A comparison between ABAcard® p30 (Abacus Diagnostics, West Hills, CA) and RSID™-Semen assays showed that the latter was more sensitive because positive results were obtained up to a 1:100000 dilution, while the ABAcard® was only able to detect p30 up to a 1:50000 dilution (33). In addition, both immunochromatographic cards demonstrated high specificities because no positive results for urine, blood, saliva, sweat, breast milk, vaginal secretions, fecal material, lubricants and spermicides were obtained (31,33,56).

1.10 Protein denaturation

When proteins are exposed to high temperatures, the denaturation, or unfolding, of proteins occurs (58-60). Denaturation of proteins can also be induced by acidic or alkaline conditions in the environment (59,60). In the case of heat, an increase in temperature will lead to an increase in the vibration of the bonds, causing a disruption of the tertiary structure and inactivation of the protein which can be an irreversible process (59,61).

1.11 Purpose of this study

Understanding the effects of higher temperatures on bodily fluids, biological stains and substrates is of interest to the forensic community. In this study blood and semen were applied to three different materials – denim, synthetic leather and carpet – and the effects of exposure to high temperature environments and direct flame contact on biological testing were explored. A better comprehension of how fire scene evidence may react with traditional presumptive and confirmatory tests for blood and semen could help determine whether the current forensic testing methods are appropriate for this type of evidence.

2. Materials and Methods

Biological samples were obtained in accordance with methods approved by the Institutional Review Board of Boston University School of Medicine.

Blood

Blood was anonymously obtained from the Boston Medical Center Blood Bank and was preserved using ethylenediaminetetraacetic acid (EDTA).

Semen

Semen was obtained from anonymous volunteers or was purchased, then pooled and stored frozen until analysis. For Part I, the pool was composed of three donors and for Part II the pool was composed of six different donors.

Substrates

Three different substrates were utilized: blue denim (98% cotton, 2% polyester), beige synthetic leather (100% vinyl) and off-white carpet (100% nylon). The substrates were cut into 5.5 x 3 cm, 5 x 2.5 cm and 4.5 x 4 cm swatches, respectively.

Phenolphthalein reagent

Phenolphthalein was prepared using the protocol published by the National Forensic Science Technology Center (NFSTC) (62).

AP Spot test

AP Spot reagent was made fresh daily following the manufacturer's instructions. (50)

Photography

Photos were taken using a PowerShot SD1400 IS camera (Canon, Lake Success, NY) in conjunction with the MK Photo-eBox™ (MK Digital Direct, Chula Vista, CA). The camera's macro setting was used and white balance was adjusted manually. A SPOT Insight™ Camera (SPOT Imaging Solutions, Sterling Heights MI) was utilized to take pictures under the microscope.

Part I - The effects of high temperatures on blood and semen stains

2.1 Screening and presumptive testing

Biological fluids were applied to three different substrates and subsequently exposed to 130°C, 150°C, 170°C and 190°C temperatures. For each trial, 10 pieces of the desired substrate were stained with 20 μL of blood or semen. The area around the semen stains on the carpet was marked using yellow colored tape in order to easily identify the stain's location; a permanent marker was utilized for semen location purposes on denim and synthetic leather. Substrates were allowed to dry at room temperature, and then placed into a laboratory oven set to the respective temperature. A sample was removed and tested at different times during a 24-hour period (1, 2, 3, 4, 5, 6, 7, 15, 20 and 24 hours). Semen stains were screened using a Crimelight® blue light (Foster + Freeman, Sterling, VA) and an orange barrier filter was used. Then each stain was rubbed for 10 seconds using a cotton swab moistened with distilled water, making sure the entire stained area was swabbed. Presumptive testing was conducted as follows:

- Phenolphthalein: Two drops of reduced phenolphthalin reagent were added to the swab followed by two drops of 3% hydrogen peroxide. A positive result was noted when a pink color was observed within one minute.
- AP Spot Test: Two drops of AP spot reagent were added to the swab. A positive result was noted when a purple color was observed within five minutes.

The time it took for a color change to occur was recorded, as well as other observations including fluorescence, color change of the stain/substrate and any other visual changes to the substrate.

All samples were tested in triplicate such that for each temperature there was a total of 30 blood samples and 30 semen samples for each substrate. Each trial within a triplicate set was placed in the oven on a different day in order to determine if there were any irregularities in the results.

2.2 Confirmatory testing

2.2.1 Detection of spermatozoa

One sample group exposed to heat on Part 2.1 was arbitrarily selected for microscopic examination. First, a 5 x 5 mm sample of the stain was cut and extracted in a microcentrifuge tube containing 250 µL of distilled water for two hours. Following extraction, the cuttings were placed in a Spin-x® Insert (Corning Inc., Corning, NY) and centrifuged for two minutes at 13,200 revolutions per minute (RPM). Carefully, without disturbing the pellet containing spermatozoa, 230 µL of the supernatant was removed, leaving approximately 20 µL for examination. The microcentrifuge tubes were briefly vortexed to resuspend the pellet material. Then, 4 µL was placed on a glass microscope slide and heat fixed using a Bunsen burner. The slide was allowed to cool, then stained using two drops of XMAS Tree Stain A (SERI, Richmond, CA) for 15 minutes, washed gently using distilled water and air-dried. Then two drops of XMAS Tree Stain B (SERI, Richmond, CA) were added for two minutes, rinsed using ethanol and air-dried. Finally, the slide was mounted using Cytoseal™ 280 (Thermo Fisher Scientific Inc., Waltham, MA) to secure a cover slip. The slides were examined for the presence of spermatozoa using an Eclipse TE2000-S light microscope (Nikon Corporation, Tokyo, Japan) at 400x magnification.

2.2.2 Immunochromatographic assays

Blood and semen sample groups (not used in Section 2.2.1) were analyzed using immunochromatographic cartridges following exposure to heat. For blood, the ABAcard® Hematrace® assay, which tests for the presence of human hemoglobin antigen, was utilized. The manufacturer's instructions were followed for sample extraction (22). First, a piece of stained substrate between 3 to 5 mm^2 was cut and placed inside a vial containing the extraction buffer (provided in kit). The samples were then agitated for five minutes using an orbital shaker. Using the disposable pipette that was provided with each assay, four drops of the extract were added to the sample well of the cartridge. Positive results were noted when a horizontal line was present in both the test and control areas within 10 minutes. Negative results displayed a horizontal red line in the control area only.

For semen testing, the RSID™- Semen assay was used. Sample extraction was performed according to the manufacturer's instructions (56). Approximately 20 mm^2 of each sample was extracted on an orbital shaker for two hours in 100 µL of RSID-Universal Buffer and then centrifuged for two minutes at 13,200 RPM. The entire extract was pipetted into the sample well on the assay card and results were read after 10 minutes. As with the ABAcard® Hematrace®, positive results were represented by a red horizontal line in both the test and control areas. Negative results were recorded when a red horizontal line was present only in the control area.

Part II: The effects of flame contact on blood and semen stains

Denim, synthetic leather and carpet were cut into 2.5 inches x 2.5 inches swatches. Blood and semen stain analysis was performed on samples exposed to a direct flame for 5 or 10 seconds. For each trial, two swatches from the same material were each placed on top of a ceramic tile and burned using a propane torch (Bernzomatic Propane, Medina, NY). The inner cone of the flame was set to four inches long. The flame was placed approximately four inches away from the substrate at approximately a 45° angle and was moved side to side across the substrate throughout the duration of the burning. Samples that caught fire were extinguished using the bottom of a beaker in order to stop the flames immediately. The first swatch was utilized for presumptive testing and the second one for confirmatory testing. This procedure was performed for each material (denim, synthetic leather and carpet) in triplicate for a total of 18 swatches per burn time.

2.3 *Screening and presumptive testing*

Swatches containing blood were tested using phenolphthalein, following the procedure described in Section 2.1.

Swatches containing semen were examined using a Crimelight® blue light with an orange filter. Screening with the light source was conducted on both sides of the substrate. These same swatches were then tested using AP Spot reagent following the procedure described in Section 2.1.

2.4 Confirmatory testing

Extraction was performed as described in Section 2.2.2, using 120 μL of RSID™-Universal Buffer. Without disturbing the pellet, 100 μL of the supernatant was removed and stored in a separate microcentrifuge tube for immunochromatographic testing. The 20 μL remaining was utilized for microscopic identification.

2.4.1 Detection of spermatozoa

This procedure was performed following the instructions described in Section 2.2.1.

2.4.2 Immunochromatographic assays

Blood and semen stains were extracted and tested as previously described in Section 2.2.2.

3. Results and Discussion

Part I: The effects of high temperatures on blood and semen stains

Control samples of denim, synthetic leather and carpet stained with blood and semen were tested at room temperature after 24 hours (24°C), and all yielded positive results with phenolphthalein, AP Spot test, ABAcard® Hematrace® and RSID™-Semen (data not shown).

Since the oven used did not maintain a constant temperature, the range of temperatures was recorded as shown in Table 1.

Temperature Set (°C)	Temperature Range (°C)
130	128 - 136
150	148 - 155
170	167 - 175
190	188 - 190

Table 1: Temperature range inside the oven

The temperature noted throughout the report is the temperature to which the oven was set.

3.1 Absorbent vs. non-absorbent materials

A porous material is one that easily absorbs any fluid. These types of materials may retain biological evidence better than non-porous materials because they provide protection from environmental effects and cleaning. Non-porous materials that don't contain any small openings on the surface can be somewhat problematic as any biological evidence present can be easily cleaned or lost and would be directly exposed to harsh exterior conditions. For this study, three different types of materials were tested: denim, a thin but absorbent material; synthetic leather (vinyl), a slightly textured non-porous material; and carpet, an absorbent material thicker than denim that could possibly offer more protection from heat degradation of the bodily fluids.

3.2 Physical changes of denim, synthetic leather and carpet

Two types of denim, synthetic leather and carpet were used, and the changes observed during Part I are summarized in Table 2.

Temp (°C)	Denim	Synthetic Leather	Carpet
130	○ Type 1: yellowing after 6 hrs. ○ Type 2: yellowing after 15 hrs. ○ From both: dye was collected when swabbed	Type 1: No visual changes	○ Type 1: No visual changes ○ Type 2: No visual changes
150	○ Type 2: yellowing after 15 hrs. ○ Dye was collected when swabbed	○ Type 1: Started to darken at 15 hrs. ○ Type 2: Darkened at 7 hrs. ○ From both: Curved at 15 hrs.	Type 2: First signs of disintegrations at 15 hrs. Changed to yellow and some release of oil observed
170	○ Type 2: yellowing of the material after 15 hrs. ○ Dye was collected when swabbed	○ Type 1: Started to darken at 15 hrs. ○ Type 2: Darkened at 6 hrs. ○ From both: Curved at 15 hrs.	Type 2: Started to melt and release oil at 3 hrs.
190	○ Type 2: yellowing at 3 hrs. ○ Dye was collected when swabbed	○ Type 1: Darkened at 2 hrs. Started to curve at 3 hrs. Darker at 6 hrs. ○ Type 2: Turned black and curved at 1 hr.	Type 2: Melted and released oil at 1hr.

Table 2: Changes observed on the materials as temperature increased

It's important to understand the physical changes of denim, synthetic leather and carpet as temperature increases because these changes could interfere with the visualization of a biological stain and its collection.

Denim was the substrate that showed the least amount of visible physical changes as temperatures increased above 130°C. One of the main observations of denim was the release of blue dye when the material was swabbed; the dye did not cause interference with the color development of phenolphthalein. Two types of visually similar denim were used in Part I: Type 1 (D1) and Type 2 (D2). D1 was used for the 130°C samples, but due to a limited availability, D2 was used for all subsequent samples. The two types of denim did not inhibit the observation of the stains at any temperature. The only difference observed between them at 130°C was that D1 yellowed after 6 hours and D2 yellowed after 15 hours.

Synthetic leather and carpet were purposely chosen in an light color because a lighter background would allow for a better observation of any physical changes to the stain. For synthetic leather, the darkening of the material was the primary change observed as the temperature increased. Two types of synthetic leather were used: Type 1 (SL1) and Type 2 (SL2). For both types when the temperature increased, the materials became darker and the edges curled up faster (Figure 1). The color of the substrate did not transfer to the swab and did not interfere with the color development of phenolphthalein or AP Spot test.

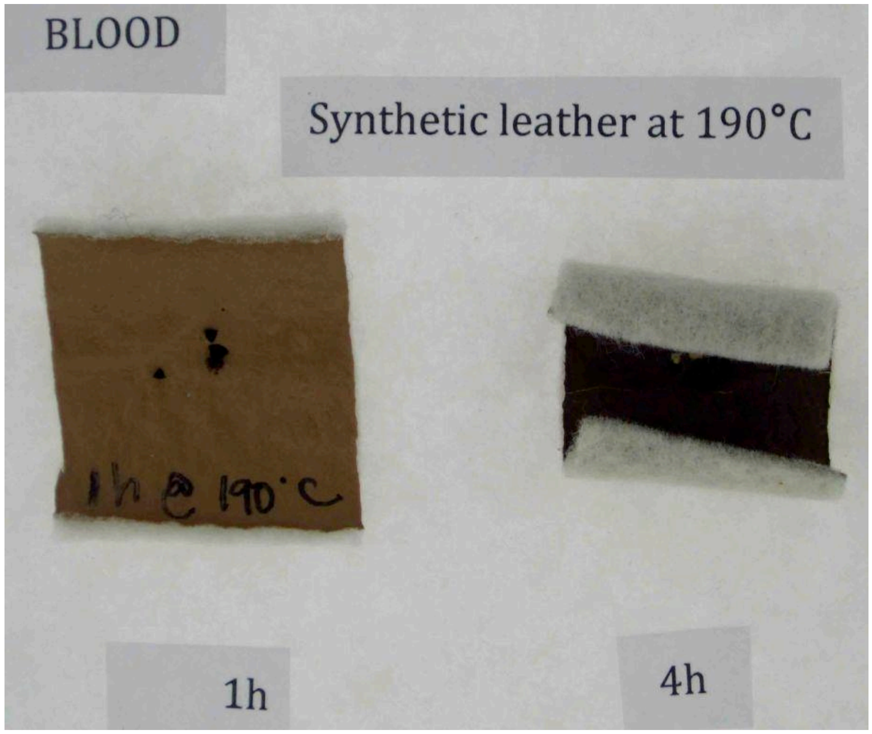

Figure 1: Bloodstain on synthetic leather (SL1) exposed to 190°C for 1 and 4 hours

There were two types of carpet used: Type 1 (C1) and Type 2 (C2). C1 was used initially, but was discontinued because the long fibers interfered with the visualization of the changes of the stains, especially for semen. Also, semen absorption into the long fibers hindered swabbing. C2 was chosen as a replacement due to its shorter fibers that were sewn tighter together and allowed for improved visualization of the semen stain. Carpet exhibited the most physical changes as temperature increased. At 150°C, carpet showed signs of disintegration starting at 15 hours (Figure 2). The carpet fibers shrunk and became fragile; that is why a careful handling was necessary to prevent further disintegration. As observed in Figure 2, the carpet coloration changed to yellow as the temperature increased, and the carpet started to release an oil-like substance which could be due to the petroleum based compounds that are often used in carpet manufacturing (63). Results obtained in this study showed carpet began to melt at 170°C after 3 hours. Carpet samples heated at 190°C exhibited melting of the carpet fibers and charring of the back at one hour, which was lower than the melting temperature range in the literature and could be due to the small size of the material tested. These changes reduced the size of the carpet pieces in the first hour, and the melting of the carpet fibers interfered with the visualization of blood and semen stains (Figure 3).

Figure 2: Bloodstain on carpet (C2) exposed to 150°C for 24 hours

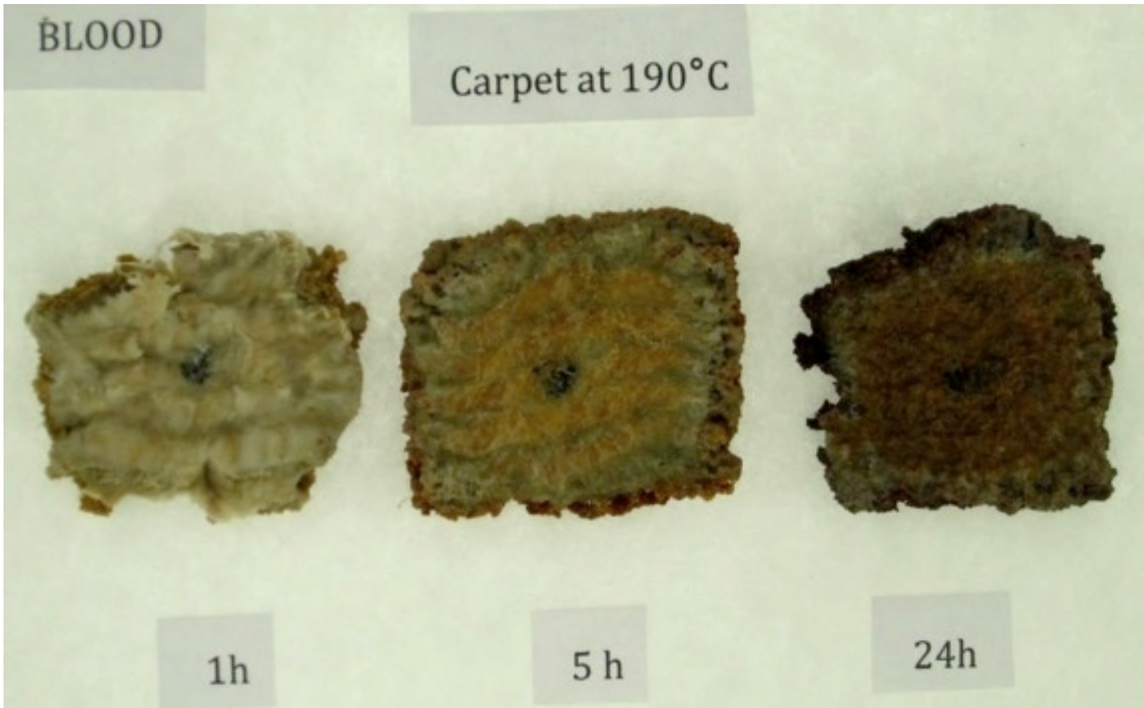

Figure 3: Bloodstain on carpet (C2) exposed to 190°C for 1, 5 and 24 hours (after scrapping)

3.3 Visual changes of blood and semen stains

At a fire scene, crime scene investigators are in charge of evidence collection. Understanding the changes that blood and semen stains undergo as temperatures increase is important for evidence screening and selection; these changes are recorded in Table 3.

Temp (°C)	Denim		Synthetic Leather		Carpet	
	Blood	Semen	Blood	Semen	Blood	Semen
24*	RBS	Whitish stain	Red-brown shiny flaky stain	Colorless stain	RBS	Colorless stain
130	RBS	Yellow (7 - 24 hrs)	Red-brown shiny flaky stain	○ Yellow (15 - 20 hrs) ○ Dark yellow (24 hrs)	RBS	N/A
150	RBS	○ Light yellow (1 - 7 hrs) ○ Dark yellow (15 - 24 hrs)	Red-brown shiny flaky stain	○ Light brown (1 - 2 hrs) ○ Brown (3 - 7 hrs) ○ Dark brown (15 - 24 hrs)	RBS	○ Orange/light brown (1 hr) ○ Light brown (2 - 24 hrs)
170	RBS	○ Yellow (1 - 5 hrs) ○ Dark yellow (6 - 7 hrs) ○ RBS (15 - 24 hrs)	Dark brown/black shiny flaky stain; Color of substrate darkened, difficulty screening	○ Brown (1 hr) ○ Dark brown (2 - 5 hrs) ○ Dark brown/black (6 - 24 hrs)	○ Dark brown (1 - 6 hrs). ○ Dark brown/ black (7 - 24 hrs)	○ Light brown (1 - 7 hrs) ○ Stain covered by melted carpet (15 - 24 hrs)
190	RBS	○ Yellow (1 - 5 hrs) ○ Dark yellow (6 - 7 hrs) ○ RBS (15 - 24 hrs)	○ Dark brown/black (1 - 24hrs) ○ Darkening of substrate difficulty screening (15 - 24 hrs)	○ Light brown (1 hr) ○ Dark brown (2 hrs) ○ Dark brown/black (5 – 24 hrs) ○ On Type 2, area stained has an irregular texture from the substrate (1 - 24 hrs)	Black and smaller in size due to engulfing by the melting carpet (1 - 24 hrs)	Difficult to visualize stain due to melted carpet; Stain light brown

*Table 3: Visual changes of the blood and semen stains as temperature increased, *= room temperature, RBS= red-brown stain*

Even as temperature increased to 190°C, bloodstains did not show significant morphological changes on denim. Bloodstain flakes on synthetic leather changed from red-brown to dark brown. The first visible changes to bloodstains on carpet were observed at 170°C as the stains began to darken and protruded from the melting carpet. Melting of the carpet that covered the stain, started after 15 hours at 170°C. Bloodstains on carpet at 190°C appeared as a dark brown/black dot, as the bloodstain was engulfed by melted carpet, decreasing in size (Figure 3).

A limited amount of information about the changes in blood coloration in extreme temperature conditions has been published in the literature. Brady et al. (64) describe the differences in visual appearance of bloodstain patterns on drywall in a cold environment (~6°C) as “frozen and crystal like” and dark red in color. In ambient temperature (~22°C), they observed that the stains were less dark red-brown than in the cold temperatures. In hot environments (~38°C), they noticed that the stains were light red-brown in color. Higher temperatures were used in the present study, but the change of blood to a lighter coloration was not observed as temperature increased. Moody et al. (65) describe the changes of blood when it was exposed to temperatures between 260°C to 871°C inside a furnace. They report that blood started to evaporate at 315°C, and at 426°C blood turned granular in texture.

Semen stains showed changes in coloration as the temperature increased with all of the materials. Yellowing of the stain was observed for the first time after 7 hours at 130°C. This change in semen color could make the stain appear to be a urine stain and thus, be tested as such. As the temperature increased the semen stain became darker at a faster rate. On denim at 170°C, the coloration of the stain turned red-brown after 15 hours. This observation fits the typical visual characteristic of blood and could cause the stain to be processed as such (Figure 4).

Figure 4: Semen stain on denim swatches (D2) exposed to 170°C for 1,7 and 24 hours

3.4 Absorption of blood and semen

Understanding how blood and semen are absorbed on the three different materials is important in order to understand the way they are exposed to high temperatures. Semen is viscous and when it was pipetted, it liquefied minutes later enabling it to be absorbed through the denim and carpet fibers. When blood was pipetted on to the absorbent materials, it spread out less across the surface than the semen. Blood on synthetic leather, a non-absorbent material, did not adhere well when it dried, instead forming flakes. Careful handling of this material was needed in order to prevent sample loss. Semen stains did adhere to the synthetic leather material; this facilitated swabbing of the stain. Blood and semen stains on synthetic leather covered a similar amount of surface area.

The collection and testing of blood and semen stains on carpet were challenging due to the drastic physical changes that occurred as the temperature increased. When blood was applied to carpet, it primarily stayed on the surface of the fibers. This detail is important especially at 190°C, when the bloodstains on the carpet were engulfed due to the rapid melting of the carpet within the first hour. In contrast to blood, semen stains were completely absorbed by carpet rather than remaining on the surface. The semen stains were covered by melted carpet in some samples exposed to 190°C. The light brown coloration of semen was observed underneath the layer of melted carpet and in some cases tested positive using AP Spot test. It is suspected that some semen stains were negative due to interference from the melted carpet fibers, making semen stains on a large melted carpet difficult to localize because they are concealed.

3.5 Phenolphthalein testing on bloodstains exposed to 130°C, 150°C, 170°C and 190°C

For each material exposed to heat (130°C, 150°C, 170°C and 190°C) the time for a positive phenolphthalein reaction to develop was recorded and the average time was calculated and plotted (Figures 5 - 8).

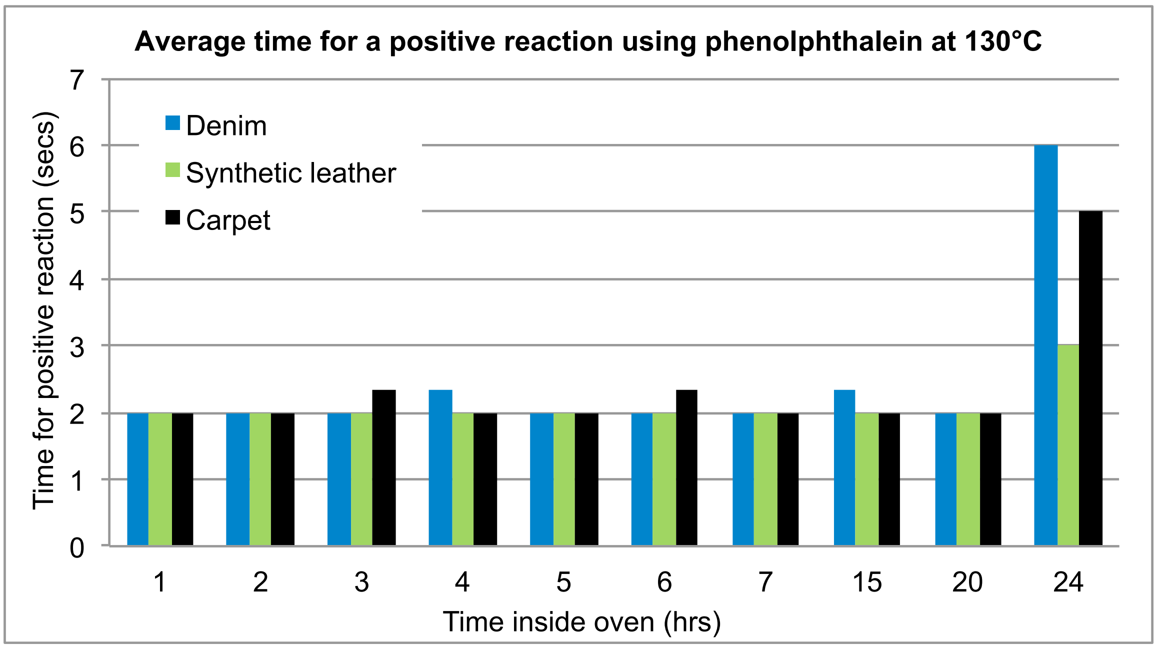

Figure 5: Average time for a positive phenolphthalein reaction on bloodstains on denim, synthetic leather and carpet exposed to 130°C

Positive results were obtained for all the materials at 130°C, indicating that bloodstains after a 24-hour exposure to heat are still capable of being presumptively identified. The average time for a positive reaction between hour one and hour 20 was around 2 seconds for all three materials. At hour 24, the average time for a positive result for synthetic leather was 3 seconds and for carpet was 5 seconds. Denim had the longest average reaction time, observed at 6 seconds. All of these values are still well below the 10 to 15 second cut-off point utilized by forensic labs to indicate a positive result (25,66). Side-by-side visual comparison of the stains tested from hour 1 to hour 24 appear similar and cannot be differentiated at 130°C.

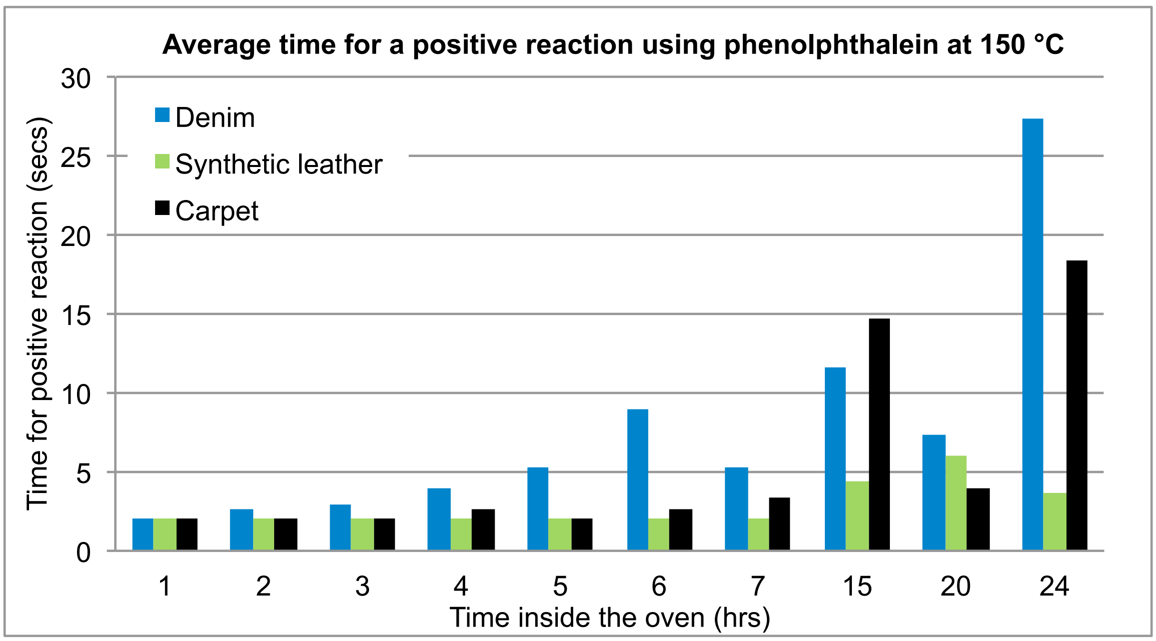

Figure 6: Average time for a positive phenolphthalein reaction on bloodstains on denim, synthetic leather and carpet exposed to 150°C

One of the first observations made at 150°C was that even after an exposure of 24 hours, bloodstains tested positive using phenolphthalein on denim, synthetic leather and carpet. At the first hour, all the materials showed the same average time of 2 seconds for a positive reaction. Denim showed a steady increase in the average time for a positive reaction from the first hour to hour 24, ranging from 2 to 27.33 seconds.

Bloodstains on synthetic leather were carefully swabbed due to the detachment of the flaky bloodstain. Of the three materials, synthetic leather had the smallest time variation of 2 to 6 seconds across a 24-hour period. At hour 24, the average time for a positive reaction was four times faster than for denim and carpet.

After a 24-hour period, carpet demonstrated a maximum average time of 18 seconds for a positive result. The collection technique used was changed from swabbing to scraping at hour 20 because when swabbing carpet at 150°C, the material would disintegrate. If negative results were obtained with swabbing, the stain was scraped and the phenolphthalein test was carried out on a ceramic spot plate. If the test gave negative results using the scrapings, a second swab was tested to ensure that results were still negative even after scraping the stain. The time was recorded when the first positive results were obtained using the scraping or swabbing technique. The increase in time for a positive reaction at hour 15 could be due to the fact that physical changes in the carpet made swabbing of the stain challenging in addition to the breakdown of heme that was likely occurring as time inside the oven increased. The decrease in reaction time at hour 20 is attributed to the fact that the scraping method, rather than the swabbing method, was used, exposing portions of the stain less damaged by the heat.

Beginning at 150°C the concern for false negative results arises if the typical 10-15 second cut-off point is used on denim and carpet samples, as some positive reactions required more time to become visible. The results indicate that the cut-off point for samples that have been exposed to a high temperature environment may need to be reconsidered in order to prevent the occurrence of false negatives.

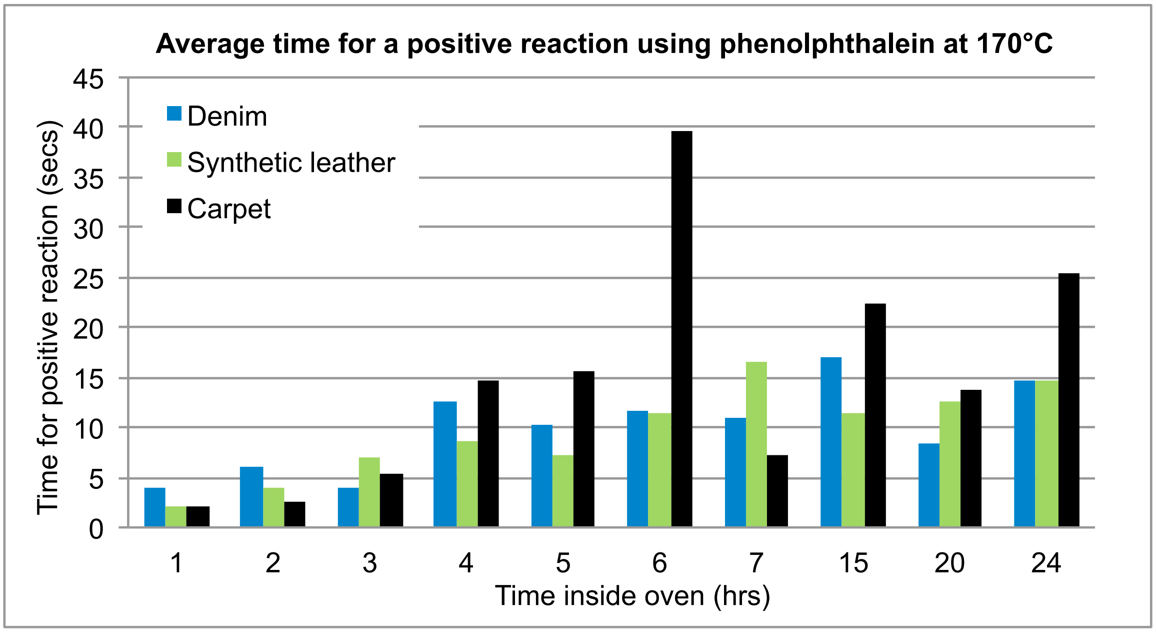

Figure 7: Average time for a positive phenolphthalein reaction on bloodstains on denim, synthetic leather and carpet exposed to 170°C

At 170°C, denim showed an average positive reaction time between 4 and 17 seconds. The scraping method was used for only one of the denim samples starting at hour 20, which gave a positive reaction time of 4 seconds; samples using the swabbing technique that were exposed for the same period of time inside the oven showed an average positive reaction at 9 and 12 seconds.

At this temperature, the curled synthetic leather was flattened to swab the stain, causing the surface of the material to split. This could be problematic at a crime scene because blood that has not adhered well to the material could be easily lost. For hours one through seven the swab technique was utilized; scraping of the bloodstain flakes was implemented after hour seven when results for the first swab were negative. One of the main advantages to scraping the flakes rather than swabbing is that the analyst doesn't have to worry about making sufficient contact with the stain to allow a transfer of solubilized biological material to occur. On the contrary, the analyst has to be cautious not to consume the entire stain during the screening process.

The time range for an average positive reaction to occur on carpet was between 2 to 40 seconds. Sample collection from carpet was changed to scraping at hour seven, as the melting carpet began to engulf the stained area, which explains why the time required for a positive reaction decreased. Scrapings were used in order to ensure direct contact with the stain. It is possible that heme degradation was hindered in the subsurface layers of the carpet because the top layer acted as a protective barrier from direct heat. When working with carpet at 170°C, scraping rather than swabbing is suggested.

At 170°C, using the cut-off point of 10 to 15 seconds would yield a false negative result when samples have been exposed to heat for a period of 15 hours on denim, for 7 hours for synthetic leather and at 5 hours for carpet. This shows that as the temperature increases, the time limit currently used would not be sufficient to presumptively test blood when heat-exposed evidence from fire scenes is examined.

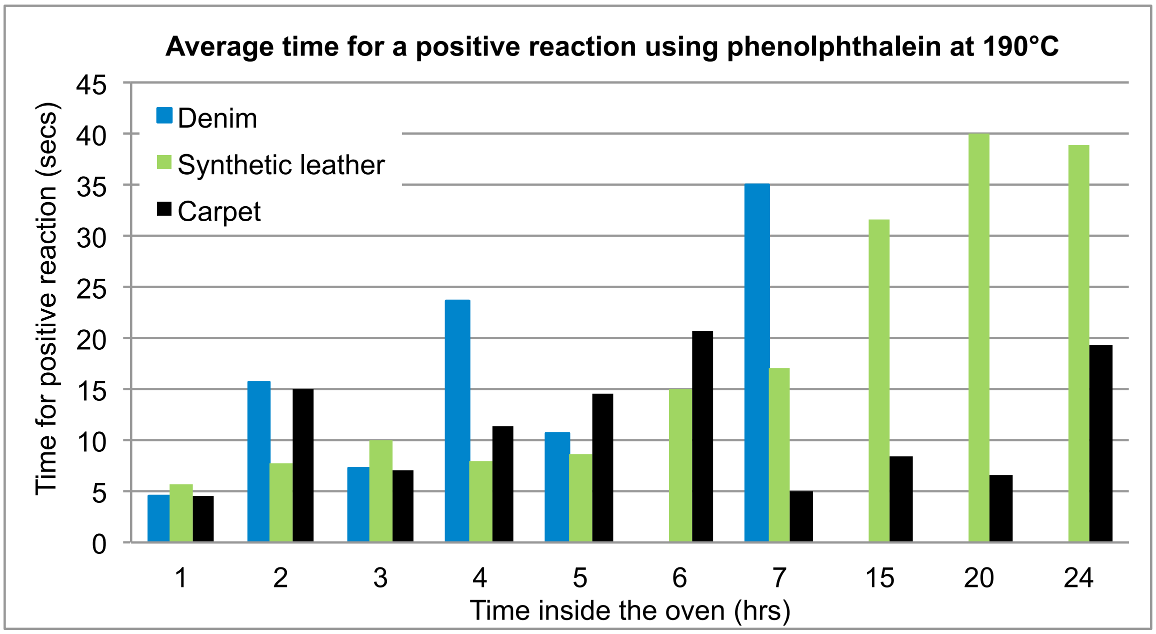

Figure 8: Average time for a positive phenolphthalein reaction on bloodstains on denim, synthetic leather and carpet exposed to 190°C

At 190°C, negative results (no reaction within one minute) for bloodstains on denim were observed starting at hour 15. In Figure 8, denim shows an irregular trend for the average time of positive reaction as low values were obtained at hours 3 and 5. Scraping the bloodstain on denim was necessary starting at hour three because the swabbing technique was no longer yielding positive results. Only one sample gave positive results at hour six and two samples gave positive results at hour seven, explaining why in no results at hour 6 are shown in Figure 8. Denim was the only material that after seven hours at

190°C gave negative results even when the samples were collected using the scraping method.

Only synthetic leather showed an overall increase in the average time for a positive result. The average minimum time for a positive reaction was 6 seconds recorded at hour one and the maximum average time was 40 seconds recorded at hour 20. One sample showed a negative result at hour 24. The observation of the first negative result for synthetic leather indicates that it is possible that negative results for this material could be observed at higher temperatures.

At 190°C, carpet had the shortest range of average positive reaction times, from 4.66 seconds to 20.66 seconds, as time inside the oven increased. This may be attributed to the fact that the scraping method was used from the first hour, when the carpet fibers began to melt.

At 190°C, the 10-15 seconds cut-off point again proved too low for samples exposed to high temperatures. At this temperature, blood could not be detected on denim and carpet after two hours and in the case of synthetic leather, after an exposure of six hours.

Data discussed in this section was further analyzed as shown in Figures 9, 10 and 11 to illustrate the time variation within each triplicate set. It was observed that individual samples exposed to the same time and temperature inside the oven demonstrated a wide range of time for a positive reaction to occur. These figures also show that if the 10-15 second cut-off is used, individual samples in a sample group may or may not yield a positive result. For example, in Figure 9 for 150°C at hour 15, the times for a positive reaction on denim in the triplicate set are 4, 11 and 20 seconds.

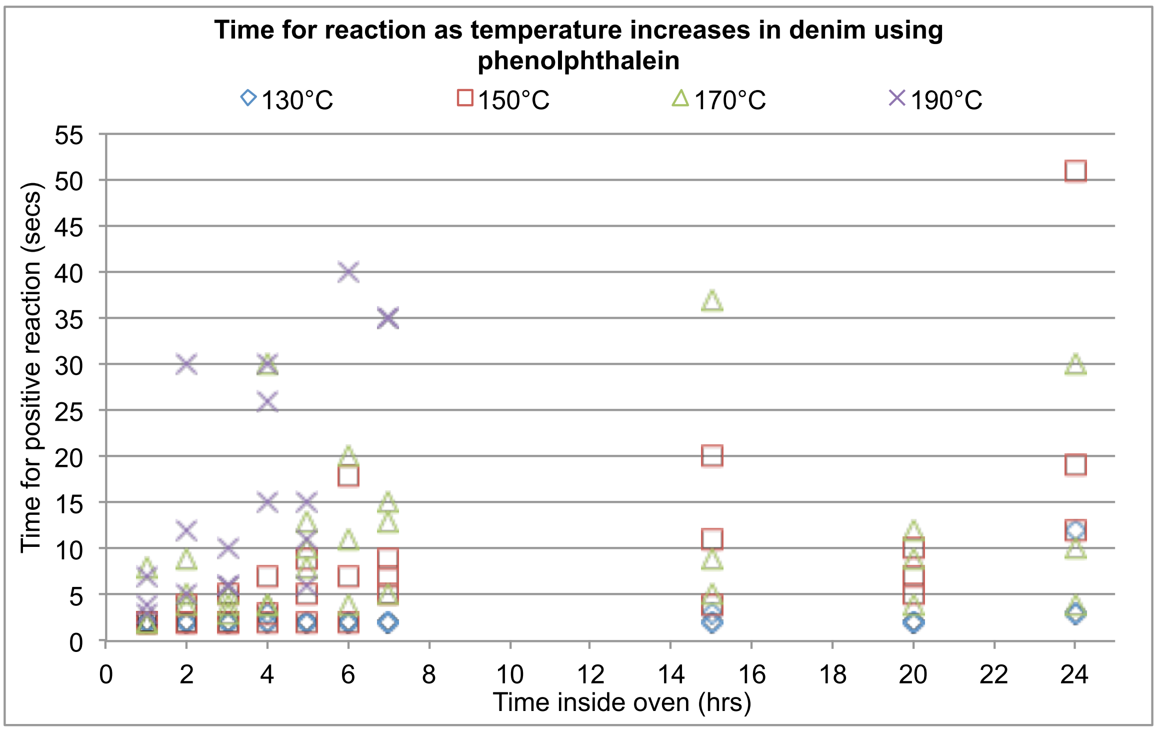

Figure 9: A comparison of time for a positive phenolphthalein reaction as temperatures increased on individual denim samples exposed for up to 24 hours.

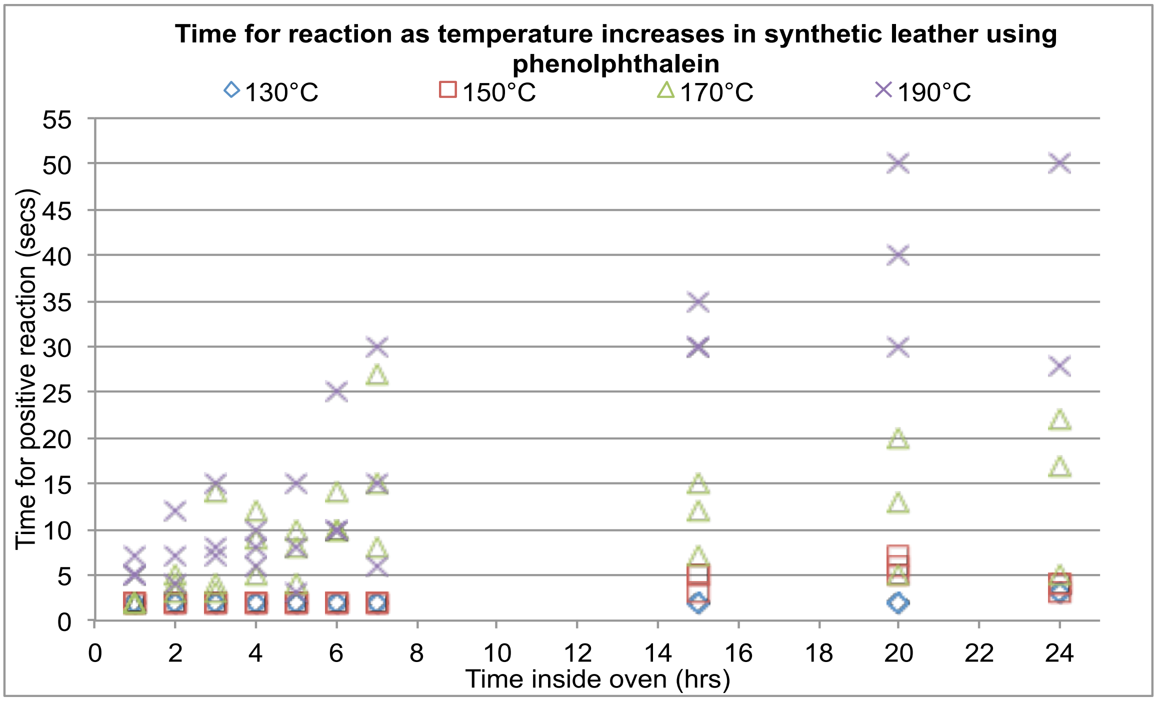

Figure 10: A comparison of time for a positive phenolphthalein reaction as temperatures increased on individual synthetic leather samples exposed for up to 24 hours.

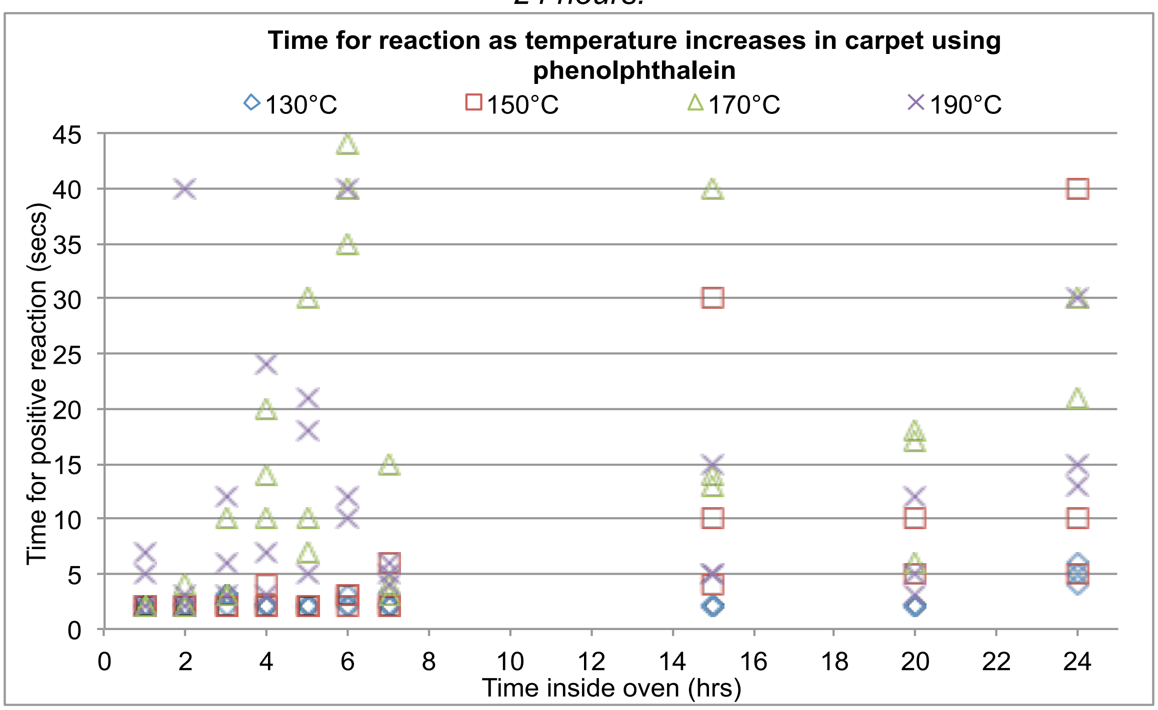

Figure 11: A comparison of time for a positive phenolphthalein reaction as temperatures increased on individual carpet samples exposed for up to 24 hours.

3.6 ABAcard® Hematrace®` testing for bloodstains exposed to 130°C, 150°C, 170°C and 190°C

Results in Table 4 show human hemoglobin was detected up to one hour on synthetic leather and carpet at 130°C. At this temperature there was no apparent physical damage to the substrate.

Temp (°C)	Maximum time for a positive ABAcard® Hematrace® result observation (hrs)		
	Denim	Synthetic Leather	Carpet
130	NEG	1	1
150	NEG	NEG	NEG
170	NEG	NEG	NEG
190	NEG	NEG	NEG

Table 4: Maximum number of hours exposed to heat for which a positive result using ABAcard® Hematrace® was obtained on denim, synthetic leather and carpet as temperature increased (n=1). NEG = negative results at all times

Comparing these results with the ones obtained in Section 3.5, it is observed that bloodstains tested using phenolphthalein gave positive results up to 190°C and bloodstains exposed to the same environment and tested with ABAcard® Hematrace® gave positive results only up to 130°C. This suggests that phenolphthalein, rather than ABAcard® Hematrace® should be used to test samples that have been exposed to high heat. Another factor that could have influenced the results is the mechanism of these two tests; phenolphthalein relies on a chemical oxidation-reduction reaction and ABAcard® Hematrace® results depend on an immunoreaction.

Since ABAcard® Hematrace® was not able to detect the presence of human blood at temperatures higher than 130°C, this type of assay may be problematic for arson cases or other high-temperature scenes. It is possible that a longer extraction time before analysis could improve the solubility of the stain, and thus may increase the detection limits for this type of assay (22).

3.7 Fluorescence on semen stains exposed to 130°C, 150°C, 170°C and 190°C

Although fluorescence was not necessary due to the darkening in semen color as the temperature increased, it was important to analyze how long fluorescence could be used as a screening method for semen stains exposed to high temperatures. The maximum time of heat exposure with visualization of fluorescence was determined by recording the time at which all samples within the triplicate set gave negative results.

Temp (°C)	Maximum time of heat exposure with visualization of fluorescence (hrs)		
	Denim	Synthetic Leather	Carpet
130	24	24	N/A
150	24	24	7
170	15	4	4
190	4	4	NEG

Table 5: Maximum number of hours exposed to heat for which fluorescence was observed in semen stains on the front of swatches of denim, synthetic leather and carpet as temperatures increased. NEG = no fluorescence

Table 5 shows that as the temperature increased, fluorescence decreased in all materials. Denim and synthetic leather showed similar fluorescence results at 130°C and 150°C, however, at 170°C samples on denim fluoresced only at the perimeter beginning at 15 hours, until fluorescence was no longer observed. Denim and synthetic leather at 190°C exhibited fluorescence for only the first four hours. At 150°C, fluorescence of the stains on carpet stopped after 15 hours, coinciding with the visible heat damage to the carpet fibers. At 170°C fluorescence stopped at hour four, an hour before scraping was needed to test the stain. At 190°C no fluorescence was observed on the carpet stains, indicating that the melting of the carpet may have affected the fluorescence of the stain. The results obtained indicate that an ALS could be used to screen denim and synthetic leather exposed to 130°C-190°C temperatures, but might be a less effective tool for carpet after exposure to 150°C or higher.

3.8 AP Spot testing on semen stains exposed to 130°C, 150°C, 170°C and 190°C

A comparison of the average reaction time for each semen-stained substrate at a given temperature indicated that higher temperatures and prolonged exposure to heat increase the time required for a color change to occur (Figures 12 - 15). Due to limited semen sample availability, the carpet samples at 130°C were not tested and samples at 150°C and 190°C were run in duplicate rather than triplicate.

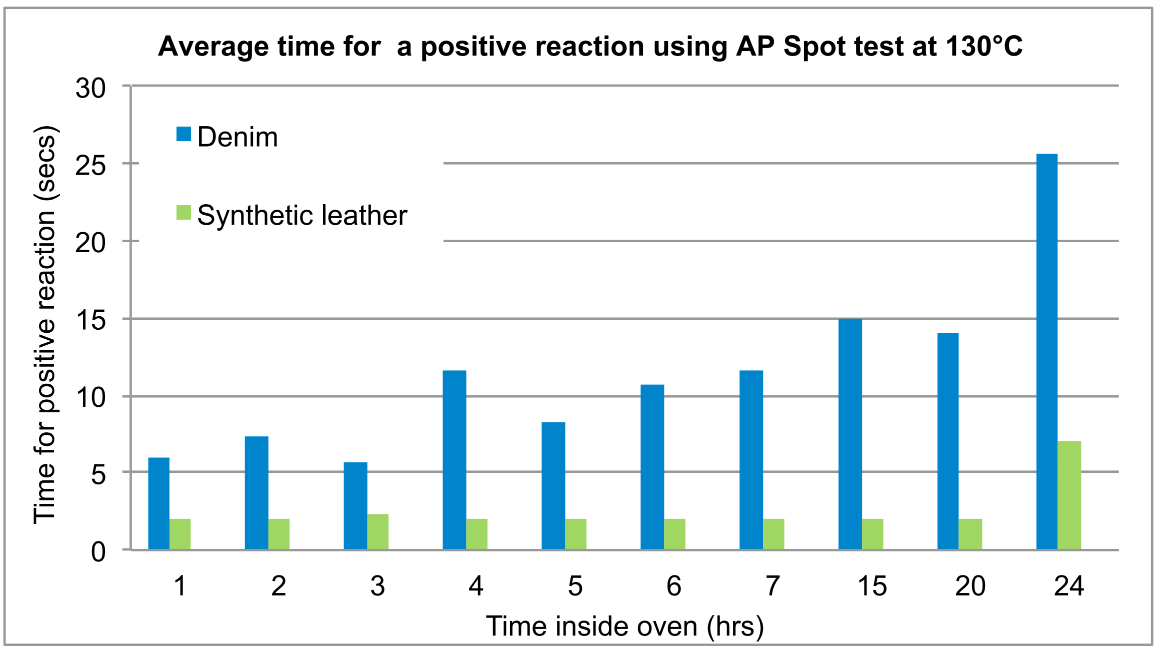

Figure 12: Average time for a positive AP Spot test on semen stains on denim, and synthetic leather exposed to 130°C

AP was detected on denim and synthetic leather showing that its detection is possible at 130°C. Figure 12 shows an average time of two seconds for a positive reaction to occur between hours 1 and 20 on synthetic leather. At the

24th hour, the time for a positive result increased to 7 seconds, which is well within the 5-minute cut-off time for the test.

The average positive reaction time for denim ranged from 6 to 26 seconds throughout the 24-hour period. The positive reaction was much faster for synthetic leather than for denim in the 24-hour period studied.

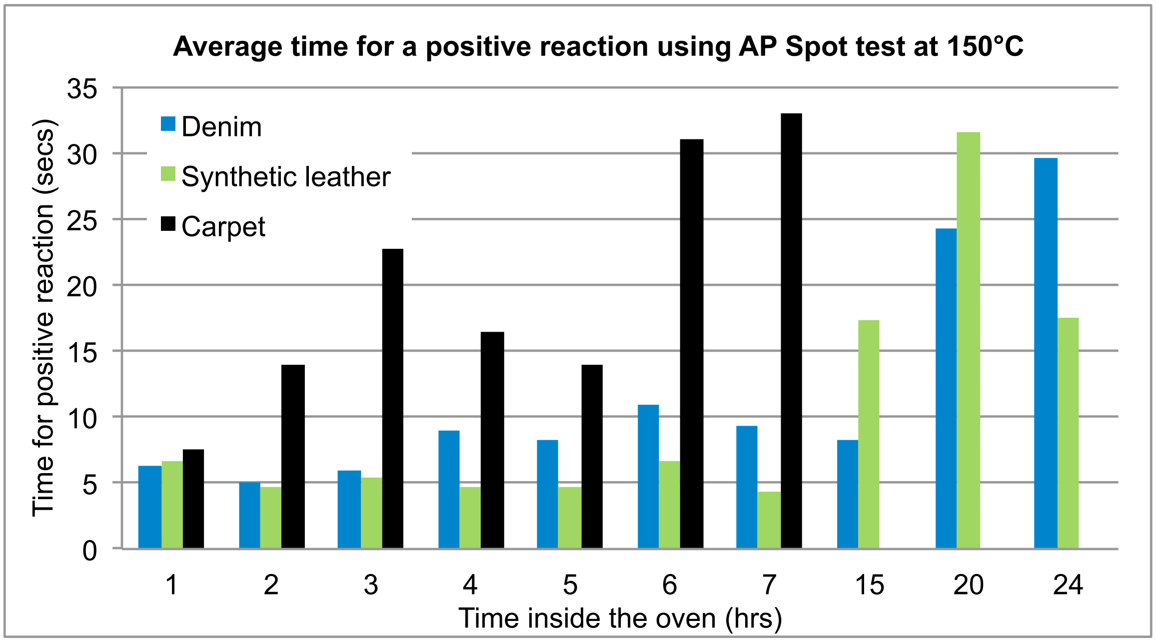

Figure 13: Average time for a positive AP Spot test on semen stains on denim, synthetic leather, carpet exposed to 150°C

At 150°C, AP was detected for all substrates except for carpet after a 15-hour period. At 150°C, the average time for a positive reaction on denim increased with time, varying from 5 to 30 seconds.

Synthetic leather showed an overall increase in average time for a positive reaction as temperatures inside the oven increased. At 24 hours, there was a small decrease in time for a positive reaction that is attributed to the small sample size of two, as one sample gave negative results.

At 150°C, carpet gave the highest average time for a positive reaction at hour 7. Swabbing was a challenge starting at hour 15 because the carpet fibers were very delicate and could easily break into pieces. An interesting observation after 15 hours was that the fibers where the semen was located showed less damage than the areas without semen. This helped with the identification of the location of the stain as well as the change in color of semen, which made the fibers look darker than the surroundings fibers. Only one of the duplicate samples gave positive results from hours 15 through 24, explaining why results are not observed in Figure 13 at these hours.

Yokota (32) tested semen stains on cotton sheets for AP under high temperatures at 80°C, 100°C, 120°C and 140°C for 10 minutes, 30 minutes and 60 minutes at each temperature. The results showed that AP could be detected at temperatures up to 140°C for 10 minutes exposure. Results obtained in the present study show a higher level of detection for AP for the three substrates tested. This may be attributed to varying starting levels of AP in the donor samples and/or substrate differences.

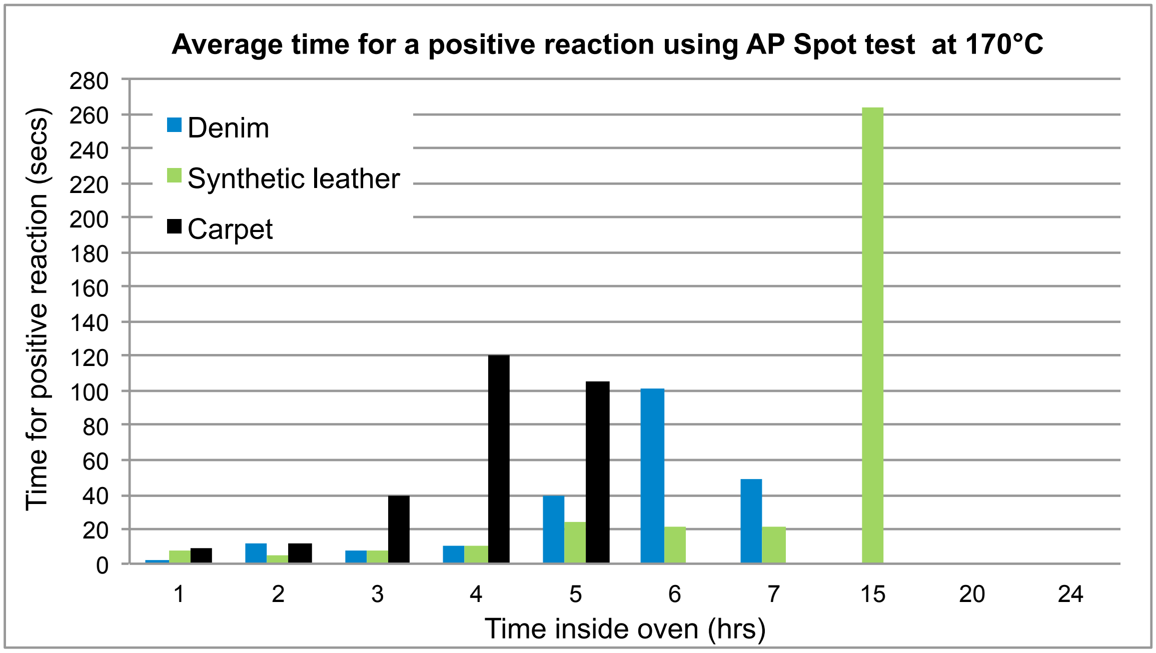

Figure 14: Average time for a positive AP Spot test on semen stains on denim, synthetic leather, carpet exposed to 170°C

At 170°C, the absorbent materials (denim and carpet) started to give negative results for AP as it was only detected up to hour seven for denim and hour five for carpet as observed in Figure 14. Synthetic leather gave positive results up to hour 15. Only one sample gave positive AP results at hour 20 and 24 for synthetic leather and that is why it is not represented in Figure 14.

At 170°C the areas on carpet thought to contain semen were scraped because these stains were covered by the melted carpet; this could be one reason why negative AP results were obtained.

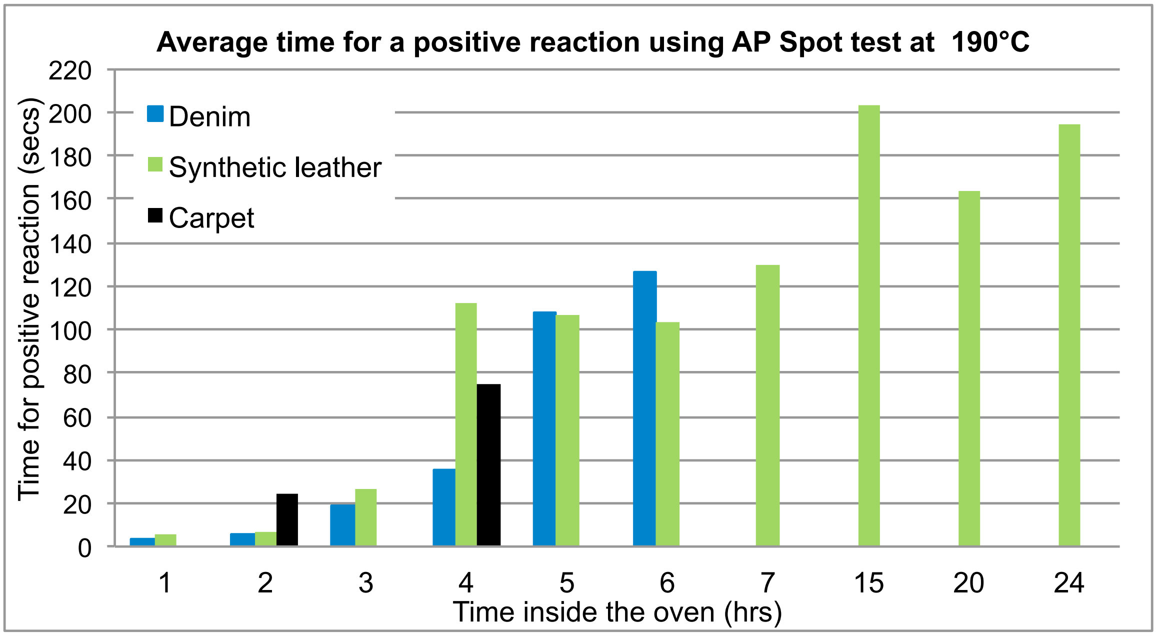

Figure 15: Average time for a positive AP Spot test on semen stains on denim, synthetic leather and carpet exposed to 190°C

Increasing the temperatures to 190°C showed drastic changes in the ability to screen for the presence of AP on denim and carpet. Denim at 190°C showed an average increase of 3 seconds to 127 seconds for a positive AP result from hour one to hour six. Scraping was used starting at hour seven and negative results were still obtained. Negative results were observed at hour four for one sample of the triplicate set; the other two samples gave positive results until hour six.

Carpet at 190°C was not a suitable material for the screening of AP as it gave irregular results, showing positive results only at hours two and four. However, the samples within each duplicate set took the same time for a positive result to occur. The fact that semen stains are difficult to visually and chemically detect on some substrates at 190°C can be problematic when dealing with fire scene evidence, both for the crime scene investigator and forensic biologist.

Positive AP results were obtained on the non-absorbent synthetic leather for the entire 24-hour period at 190°C (Figure 15).

Sample groups discussed in this section were individually plotted in order to analyze the difference in positive reaction times for each individual sample, rather than as average times per group. It was observed at 150°C, 170°C and 190°C that individual samples exposed to the same time inside the oven had a wide range of times for a positive reaction to occur (Figures 16 - 18).

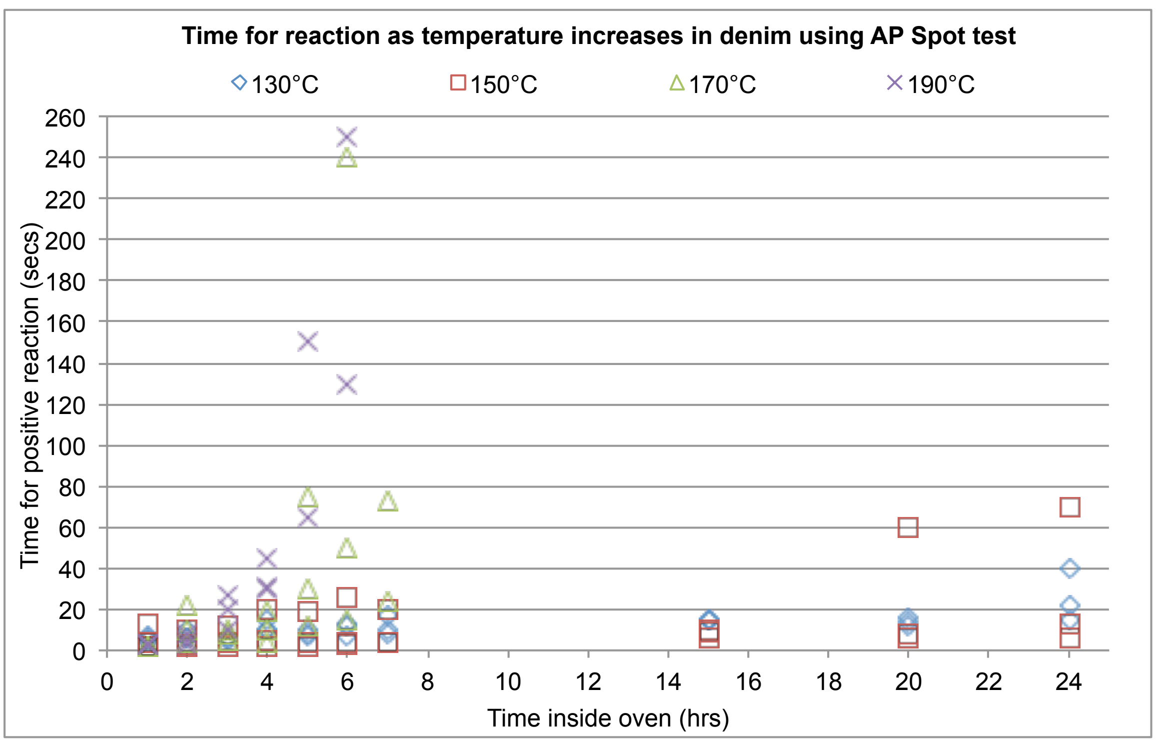

Figure 16: A comparison of time for a positive AP Spot test as temperatures increased on individual denim samples exposed up to 24 hours.

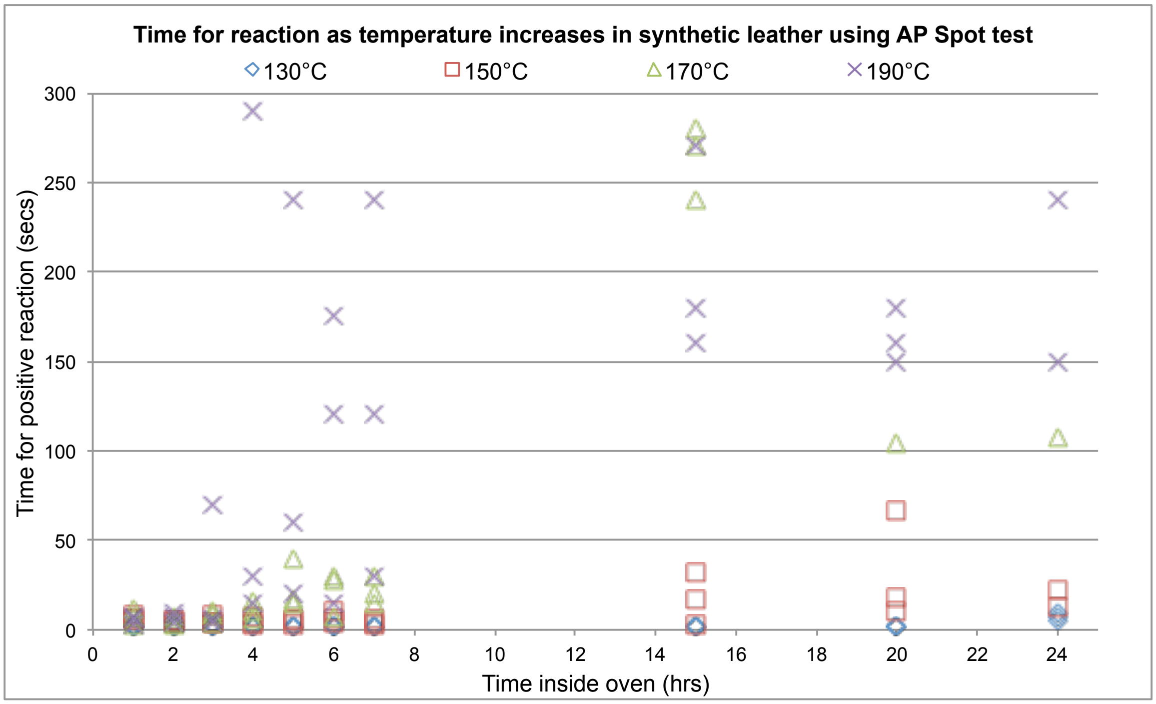

Figure 17: A comparison of time for a positive AP Spot test as temperatures increased on individual synthetic leather samples exposed for up to 24 hours.

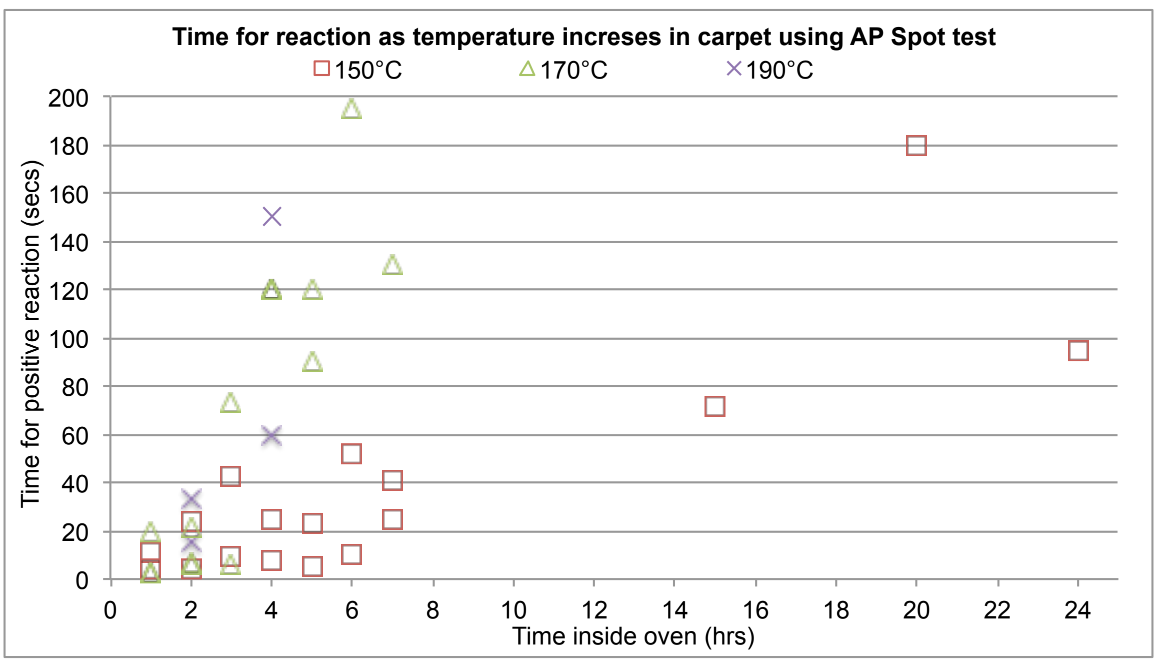

Figure 18: A comparison of time for a positive AP Spot test as temperatures increased on individual carpet samples exposed for up to 24 hours.

3.9 Microscopic identification of spermatozoa on semen stains exposed to 130°C, 150°C, 170°C and 190°C

Identifying the presence of spermatozoa confirms the presence of semen, something that AP cannot do because it is present in other biological fluids (11,25,27,50). Only one sample group was tested to determine if microscopic identification of spermatozoa was possible in heated samples.

Temp (°C)	Maximum time of heat exposure for identification of spermatozoa (hrs)		
	Denim	Synthetic Leather	Carpet
130	24	20	N/A
150	24	1	15
170	15	NEG	2
190	7	NEG	NEG

Table 6: Maximum number of hours exposed to heat for which spermatozoa were identified on denim, synthetic leather and carpet as temperatures increased (n=1). NEG = no spermatozoa found

Results showed that as the temperature increased, the identification of spermatozoa decreased (Table 6). Substrate type also influenced the results, with denim allowing for the greatest sperm detection and synthetic leather the least. Denim showed the best capability for identification of spermatozoa up to 15 hours at 170°C and seven hours at 190°C. The presence of spermatozoa was detected after longer periods of heat exposure than AP which was only detected up to hour seven at 170°C and hour six at 190°C. The identification of spermatozoa on synthetic leather was only possible at hour one at 150°C.

Spermatozoa on carpet were detected until hour 15 at 150°C and up to hour two at 170°C; both of these results were lower than the detection of AP, which was detected after 24-hours of heat exposure at 150°C and until hour 7 at 170°C. These results are consistent with the findings of Yokota et al., who reported that spermatozoa on cotton sheets were detected at temperatures up to 140°C for 60 minutes exposure (32).

During the analysis of the denim slides it was observed that as the temperature increased the contrast of the sperm heads decreased. The staining time for NFR was increased in an attempt to achieve a better contrast for faster spermatozoa screening. Extracts from semen stains that had not been exposed to heat were stained for 15, 20 and 25 minutes (Figure 19). The sperm heads appeared brighter as the NFR absorption time increased, making them more easily visualized. For all of the three absorption times, the acrosomal cap was pink and well defined.

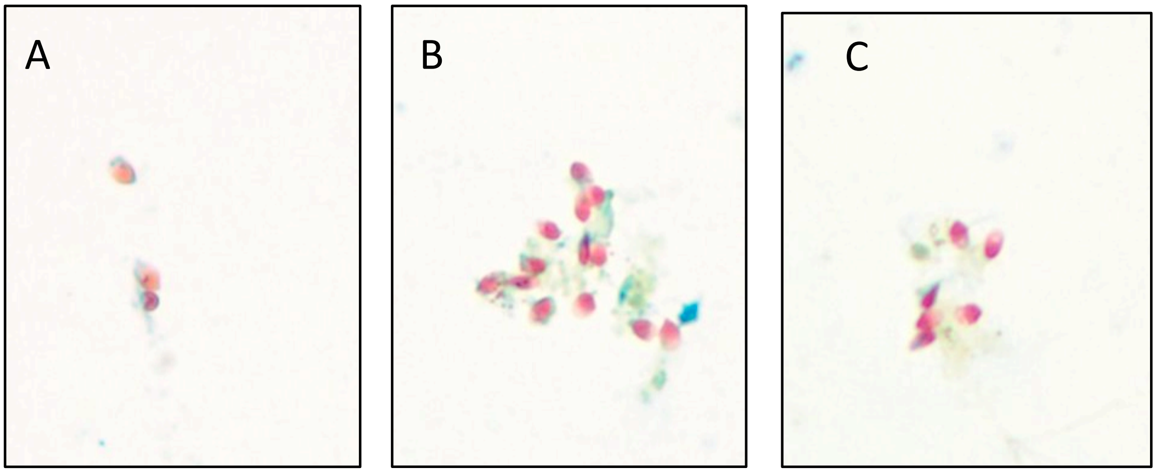

Figure 19: Control slides stained with KPIC for 15 minutes (A), 20 minutes (B) and 25 minutes (C) at 400x

The effects of the extension of NFR staining time were apparent at 190°C where at five hours the sperm heads were barely noticeable and the acrosomal cap was not clearly identified when the stain was applied for 15 minutes. When NFR was absorbed for 25 minutes, a better visualization of sperm heads was possible and the acrosomal cap was more easily identified. This is advantageous in cases where the presence of spermatozoa is limited or potentially masked by the presence of cellular debris.

3.10 RSID™-Semen testing on semen stains exposed to 130°C, 150°C, 170°C and 190°C

A sample group from denim, synthetic leather and carpet was chosen arbitrarily and tested using RSID™-Semen to confirm the presence of Sg. Carpet provided the best environment for the preservation of Sg (Table 7).

Temp (°C)	Maximum time of heat exposure for a positive Sg result (hrs)		
	Denim	Synthetic Leather	Carpet
130	24	20	N/A
150	2	NEG	24
170	1	NEG	24
190	NEG	NEG	24

Table 7: Maximum number of hours exposed to heat for which semenogelin detection was possible on denim, synthetic leather and carpet as temperatures increased (n=1). NEG= negative results at all times

Table 7 shows that on denim, Sg was able to be detected up to a 24-hour period at 130°C but the identification of Sg decreased to only two hours of exposure at 150°C, one hour at 170°C and was not detected at 190°C. The detection of Sg on synthetic leather was possible up to 20 hours at 130°C and negative results were obtained at 150°C and higher. The limits of Sg detection on synthetic leather were in contrast to AP, which could be detected for a 24-hour period at 190°C. Since this material is non-absorbent and all of the semen stain was exposed, it is suggested that Sg has a lower thermal stability than AP when no protection is provided by the material.

On carpet, Sg could be detected at all temperatures after 24 hours inside the oven. Conversely, neither AP nor fluorescence was detected at the maximum time and temperature studied. In forensic cases, fluorescence screening and AP presumptive testing are often performed before utilizing immunochromatographic assays like RSID™- Semen, and therefore, samples with negative screening results may be deliberately withheld from further testing. Furthermore, Sg is more specific to semen than AP because although it is not exclusively found in semen, the other biological materials in which it can be detected are not typically forensically relevant (11,40,41).

Sato et al. discussed the thermo-stability of Sg in a study in which seminal stains were dried on filter paper (31). Results showed that Sg was stable for one hour at 150°C, similar to the results for denim in this study. Sato did not test any higher temperatures or periods of time longer than one hour.

Part II: The effects of flame contact on blood and semen stains

Results obtained in Part I provided a guideline to understanding the effects of high temperatures on the detection of blood and semen. For Part II of this study, blood and semen stains were exposed to direct flame contact for 5 and 10 seconds followed by the same presumptive and confirmatory tests used in Part I. To burn the materials, a propane torch that reaches between the temperature of 482-632°C was used (5); these temperatures were 3 times higher than the temperatures tested in Part I.

Before burning the materials containing blood or semen samples, the damage to the substrate was examined to see if physical changes to the material itself would be problematic for the subsequent testing. For this part of the study, the types of materials used were: D2, SL2 and C2.

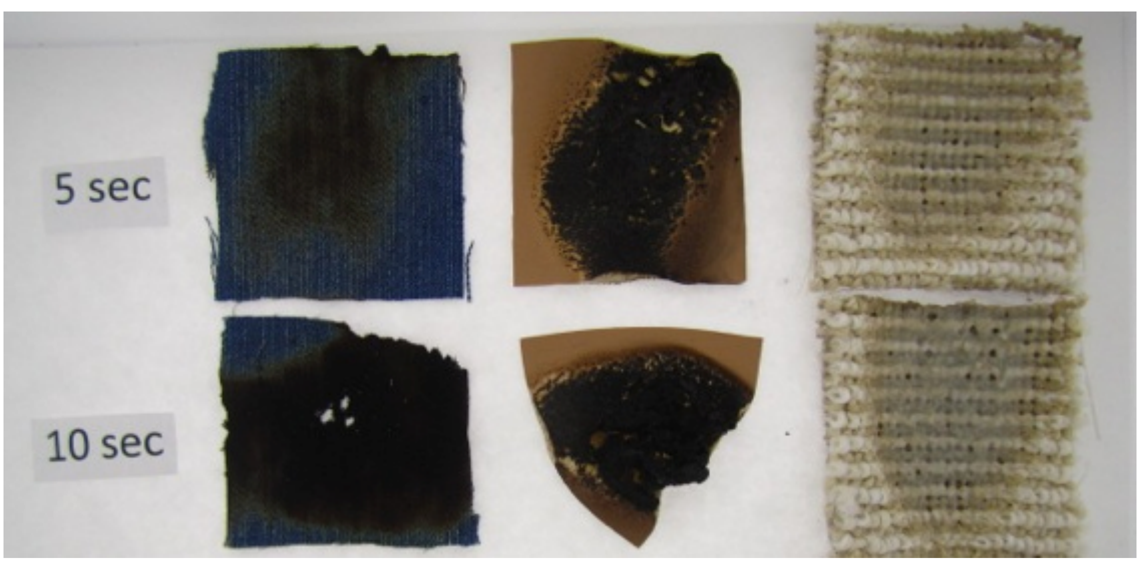

Figure 20: Denim, synthetic leather and carpet burnt for 5 and 10 seconds (side exposed to direct flame is pictured)

Figure 20 shows the difference in physical characteristics after materials were burnt for 5 and 10 seconds. After being exposed to the flame, the side of carpet exposed to the flame became very hard due to the melting of the carpet fibers. When the material was swabbed it did not show any darkening of the cotton. No damage was observed on the side of the carpet not in direct contact with the flame. Carpet showed the smallest degree of physical damage in contrast to Part I. When carpet was burnt, it didn’t catch on fire like denim or synthetic leather. This is likely attributed to the standards set by the U.S. Consumer Product Safety Commission and ASTM regarding flammability resistance in finished textile floor covering materials (67).

The side of denim exposed to direct flame and the side not exposed to direct flame both showed darkening in coloration due to the physical changes after contact with the flame. Also the blue dye of the material was transferred to the surface underneath the denim swatch. When this material was exposed to the flame, the perimeter of the swatch caught on fire first, causing the perimeter to appear irregular. When the burnt area of the material was swabbed, soot was transferred to the swab.

Synthetic leather showed the most physical damage from flame contact as compared to denim and carpet. This material caught on fire easily, changing drastically in shape. When the material was touched it felt spongy and the charred areas easily disintegrated. The backing on the synthetic leather held the material together and prevented it from separating into pieces. When swatches exposed to the flame for 5 and 10 seconds were swabbed, the black charred debris was transferred to the swab as with the denim. An increase from 20 μL to 100 μL of blood and semen was utilized with the test samples to help ensure that the stains could still be visualized after flame contact despite the damage to the material.

To determine if the charred debris/soot would interfere with the color development of phenolphthalein, AP Spot test, or the immunochromatographic cards, the three materials were burnt and then spiked with blood or semen on the side where the flame was in contact. When bloodstains on the materials were tested using phenolphthalein, all of the stains gave immediate positive results as expected. Similarly, positive ABAcard® Hematrace® results were obtained and the development of the red line on the membrane strip was not inhibited by the sooty material.

Semen was added to burnt substrates in a similar manner and the semen stains were easy to locate. On denim and synthetic leather, the semen stain appeared whitish, and on carpet it appeared yellowish. All materials gave positive results using AP Spot test. Purple coloration development was easily observed on the edges of the black charred debris on the swabs so inhibition or masking caused by the charred material or soot was not a concern. Fluorescence was observed on all of the stains. Using RSID™-Semen, Sg was detected on all three materials, demonstrating that charred particles and soot present in the extract did not cause interference with the visualization of colored band that is indicative of a positive result.

3.11 Visual observation of bloodstains burnt for 5 and 10 seconds

Bloodstains on the side exposed to direct flame appeared circular and dark-brown (Figure 21), while the bloodstains that had soaked through to the side not exposed to direct flame were red-brown. A ring was observed around the bloodstains on the backside of the denim that appeared to be an area not affected by the flames (Figure 22).

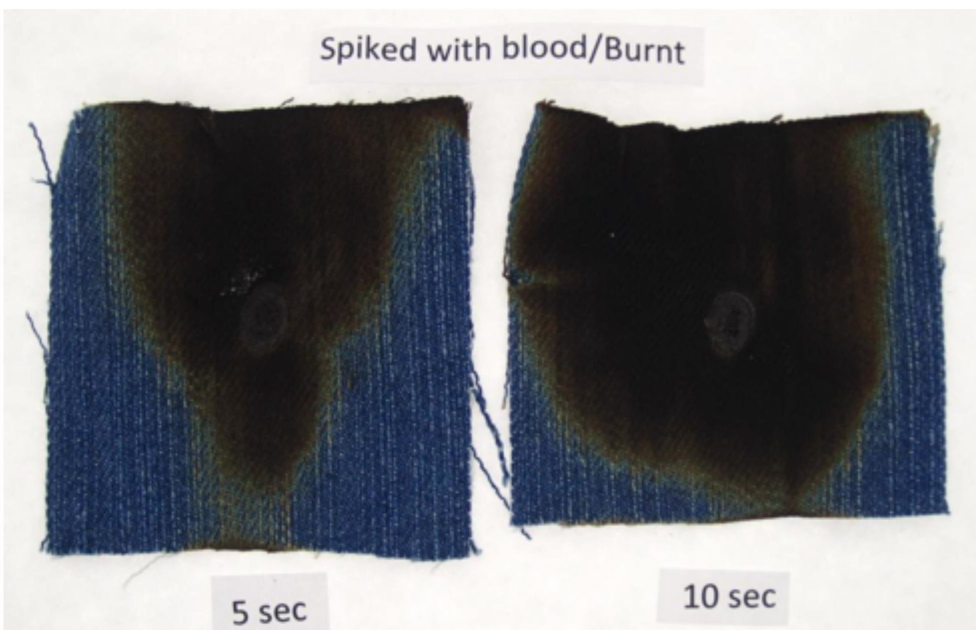

Figure 21: Bloodstains on denim swatches burnt for 5 and 10 seconds (side exposed to direct flame is pictured)

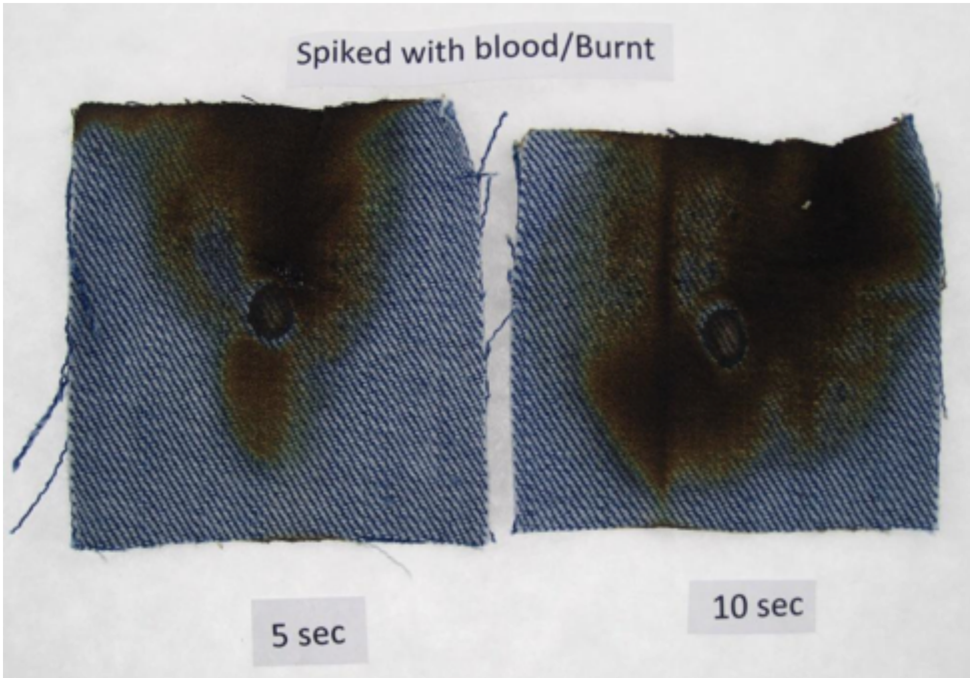

Figure 22: Bloodstains on denim swatches burnt for 5 and 10 seconds (side not exposed to direct flame is pictured)

After 5 seconds under the flame, bloodstains on synthetic leather appeared irregular in shape and dark in color because of charring (Figure 23). After 10 seconds of flame contact, the bloodstain could not be located using the naked eye because the area was charred.

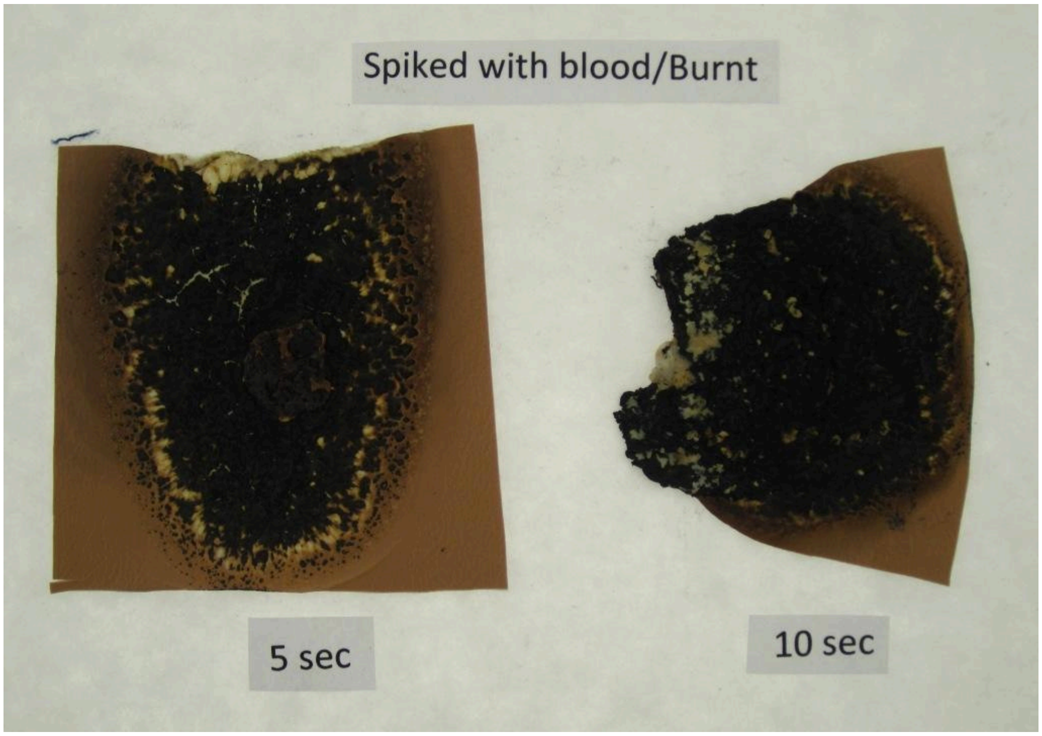

Figure 23: Bloodstains on synthetic leather swatches burnt for 5 and 10 seconds (side exposed to direct flame is pictured)

After 5 seconds under the flame, bloodstains looked black and protruded from the rest of the carpet. These characteristics were also observed in Part I of the study in which blood and carpet went through different physical changes. The black coloration of blood was transferred to the swab, but no interference with the development of phenolphthalein coloration was observed. When carpet was burnt for 10 seconds, the bloodstain changed to an irregular shape, appearing scattered (Figure 24). The change in shape could have been caused by melting of blood as it was exposed for a longer period of time under the flame. In some samples the blood travelled through the carpet and the back of the swatch was swabbed. The carpet fibers in the area surrounding the melted region were easily removed when swabbed. When the backing of the carpet was examined, no physical changes were observed.

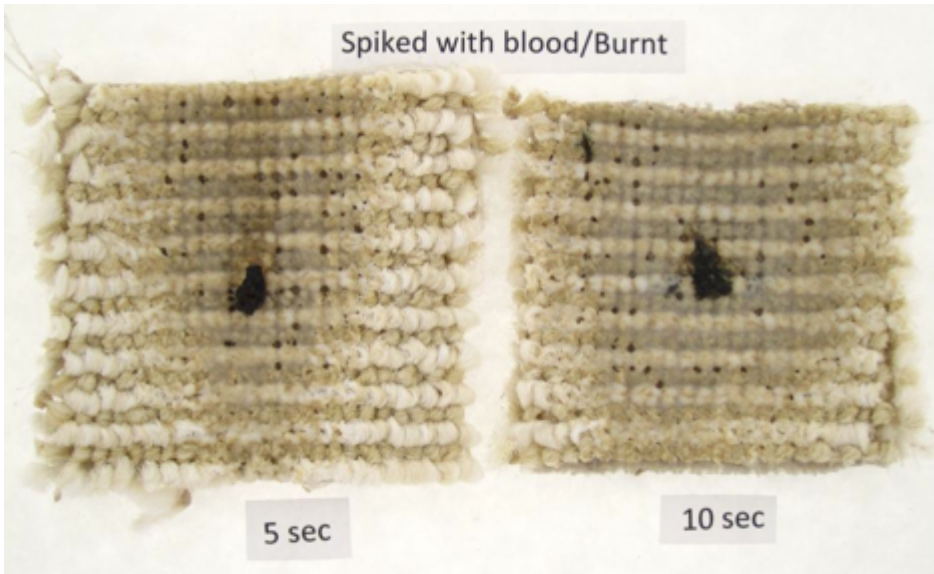

Figure 24: Bloodstains on carpet swatches burnt for 5 and 10 seconds (side exposed to direct flame is pictured)

3.12 Phenolphthalein testing on burnt bloodstains

As the bloodstains on absorbent materials were also observed on the side not exposed to the flame, both sides of the substrate were presumptively tested with phenolphthalein. Results are shown in Table 8.

Substrate	5 seconds (direct contact with flame)	5 seconds (no direct contact with flame)	10 seconds (direct contact with flame)	10 seconds (no direct contact with flame)
Denim	4.33±0.57	2.67±1.15^{*3}	4.67±0.57	8.67±7.37^{*3}
Synthetic Leather	2.67±0.57	NEG	13.67±16.97	NEG
Carpet	4.33±0.57	17±18.38^{*1}	4.67±0.57	2^{*2}

*Table 8: Average time for a positive phenolphthalein reaction for the side in direct contact with flame and the side not in direct contact with flame for 5 and 10 seconds (n=3). Results in seconds, ^{*1}= two swatches visually stained, ^{*2}= one swatch visually stained, *^{3}= all swatches visually stained, NEG= no color reaction at 1 minute*

Results showed that all bloodstains in contact with the flame tested presumptively positive for blood for all the materials. Denim and carpet yielded positive phenolphthalein results on the side exposed to the flame and the side not exposed to direct contact with flame. The average time for a positive reaction was the same for both materials on the side exposed to the flame and burnt for 5 and 10 seconds. Synthetic leather was the only material that showed an increase in time for the phenolphthalein positive reaction to occur as time under the flame increased. Bloodstains burnt for 10 seconds were difficult to visualize so the area thought to contain the stain was swabbed. This poses a problem in forensic cases as biological evidence could be overlooked even when presumptive testing could still be successfully carried out. The back of the synthetic leather swatches gave negative results due to the non-absorbent nature of the substrate. All positive results obtained were within the typical 10-15 second cut-off point except for the side of the carpet that was not in direct contact with the flame for 5 seconds and the synthetic leather sample in direct contact with flame for 10 seconds.

3.13 ABAcard® Hematrace® testing on burnt bloodstains

Denim and carpet provided the best protection for human hemoglobin exposed for 5 and 10 seconds under the flame, yielding positive results (Table 9). Human hemoglobin on synthetic leather was identified on only one sample exposed for 5 seconds under the flame.

Time (secs)	Denim	Synthetic Leather	Carpet
5	3	1	3
10	3	0	3

Table 9: Number of bloodstains yielding positive results using ABAcard® Hematrace® for swatches exposed for 5 and 10 seconds to flame contact (n=3)

Absorbent materials (denim and carpet) gave positive results for all samples exposed for 5 and 10 seconds. The ability to detect human hemoglobin under these harsh conditions may be attributed to the protection provided by the material. For example, when a piece of carpet was cut for extraction, red-brown coloration was observed in between the front and back layer, showing that the top layer protected the layers underneath. Confirmatory results for synthetic leather were negative for the presence of human hemoglobin, except in one sample exposed for 5 seconds under the flame. When comparing these results with the results using phenolphthalein, the latter is a more reliable testing method on burned items than ABAcard® Hematrace®, as it was able to detect blood on all the materials after 5 and 10 seconds of direct flame contact.

A validation study using ABAcard® Hematrace® reported negative results for a bloodstained cotton swatch that was burnt completely to ashes (18). Another study (21) reported that extreme high temperatures could ultimately lead to negative results using ABAcard® Hematrace®, but no details about other factors that could affect the results, like the total time that the item was exposed to fire, the temperature reached or the usage of different substrates was provided.

Tontarski et al. recommend that the practice to only send evidence to further DNA analysis if it gives positive presumptive results is not an adequate approach as their study showed that 64 samples collected from a mock fire scene that reached 297°C gave full DNA profiles, while only 55 of these samples gave positive presumptive results using phenolphthalein/tetramethylbenzidine (P/TMB) (4). Those results show some similarities with this study, as positive results were obtained for all the bloodstains exposed directly to the flame using phenolphthalein.

3.14 Visual characteristics of semen stain burnt for 5 and 10 seconds

As shown in Figure 25, semen stains on the side with direct flame contact were observed as black stains on denim that would be difficult to recognize if the location of the stain was unknown. On the side of the denim not in direct contact with the flame, the semen stains also looked black but fluorescence was observed in some areas (Figure 26 and 27).

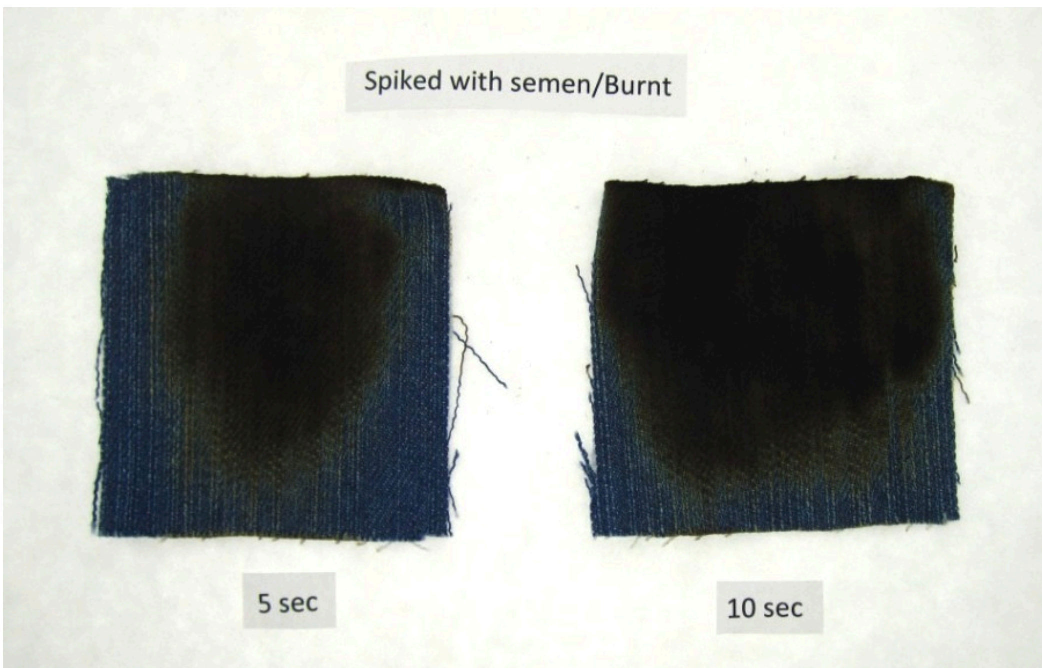

Figure 25: Semen stains on denim swatches burnt for 5 and 10 seconds (side exposed to direct flame is pictured)

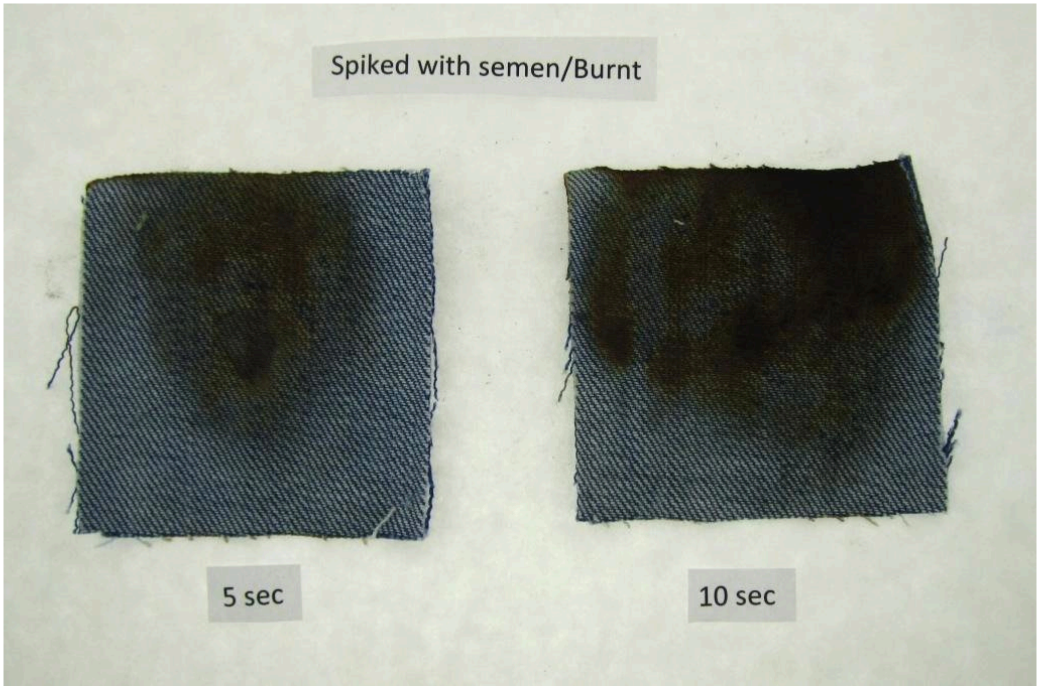

Figure 26: Semen stains on denim swatches burnt for 5 and 10 seconds (side not exposed to direct flame is pictured)

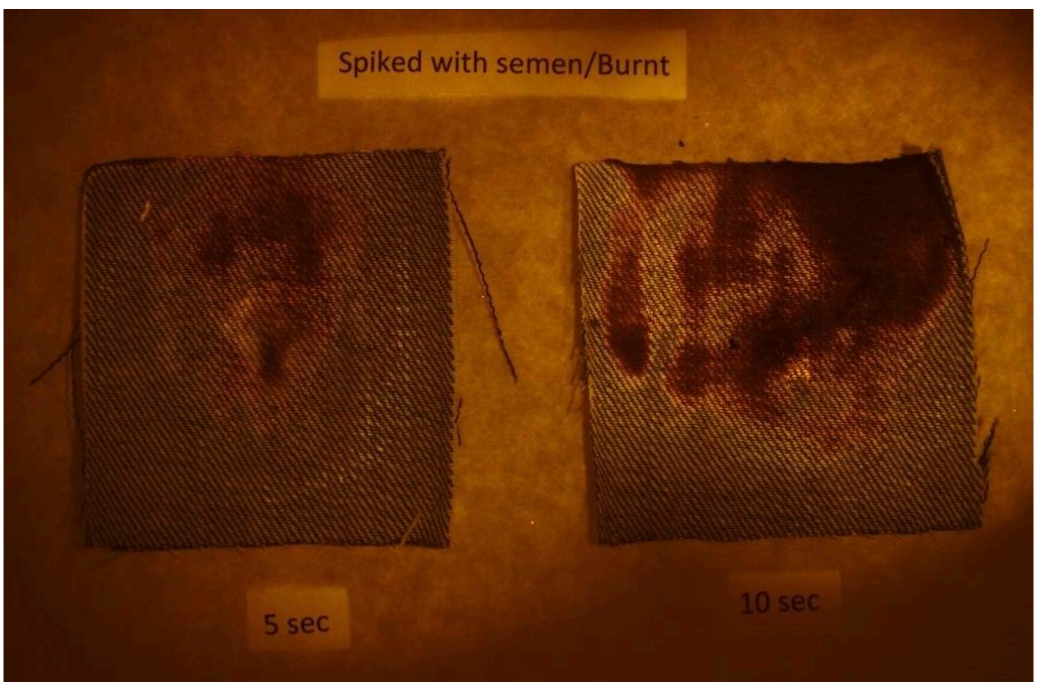

Figure 27: Semen stains on denim swatches burnt for 5 and 10 seconds using Crimelight® with an orange barrier filter (side not exposed to direct flame is pictured)

Semen stains exposed to direct flame contact for 5 seconds could still be visually identified, as the area was circular in shape, and had a different texture from the rest of the charred synthetic leather. Visual identification of semen stains at 10 seconds was not possible. These results were similar to bloodstains on synthetic leather, since the bloodstains could be visualized when exposed to 5 seconds under the flame but not at 10 seconds due to the physical changes that the material undergoes.

Semen stains on carpet exposed to direct flame for 5 seconds and 10 seconds looked very similar in shape but different in coloration. As observed in Part I, semen stains changed to a darker color with increased heat exposure—swatches exposed for 5 seconds appeared light brown and swatches exposed for 10 seconds appeared dark brown (Figure 28). Fluorescence was observed on the side not exposed directly to the flame even when the stain was not identifiable (Figure 29).

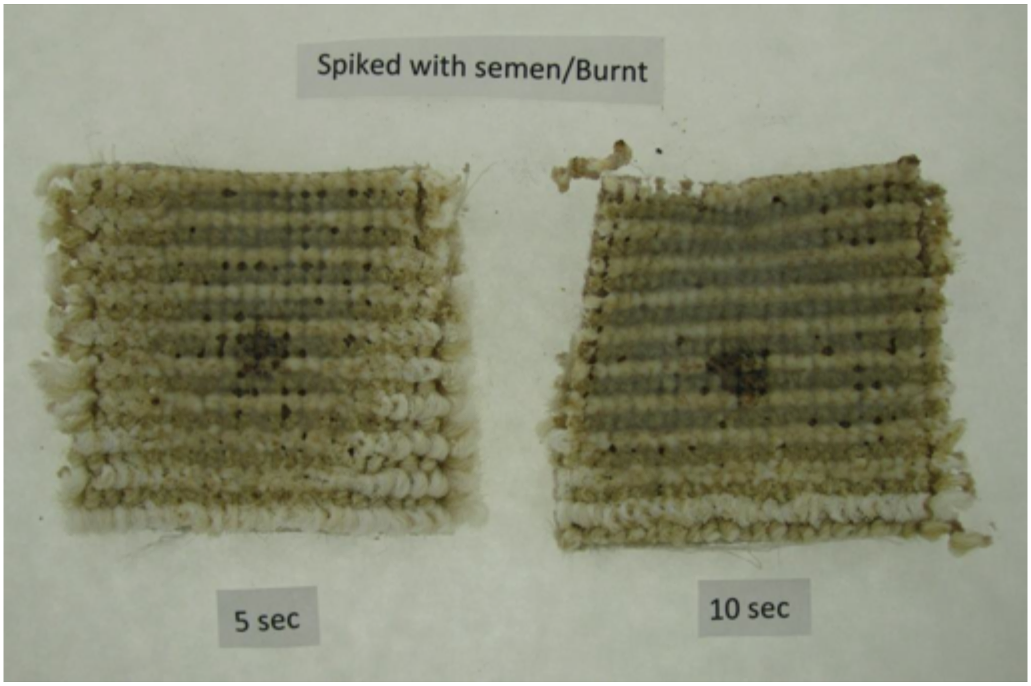

Figure 28: Semen stain on carpet swatches burnt for 5 and 10 seconds (side exposed to direct flame is pictured)

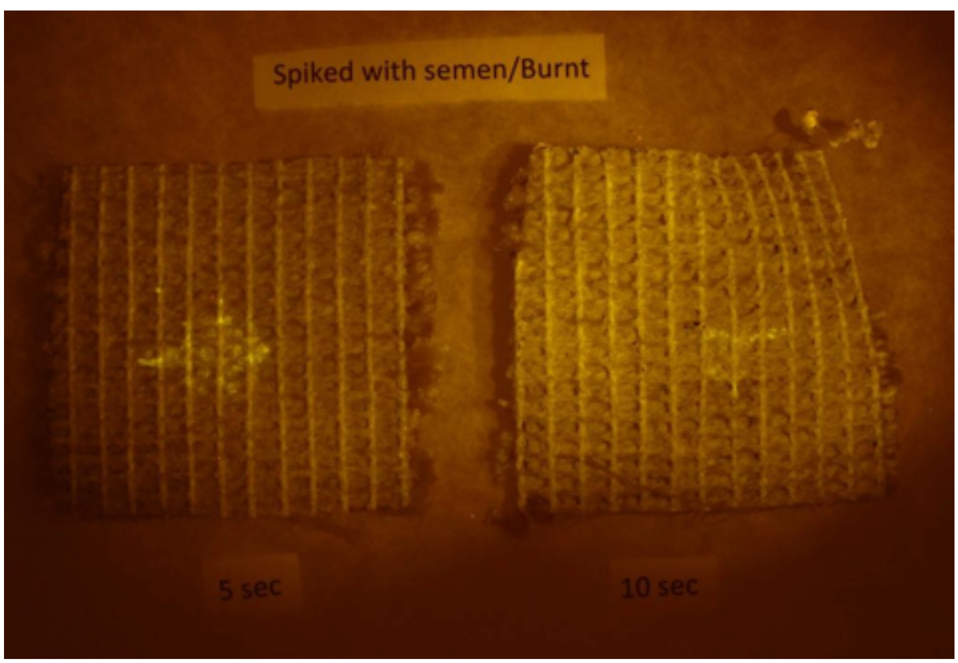

Figure 29: Semen stains on carpet swatches burnt for 5 and 10 seconds using Crimelight® with an orange barrier filter (side not exposed to direct flame is pictured)

A side-by-side comparison between carpet swatches stained with blood and semen exposed for 10 seconds to the direct flame showed a similarity in visual characteristics (Figure 30). This similarity in coloration of semen and blood after being exposed to high temperatures is relevant during evidence collection, as changes in semen coloration must be considered when observing stains exhibiting visual characteristics typical of blood. Fluorescence screening of the back of the carpet may provide useful information to the analyst in these instances.

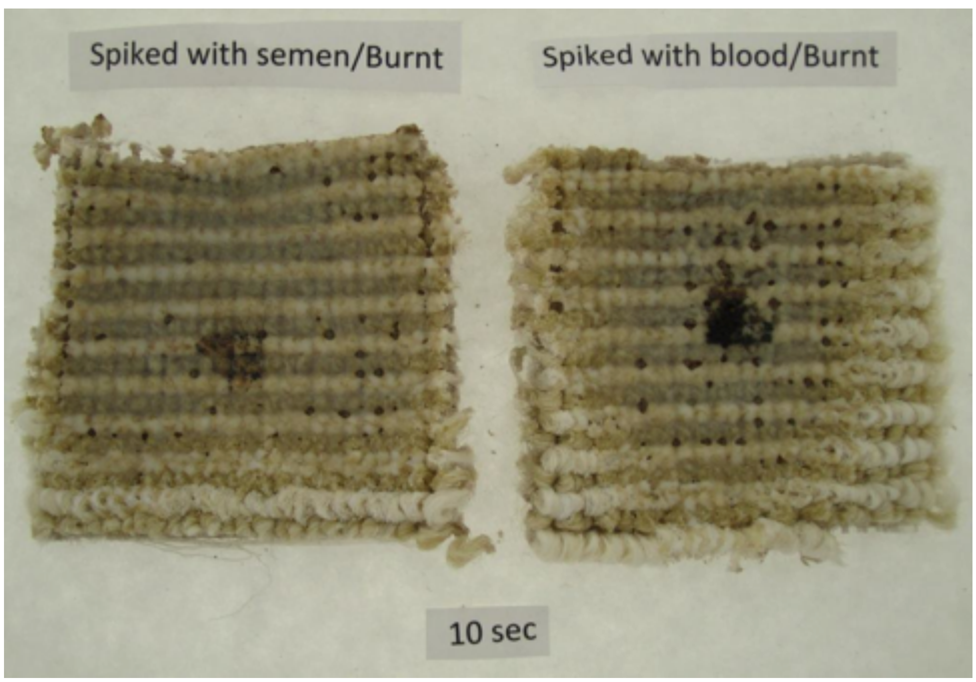

Figure 30: Semen stain (left) and bloodstain (right) on carpet swatches burnt for 10 seconds (side exposed to direct contact to flame is pictured)

3.15 AP Spot testing on burnt semen stains

AP Spot results are recorded in Table 10. All of the positive results for this section were obtained prior to the 5-minute cut-off point for AP Spot test.

Substrate	5 seconds (direct contact with flame)	5 seconds (no direct contact with flame)	10 seconds (direct contact with flame)	10 seconds (no direct contact with flame)
Denim	NEG	NEG	NEG	240*
Synthetic Leather	NEG	NEG	NEG	NEG
Carpet	16±20.81	9±2.65	8.67±2.31	9.33±1.53

*Table 10: Average time for a positive AP Spot reaction for side exposed to direct contact and not direct contact with flame for 5 and 10 seconds (n=3). Results in seconds, *= only one sample tested positive, NEG = no color reaction at 5 minutes*

Table 10 shows the results obtained after semen stains were exposed for 5 and 10 seconds to direct flame on one side. All samples gave negative results for denim except for one sample exposed to 10 seconds of flame, which gave positive results after 240 seconds. This indicates that if semen stains are being screened on this material, searching for them from each side is a better approach, even if it is presumed that the stains were deposited on a certain side of the fabric. Another factor that must be taken into account is that heavy black charring on the swab could potentially obscure the development of a weak purple coloration.

Semen stains on synthetic leather gave negative results for the presence of AP when exposed to direct flame for 5 and 10 seconds.

Semen stains on carpet gave positive results for the presumptive identification of semen on both sides tested. Semen fluorescence was observed on only the side not in direct flame contact on the carpet swatches, as shown in Figure 29. Due to this, it can be assumed that the thick fibers on the front side protected the backside from denaturing the AP and the fluorescence component. This indicates that the screening of semen stains from the side not exposed to direct flame contact may sometimes be a more successful approach. Although, if semen does not absorb through the carpet, the screening of the opposite side will not provide any results.

3.16 Microscopic identification of spermatozoa on burnt semen stains

Carpet was the only material in which spermatozoa were identified at 5 and 10 seconds. Synthetic leather gave negative results for the presence of spermatozoa and Sg.

	Denim		Synthetic leather		Carpet	
Time (secs)	Microscopic identification	RSID™-Semen	Microscopic identification	RSID™-Semen	Microscopic identification	RSID™-Semen
5	0	3	0	0	3	3
10	0	3	0	0	3	3

Table 11: Number of semen stains yielding positive results for microscopic identification of spermatozoa and RSID™- Semen for denim, synthetic leather and carpet exposed for 5 and 10 seconds to flame contact (n=3)

Results in Table 11 show that spermatozoa could be identified only on carpet; this is attributed to the thickness of the material providing more protection from the flame than denim or synthetic leather, as it did with AP and fluorescence. Also the flame resistant properties of carpet might have provided further protection, impeding spermatozoa degradation. The extract from each substrate exhibited a particular coloration after being exposed to direct contact with flames: yellowish brown for denim, black for synthetic leather due to the charred material and colorless for carpet. As observed in Figure 31, the discoloration of the liquid extract did not interfere with microscopic observation.

Due to the spermatozoa being easily found on carpet, more research should be performed on similar substrates to understand how long a semen stain can be exposed to a flame contact before microscopic identification of spermatozoa is no longer possible. It is likely that most of the spermatozoa detected were recovered from the lower layers of the carpet that were in less contact with the flame, and future studies could use a sequential sampling technique to help determine this.

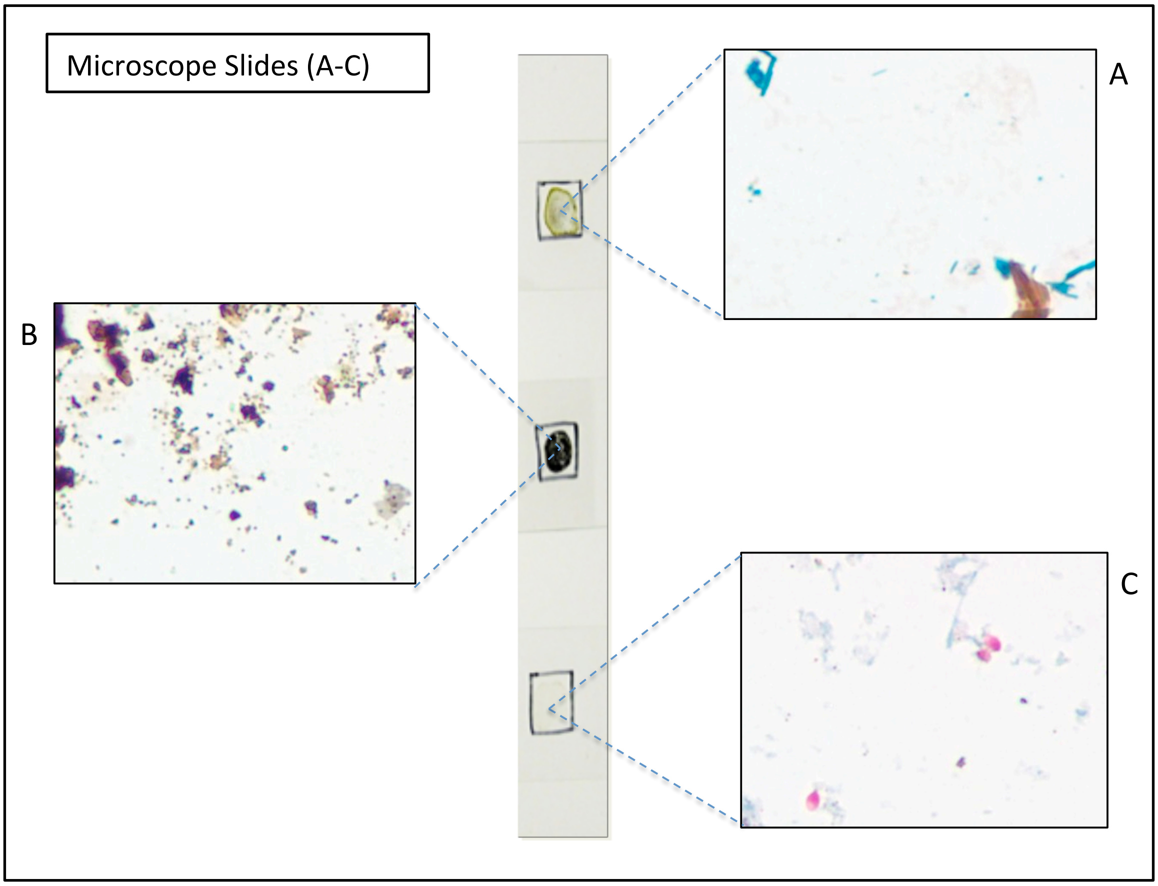

Figure 31: Denim (A), Synthetic leather (B), Carpet (C) extraction burnt for 10 seconds observed at 400x stained using KPIC

3.17 *RSID™- Semen testing on burnt semen stains*

The yellow extract obtained from denim was absorbed through the test strip without inhibiting the visualization of the red bands. For the synthetic leather extract, the black particles stayed in the sample well, and as with the carpet extract, the development of the red band on the immunochromatographic card was easy to visualize.

As shown in Table 11, RSID™- Semen results for denim and carpet were positive for the presence of human Sg which confirms that absorbance does offer some protection to the antigen when exposed for 5 and 10 seconds, though it must be kept in mind that these samples were exposed to direct contact with flame for a very brief period of time. To fully understand up to what point RSID™-Semen can detect the presence of Sg, samples should be exposed for longer periods of time than those tested in this study. However, no further testing is required for synthetic leather, as all samples gave negative results for the presence of Sg at 5 and 10 seconds.

4. Conclusions

When bloodstains were exposed to high temperatures, their detection using phenolphthalein was greatest on synthetic leather when compared to denim and carpet. It was determined that scraping is a better collection method than swabbing to test samples exposed to higher temperatures, and that the 10-15 second cut-off for phenolphthalein needs to be reconsidered (extended) when testing fire scene evidence in order to prevent the occurrence of false negatives. Detection of human hemoglobin using ABAcard® Hematrace® was not possible after 1 hour at 130°C, making it a less sensitive test for bloodstains exposed to high temperatures than phenolphthalein.

Identification of AP in semen stains was more difficult on denim and carpet, and positive results were not obtained after 7 hours at 170°C. Semen stains collected from synthetic leather yielded better results at the highest oven temperatures and longest exposure times. ALS fluorescence was visible on denim and synthetic leather but not for carpet due to the melting fibers that covered the stain and prevented the light from reaching it. Microscopic identification of spermatozoa was most successful on stains collected from denim; in some instances, spermatozoa were observed when AP detection was negative. For synthetic leather and carpet, the identification of spermatozoa wasn't as successful as AP. Microscopy staining time should be extended to at least 25 minutes because spermatozoa appeared to require a longer absorption time for NFR after semen stains on denim had been exposed to high temperatures. Sg recovery from carpet was successful up to the maximum time (24 hours) and temperature (190°C) inside the oven, and Sg testing is recommended for evidence recovered from fire scenes because AP and spermatozoa were not detected at temperatures higher than 170°C.

When bloodstains were exposed to direct contact with a flame, phenolphthalein was an appropriate presumptive test for the three materials tested. Identification of human hemoglobin using ABAcard® Hematrace® yielded better results on the absorbent materials than on the non-absorbent synthetic leather.

Presumptive identification of semen using AP Spot test was a challenge on burnt denim and synthetic leather because negative results were obtained except for one denim sample exposed to a flame for 10 seconds. On carpet, AP Spot test was an appropriate method for samples exposed to 5 and 10 seconds of direct flame contact. During this part of the study, screening and testing of the backside of the swatch was included, showing that positive results could be obtained from testing the reverse side of the substrate from which the stain was deposited if the reverse had not been in contact with the flame. Confirmatory testing (microscopic identification of spermatozoa and RSID™- Semen) of semen stains on carpet was successful, indicating that this material was better at protecting seminal constituents when semen is exposed to 5 or 10 seconds of direct contact with flame.

Future directions of this research include a further analysis of how long blood and semen stains can be exposed to a flame contact and still be identified using presumptive or confirmatory methods. In arson cases, perpetrators may use an ignitable liquid; that is why it would be relevant to investigate how the addition of an accelerant would affect the analysis of blood and semen stains.

In summary, forensic scientists must be aware that blood and semen detection on burnt or heat-exposed items is possible, even when the stains appear discolored or are masked by soot/charred material. Presumptive and confirmatory assays currently in use by forensic laboratories can be used to successfully test evidence samples exposed to high heat and flame contact in some cases. This knowledge may lead to increased detection of biological fluids on fire scene evidence.

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