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Author: Jack Ballantyne, Ph.D.

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Determination of the Age (Time since Deposition) of a Biological Stain

FINAL REPORT

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Principal Investigator:
Jack Ballantyne, PhD
Associate Professor
Department of Chemistry
Associate Director for Research
National Center for Forensic Science
4000 Central Florida Blvd, Bldg #5
University of Central Florida
Orlando, FL 32816-2366
Phone: (407) 823 4440
Fax: (407) 823 2252
e-mail: jballant@mail.ucf.edu

EXECUTIVE SUMMARY

1. With current technologies, a significant amount of genetic information can be obtained from an individual biological stain found at a crime scene. Not only is it possible to routinely obtain a DNA profile of the donor of the stain but also new methods are being developed to allow for determination of the body fluid of origin using PCR based methods rather than crude serological testing, and also physical characteristics including eye color, hair color, skin pigmentation, height, weight, and relative age. The probative information obtained from these samples can aid law enforcement investigators in cases where there are no known suspects. With the currently available technologies in molecular biochemistry it is becoming possible to produce a “genetic eyewitness” that is not constrained by human recollection or subjective accounts. The information obtained from biological stains can potentially provide an unbiased genetic description of the perpetrator when no true eyewitnesses are available. However, even with the wealth of new information being obtained from biological stains, the possibility for further characterization of biological stains exists. The ability to determine the relative time since deposition (TSD) of biological stains will provide law enforcement investigators with novel probative evidence by establishing an approximation of the time of commission of criminal offenses. While a small number of methods for the determination of time since deposition have been proposed, none of these methods have been widely accepted due to poor analytical sensitivity (large sample consumption) and inadequate resolution between stain ages.

2. In the work described herein we examined changes to hemoglobin spectral profiles, changes in enzymatic activity and degradation to RNA in order to determine the time since deposition (TSD) or the age of dried bloodstains. We sought to identify novel “molecular estimators” that would allow investigators to more accurately assess the time since deposition of dried bloodstains. The assays developed in this work were designed to accommodate low template samples in order to demonstrate their suitability for use in forensic casework.

3. The specific aims of the project were as follows:

Aim 1. Provide an accurate estimate of TSD of a biological stain by assessment of nucleic acid degradation and oxidative base damage?

Aim 1A (i) Correlate DNA degradation and oxidative base damage with TSD

Aim 1A (II) Effects of the environment (heat, light, relative humidity, and microbial growth) on DNA-based TSD estimation

Aim 1B (i) Correlate mRNA degradation and oxidative base damage with TSD

Aim 1B (ii) Effects of the environment (heat, light, relative humidity and microbial growth) on RNA-based TSD estimation

Aim 2. Provide an accurate estimate of TSD by assessment of the UV-visible spectrum of hemoglobin?

Aim 2A Correlate TSD and UV-visible spectrophotometric properties of hemoglobin from bloodstains

Aim 2B Effects of the environment (heat, light, relative humidity and microbial growth) on UV-Visible spectroscopy-based TSD estimation

Aim 3. Provide an accurate estimate of TSD by assessment of loss of enzyme activity in dried bloodstains

4. We examined the UV-Visible spectral profile of hemoglobin to identify possible regions of interest where noticeable differences between aged and fresh stains were present. No significant differences were observed in the UV region of the spectra. However, several areas of interest were observed in the visible region of the spectra and included the following:

- a. $\Delta\text{Abs}_{\text{Soret}(\sim 414\text{nm})}$ – a possible decrease in the Abs maximum.
- b. $\Delta\lambda_{\text{Soret}}$ – a possible shift to short wavelength (hypsochromic shift).
- c. $\Delta\text{Abs}_{\beta(541-560\text{nm})}$ - a possible decrease in the Abs maximum of the β peak.
- d. $\Delta\text{Abs}_{\alpha(576-560\text{nm})}$ - a possible decrease in the Abs maximum of the α peak.
- e. $\Delta\text{Abs}_{\alpha(576-560\text{nm})} / \Delta\text{Abs}_{\beta(541-560\text{nm})}$ - a possible relationship between the ratio of the changes in the absorption maxima of the α and β peaks.

5. An initial evaluation of changes in the absorption maxima of the Soret band and the α and β peaks indicated a possible correlation between the age of the stain and absorption maxima. However, months to year differences in stain age were needed in order to identify a possible correlation. Significant intra- and inter- sample variation was also observed. The use of the ratio of absorption maxima changes ($\Delta\text{Abs}_{\alpha(576-560\text{nm})} / \Delta\text{Abs}_{\beta(541-560\text{nm})}$) allowed for a stronger correlation with the age of the stain to be developed. However, months to year differences in stain age were still needed. Therefore, the use of

changes in absorption maxima of the characteristic hemoglobin spectral bands Soret_{414nm}, β _{576nm}, α _{541nm}) did not provide sufficient resolution to be useful in forensic casework.

6. A previously unidentified hypsochromic shift (shift to shorter wavelengths) of the hemoglobin Soret band was observed that demonstrated a high correlation to the time since deposition of dried bloodstains. The extent of this shift permits a distinction to be made between stains that were deposited minutes, hours, days and months prior to analysis. This method therefore provides a higher resolution than any previously developed TSD methodology. The sensitivity of this method has been determined and as little as the equivalent of 20-40 nanoliters of blood can be used from bloodstains as small as 0.5 μ l. The effects of temperature and humidity have also been evaluated.

7. For the hypsochromic shift methodology to be of greater use in forensic casework, the ability to perform such measurements at the scene would be useful. This would allow investigators to identify if a stain was blood and possibly how old the stain was on-site. Recently, a portable spectrophotometer has become commercially available from Implen called the NanoPhotometerTM. The instrument has no moving parts making it more durable during transport to a crime scene and weighs less than 10 lbs. This instrument was evaluated for use in collecting hemoglobin spectral data and its accuracy was compared to the data collected using the other non-portable spectrophotometers used in this study. Initial results demonstrate a potential future use of the NanoPhotometerTM for the “on-site” sample-to-answer determination of the age of the stain. Further optimization of this instrument is needed to determine optimal sample volumes and concentrations.

8. We determined if enzymatic activity, and possible loss of activity as the age of the stain increased, could be detected in dried biological stains. Initial work involved the use of dried bloodstains. However, unlike the Soret band hypsochromic shift method, the use of enzymatic activity changes offers the advantage of the ability to determine the time since deposition of other non-blood biological fluids. Over 30 enzyme candidates were initially evaluated. Enzyme activity was measured using simple colorimetric activity assays performed in a 96-well plate. Differing levels of activity and differing rates of activity loss were observed for six enzymes candidates (lactate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, glycerate dehydrogenase, alcohol dehydrogenase and phosphogluconate dehydrogenase) as the age of the stain increased. The initial data demonstrated that an enzymatic activity profile for an unknown bloodstain could be obtained and utilized to determine the time since deposition. Further work would be needed to identify additional enzyme candidates, as the current candidates do not provide sufficient resolution (month-year differences in stain age).

9. We were unable to demonstrate a correlation between messenger RNA degradation and the time since deposition of dried bloodstains using a variety of different analytical approaches.

ABSTRACT

The ability to determine the time since deposition of a biological stain found at a crime scene could prove invaluable to investigators, defining the time frame in which the individual depositing the evidence was present. Although various methods to accomplish this in bloodstain have been proposed in the past, none has gained widespread use in the forensic community due primarily to limitations in the predictive power of the test and poor analytical sensitivities. However molecular correlates of stain age probably exist and perhaps re-visiting the problem with more sensitive and accurate methods would be worthwhile.

The fundamental assumption of this work was that biochemical reactions still occur in the dried state and as dried stains age, damage and degradation to macromolecules such as DNA, RNA and protein occur. We sought to investigate whether macromolecular degradation intermediates and/or specific nucleic acid base damage could serve as molecular clocks for time since deposition (TSD) estimation. DNA and RNA exhibit varying degrees of stability and their degradation over time is manifested by a reduction in the number average molecular weight. Specific oxidative damage to the purine and pyrimidine bases is well characterized in DNA, and more recently, has been observed in RNA. The original goal of this study was to investigate whether degradation and oxidative damage to DNA and mRNA in bloodstains can be correlated with TSD and whether this correlation was sufficiently robust to be useful for routine use. The general concept was that although nucleic acid degradation intermediates and the subtle oxidative changes would be more representative of longer and shorter time spans respectively, fine resolution estimates of TSD may be possible by a combination of both. Initial work was

focused on an examination of degradation to mRNA in dried bloodstains with attempts to measure a progressive increase in the amount of degradation to determine the time since deposition. However, all RNA-based methods provided inconsistent results and were therefore unsuccessful.

The remainder of the work was focused on an examination of spectral changes to proteins, namely hemoglobin, and an examination of changes in enzyme activity as the age of the stain increased. A previously unidentified hypsochromic shift (shift to shorter wavelengths) of the Soret band of hemoglobin was observed which allowed for bloodstains differing in age by minutes, hours, days, weeks and months to be distinguished. The resolution and sensitivity of this method make it particularly suited for use with forensic casework bloodstains. Additionally, the loss of activity for six enzyme candidates was used to determine the age of dried bloodstains. While this method required month differences in stain age, it does provide initial support for the use of changes in enzyme activity to determine the age of a stain. It is possible that additional enzyme candidates could be identified to improve the resolution of this method.

The use of the novel methodologies described in this report could be used to provide investigators with novel probative information from a crime scene stain, namely the time since deposition of a bloodstain.

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CHAPTER 1: INTRODUCTION

With current technologies in forensic science, a significant amount of genetic information can be obtained from an individual biological stain found at a crime scene. Not only is it possible to routinely obtain a DNA profile of the donor of the stain, but new methods are being developed to determine body fluid origin using RNA based methods (rather than conventional serological testing), and also to predict physical characteristics including eye color, hair color, skin pigmentation, height, weight, and relative age [1-28]. The probative information obtained from these samples can aid law enforcement investigators in cases where there are no known suspects. With the currently available technologies in molecular biochemistry it is, in theory, becoming possible to produce a “genetic eyewitness” that is not constrained by human recollection or subjective accounts. The information obtained from biological stains can potentially provide an unbiased genetic description of the perpetrator when no true eyewitnesses are available.

However, even with the wealth of new information being obtained from biological stains, the possibility for further characterization of biological stains exists. The ability to determine the relative time since deposition (TSD) of biological stains will provide law enforcement investigators with novel probative evidence by establishing an approximation of the time of commission of criminal offenses. The establishment of a time line of events in criminal offenses is often limited to eyewitness or victim accounts. If the crime involves murder it is possible to determine an approximate time of death, and therefore time of commission of the criminal offense, if a body is found at the crime scene. However, many criminal investigations do not include eyewitnesses or bodies for time of commission determinations. Evidence found at crime scenes is often limited to

dried biological stains or tissues. Currently, few reliable and accurate methods exist to approximate the time of deposition of these dried biological stains. The few studies that have been conducted previously have relied solely on measurements of the degradation to macromolecules, or a progressive reduction in molecular weight [29-35].

Early methods to determine an approximate age of a stain focused on deterioration of the visible spectral patterns for hemoglobin in bloodstains. One such method used to determine approximate age of a bloodstain involved obtaining HPLC chromatographs for measurement of α -chain area to heme area [31]. With this method, a linear decrease in the α -chain area/heme area was observed, on a logarithmic scale, as stain age increased. In a subsequent study, a peak designated as "X" was detected only in aged stains, and the area of this peak increased as the age of the stain increased [36]. Various other studies have utilized HPLC analysis of hemoglobin to determine the age of bloodstains [37-39]. While these studies all produce a linear relationship with the age of a stain and the deterioration of hemoglobin, they are limited in methodology and applicability to other body fluids. No studies have examined whole protein populations within bloodstains or other body fluids to determine the extent of degradation to proteins in dried biological stains. This could be accomplished through the use of one-and two-dimensional SDS-PAGE electrophoresis to look for degradation products that may occur only after a specific time since deposition. In addition, no studies have included UV spectral analysis of whole protein populations to again look for more significant and characteristic degradation patterns correlating to the age of a stain.

More recent studies involving attempts to determine approximate age of biological stains have examined RNA degradation, using both mRNA and rRNA [29,40].

One such study utilized a semi-quantitative duplex and competitive real-time PC method to estimate approximate age of bloodstains, with an assumption that degradation to mRNA occurs from the 5' end. However, this study provides no attempt to investigate mRNA degradation in dried biological stains in order to verify that this assumption is correct, thus validating their proposed method. It was reported in this study that a difference of 4-5 years between samples in order to detect significant differences in RNA. The estimations produced using this method are too large to be forensically useful. Another study examined ratios of β -actin mRNA and 18S rRNA as a function of time using real-time PCR. While this method has a greater applicability in that it is more compatible with the current capabilities of most crime laboratories, the analysis is again limited to only bloodstains and produces only crude time estimates, examining samples only every four weeks. This study is rudimentary in its approach, in that it only measures ratios of β -actin and 18S rRNA using the Ct value from RT-PCR. It provides no analysis detailing the extent or type of degradation occurring to the RNA species that may prove useful in obtaining a more accurate estimation of time of deposition.

These studies, while limited in their approaches, methodologies, and applicability to current capabilities of forensic crime laboratories, have established a necessity for future research in order to more accurately determine the time of deposition of forensic biological stains. The approach taken for this current project included a more comprehensive assessment of the biological processes occurring in dried biological stains in order to produce novel methods of estimating approximate age of stains through examination of degradation and damage occurring to all macromolecules: DNA, RNA, and proteins.

Numerous studies have been conducted that demonstrate the ability to obtain genetic information from samples stored under various conditions for considerable amounts of time [41-43]. However, no studies have attempted to quantify the amount of degradation to DNA or the amount of damage to DNA in dried biological stains in an attempt to establish a correlation with age of a stain. In addition to DNA, no studies have critically examined the specific mechanism of degradation to RNA in dried stains, nor offered any information on the extent or quantity of degradation. This study included an assessment of the mechanism of RNA degradation (5'-end vs. 3'-end) and the extent of degradation to tissue specific RNA molecules by measurement of truncated products in stains of various ages. Damage to RNA molecules may occur at a much higher rate than that found in DNA due to the fact that RNA exists in a single-stranded form and is not associated with as many proteins. In dried biological stains, it may be possible to observe an accumulation of RNA damage as the age of stain, or time since deposition, increases. Another purpose of the current study was also to provide a more thorough evaluation of degradation to proteins in biological stains. Thus we took a unique approach to trying to more accurately determine the time of deposition of biological stains by examining the degradation of multiple types of macromolecules (nucleic acids and proteins).

CHAPTER 2: MATERIALS AND METHODS

Preparation of Body Fluid Stains

Body fluids were collected from volunteers using procedures approved by the University of Central Florida's Institutional Review Board. Informed written consent was obtained from each donor. Blood samples were collected by venipuncture into additive-free vacutainers and 50 μ l aliquots were placed onto non-sterile cotton cloth. The bloodstains were stored at various temperatures including room temperature (22°C, 50% humidity) and 37°C for varying lengths of time (15 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 12-18 hours, 24 hours, 48 hours, 1 week, 1 month, 3 months, 6 months, 1 year, and 2 years).

Environmental Samples

50 μ l aliquots of human blood were dried onto cotton cloth. These samples were exposed to different environmental influences including heat, light, humidity and rain. Two sets of bloodstains were placed outside. One of the sets was placed outside and covered exposed to heat, light and humidity (HLH), and the other set was placed outside and uncovered exposed to heat, light, humidity and rain (HLHR). Samples from both sets of conditions were collected at varying lengths of time.

Humidity Studies

Blood samples were collected by venipuncture into additive-free vacutainers and 50 μ l aliquots were placed onto non-sterile cotton cloth. The bloodstains were placed in a MicroClimate[®] Humidity Chamber MCH-3 (Cincinnati Sub-Zero, Cincinnati, OH) at

22°C and both 50% and 75% humidity for one week. Samples were collected at the following intervals: 15 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 18 hours, 24 hours, 48 hours and 1 week.

RNA Isolation

Total RNA was extracted from blood, semen, saliva, vaginal secretions and menstrual blood with guanidine isothiocyanate-phenol:chloroform and precipitated with isopropanol. Briefly, 500 µl of pre-heated (56°C for 10 minutes) denaturing solution (4M guanidine isothiocyanate, 0.02M sodium citrate, 0.5% sarkosyl, 0.1M β-mercaptoethanol) was added to a 1.5mL Safe Lock tube extraction tube (Eppendorf, Westbury, NY) containing the stain or swab. The samples were incubated at 56°C for 30 minutes. The swab or stain pieces were then placed into a DNA IQ™ spin basket (Promega, Madison, WI), re-inserted back into the original extraction tube, and centrifuged at 14,000 rpm (16,000 x g) for 5 minutes. After centrifugation, the basket with swab/stain pieces was discarded. To each extract the following was added: 50 µl 2 M sodium acetate and 600 µl acid phenol:chloroform (5:1), pH 4.5 (Applied Biosystems/Ambion). The samples were placed at 4°C for 30 minutes to separate the layers and then centrifuged for 20 minutes at 14,000 rpm (16,000 x g). The RNA-containing top aqueous layer was transferred to a new 1.5ml microcentrifuge tube, to which 2 µl of GlycoBlue™ glycogen carrier (Applied Biosystems/Ambion) and 500 µl of isopropanol were added. RNA was precipitated for 1 hour at -20°C. The extracts were then centrifuged at 14,000 rpm (16,000 x g). The supernatant was removed and the pellet was washed with 900 µl of 75% ethanol/25% DEPC-treated water. Following a centrifugation for 10 minutes at 14,000 rpm (16,000 x

g), the supernatant was removed and the pellet dried using vacuum centrifugation (56°C) for 3 minutes. Twenty microliters of pre-heated (60°C for 5 minutes) *RNAsecure*TM solution (Applied Biosystems/Ambion) was added to each sample followed by an incubation at 60°C for 10 minutes. Samples were used immediately or stored at -20°C until needed.

DNase I digestion

Six units of *TURBO*TM DNase I (2U/μl) (Applied Biosystems/Ambion, Inc.) and 2.2 μl of Turbo DNase I Buffer (10X) were added to each RNA extract and incubated at 37°C for 1 hour. The DNase was inactivated at 75°C for 10 minutes. The samples were used immediately or stored at -20°C until needed. Alternatively, DNase digestion was performed using the *Turbo DNA-free*TM kit (Applied Biosystems/Ambion) according to the manufacturer's protocol.

RNA quantitation

RNA extracts were quantitated with *Quant-iT*TM *RiboGreen*[®] RNA Kit (Invitrogen, Carlsbad, CA) as previously described. Fluorescence was determined using a *Synergy*TM 2 Multi-Mode microplate reader (BioTek Instruments, Inc., Winooski, VT).

cDNA Synthesis

For the reverse transcriptase (RT) reaction, the *miScript Reverse Transcription Kit* (Qiagen, Valencia, CA) was used according to manufacturer's protocols. One nanogram of total RNA from blood, semen, vaginal secretions and menstrual blood

extracts and 5 ng of total RNA from semen extracts were used in the RT reactions. A reverse transcription negative reaction (containing total RNA and reaction buffer but no reverse transcriptase enzyme mix) was performed for each sample.

Polymerase chain reaction

A volume of 2 μ l of the RT-reaction was amplified in a final reaction volume of 25 μ l. The reaction mixture included buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5mM MgCl₂) (Applied Biosystems, Foster City, CA), 0.125 mM each dNTP (applied Biosystems), 0.8 μ M PCR primer (Invitrogen, Grand Island, NY), and 1.25 units AmpliTaq Gold[®] DNA Polymerase (5U/ μ l). PCR conditions consisted of a denaturing step (95°C 11 min) followed by 35 cycles (94°C 20 sec; 55°C 30 sec; 72°C 40 sec) and a final extension step (72°C 5 min).

Gel Electrophoresis

Amplified RT-PCR products were separated on 2.5% NuSieve agarose gels (Cambrex, Rockland, ME). Electrophoresis was carried out at 100 volts for 60 minutes in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA). The gel was stained with SYBR[®] Gold nucleic acid stain (Molecular Probes) and photographed under UV transillumination.

Protein extraction

UV-Visible spectroscopy

Approximately a 50 μ l bloodstain was placed in a 1.5mL microcentrifuge tube with 750 μ l of 0.2M Tris-HCl, pH 8.0. The samples were allowed to extract overnight at

room temperature (protected from light). After the overnight incubation, the stain pieces were placed in a spin basket and the samples were centrifuged at 14,000 rpm (16,000 x g) for 3 minutes. The stain pieces and basket were then discarded. All extracts were stored at -20°C until needed.

Enzyme Activity

Approximately one-half a 50 µl bloodstain was placed in a 1.5mL microcentrifuge tube with 500 µl of appropriate buffer (see enzyme activity section below). The samples were allowed to extract at room temperature for 30 minutes (protected from light). After incubation, the stain pieces were placed in a spin basket and the samples were centrifuged at 14,000 rpm (16,000 x g) for 3 minutes. The stain pieces and basket were then discarded. All extracts were stored at -20°C until needed.

Protein Quantitation

All bloodstain extracts were quantitated using the Quant-It™ Protein Assay Kit according to manufacturer's recommended conditions. The quantitation was performed using a Synergy 2 Microplate Reader (BioTek, Winooski, VT). All samples were run in duplicate and an average of the two measurements obtained.

UV-Visible Spectroscopy

Microplate Reader

UV-Visible spectral profiles were obtained using a Synergy 2 Microplate Reader. Spectral data was collected from 200-700nm in 1nm increments. Samples were run in a

clear, flat-bottomed 96-well reaction plate using 7.5 μg of total protein and brought to a final reaction volume of 75 μl per well using 0.2M Tris-HCl, pH 8.0. All spectral data was blank corrected using 75 μl of 0.2M Tris-HCl. All data was run in triplicate and an average of the data was used in subsequent analysis.

Standard Bench-Top Spectrophotometer

UV-Visible spectral profiles were also obtained using a U-0080D Photodiode Array Spectrophotometer (Hitachi, Pleasanton, CA). Spectral data was collected from 200-700nm in 1 nm increments using a 5 μl cell (7.5 μg total protein used for analysis). All spectral data was blank corrected using 5 μl of 0.2M Tris-HCl. All data was run in triplicate and an average of the data was used in subsequent analysis.

Portable "Point-of-Use" Spectrophotometer

UV-Visible spectral profiles were also obtained using the portable NanoPhotomerTM (Implen, Inc., c/o LABREPCO, Horsman, PA). Spectral data was collected from 350-600nm in 1 nm increments. A 1 μl aliquot of the bloodstain extracts was added directly to the spectrophotometer for analysis. All data was run in triplicate and an average of the data was used in subsequent analysis.

Enzyme Activity Assays (MTT-PMS colorimetric assays)

All assays were run on the Synergy 2 Microplate Reader using a kinetic absorbance program: 3 minutes hold, absorbance read: 565nm for 30 minutes in 1 minute intervals. Each reaction was run in duplicate and the average maximum velocity

of each reaction was obtained. Two negative controls, including a no-substrate control and a no-NAD/NADP control, were run with each assay in order to ensure that the measured activity was resulting from the enzyme itself and not other non-specific interaction between reaction components. All enzyme assays were performed in 200 μ l reactions using 3 μ g of total protein, 7 mM MTT and 26 mM PMS. The reaction mixtures for each enzyme assay were prepared as follows: *Lactate dehydrogenase* – 16 mM calcium lactate, 15 mM NAD in 0.05M Tris-HCl; *Malate dehydrogenase* – 100 mM L-malic acid, 15 mM NAD in 0.1M Tris-HCl; *Alcohol dehydrogenase* – 60% Absolute ethanol, 15 mM NAD in 0.05M Tris-HCl; *Glycerate dehydrogenase* – 160 mM DL-Glyceric acid hemicalcium salt hydrate, 15 mM NAD in 0.1M Tris-HCl; *3-hydroxybutyrate dehydrogenase* – 150 mM (\pm) Sodium 3-hydroxybutyrate, 50 mM $MgCl_2$, 300 mM NaCl in 0.1M Tris-HCl; *Glucose-6-phosphate dehydrogenase* – 16 mM D-Glucose 6-phosphate disodium salt hydrate, 25 mM $MgCl_2$, 4 mM NADP in 0.2M Tris-HCl; *Phosphogluconate dehydrogenase* – 24 mM 6-Phosphogluconic acid trisodium salt, 30 mM $MgCl_2$, 4 mM NADP in 0.5M Tris-HCl; *Isocitrate dehydrogenase* – 38 mM DL-Isocitric acid trisodium salt, 30 mM $MgCl_2$, 4 mM NADP in 0.5M Tris-HCl; *Phosphoglucomutase* – 30 mM glucose-1-phosphate dipotassium salt hydrate, 2U glucose-6-phosphate dehydrogenase, 30 mM $MgCl_2$, 4 mM NADP in 0.05M Tris-HCl; *Gluconate dehydrogenase* – 15 mM 6-phosphogluconic acid, 30 mM $MgCl_2$, 4 mM NADP in 0.2M Tris-HCl; *L-xyulose reductase* – 33 mM xylitol, 4 mM NADP in 0.5M Tris-HCl. All reagents used in the enzyme assays were obtained from Sigma-Aldrich (St. Louis, MO), except for NADP which was obtained from USB Corporation (Cleveland, OH).

CHAPTER 3: RESULTS AND DISCUSSION

Aim I: Assessment of nucleic acid degradation and oxidative base damage

mRNA degradation

The goal of the first phase of the current work was to determine if degradation and damage to DNA and RNA could be correlated to the time since deposition of forensic biological stains. The focus of the initial work involved an examination of the extent of degradation to mRNA in dried bloodstains. Although we and others have demonstrated that mRNA persists in dried stains, the RNA molecule is inherently less thermodynamically stable than DNA and degradation products are, like DNA, readily observed by electrophoresis. However, unlike DNA, there are numerous mRNA species present in each sample and, depending upon the structure each could be degraded at differing rates. In order to determine which mRNAs may be more useful in degradation studies, mRNA from bloodstains stored at room temperature for 5 minutes and 3 months was isolated and reverse transcribed using random decamers. The cDNA was then amplified using primers specific to each of the fifteen blood-specific genes (ALAD, ALAS2, AQP1, CPOX, CSF1R, GATA1, GYPA, GYPB, GYPE, NFE2, PBGD, SPTB, TFR2, UROD, and URO2) and the products visualized using 2.5% NuSieve gels. The products for each gene from the 5 minute and 3 month samples were run side by side to determine if there was any decrease in intensity of the obtained products (data not shown). While products were obtained for all genes except for two (URO2 and ALAD), no significant differences were observed between the 5 minute and 3 month samples. It

was difficult to determine if slight differences in intensity existed since this gel based assay is not a quantitative approach.

The previous experiment utilized random decamers for reverse transcription of the mRNA samples. The use of random decamers may not be able to identify degradation to specific regions of mRNAs such as the polyA tails. Reverse transcription can also be accomplished by the use of oligo-dT primers which specifically bind to the polyA tails. If the polyA tail of specific mRNAs were degraded in older stains, reverse transcription with an oligo-dT primer may not be possible if the tail was very degraded, or may be less efficient if the degradation was not as extensive. RNA from the 5 minute and 3 month samples was reverse transcribed with oligo-dT primers and amplified using primers for the same blood specific genes. The products were visualized on 2.5% NuSieve gels (data not shown). For most of the genes, no product was detected for either of the time intervals. It was determined that this method may simply be an inefficient method for reverse transcription and not necessarily indicate degradation of polyA tails. More specific methods for examining the length of polyA tails would therefore be necessary.

A method known as RACE-PAT (rapid amplification of cDNA ends polyA tail) was examined for determination of the length of polyA tails [44-46]. This method utilizes a specific primer for reverse transcription that is comprised of an oligo-dT (usually 12 T's) primer with a random GC-rich anchor sequence. The oligo-dT portion of the primer can bind throughout the polyA tail, priming cDNA products of various lengths. The oligo-dT primer would have more places to bind to with longer polyA tails, and less places to bind with a shorter polyA tail. The products can then be amplified with the oligo-dT primer with attached anchor sequence and a primer specific to the gene of interest. A smear with

larger sized products should be observed for those mRNAs with longer polyA tails, since there were more places along the polyA tail for the primer to bind. It was hypothesized that the sensitivity of this assay may detect degradation of the polyA tails that may be occurring in dried bloodstains.

A GC rich anchor sequence (to attach to the oligo-dT primer) was generated that would have a similar T_m to the blood specific gene primer that would be used in conjunction with the RACE-PAT primer in subsequent amplifications. It was used to reverse transcribe RNA isolated from the 5 minute and 3 month samples. While very similar patterns were observed between the 5 minute and 3 month samples, each of the 5 minute samples showed a higher MW band that was not seen in the 3 month samples (data not shown). This indicated a possible degradation to specific mRNAs in dried bloodstains.

Since a reduction in a higher MW band was observed between the 5 minute and 3 month samples, bloodstains stored at time intervals between 5 minutes and 3 months were examined in order to determine at what time interval this higher MW band (seen in the 5 minute sample) was no longer observed. The same donor was used to observe products from 5 minutes, 15 minutes, 30 minutes, 1 hr, 3 hrs, 6 hrs, 12 hrs, 24 hrs, 48 hrs, 1 week, 4 weeks, and 3 months, using the PBGD gene as an example. The higher MW products observed in the 5 minute to 1 week samples were not observed in the 4 week and 3 month samples (data not shown). There was a decrease in the MW of the recovered products in the 4 week and 3 month samples (data not shown). In order to confirm the time interval at which this higher MW band was lost, a second set of bloodstains stored at room temperature for intervals longer than 3 months was examined. This set of

bloodstains ranged in age, including 1 day, 3 days, 1 week, 4 weeks, 3 months, 6 months, 1 year, and greater than 1 year (18 months). RNA was extracted from these samples and examined using the RACE-PAT assay, again using the PBGD gene, in order to confirm the previous findings. Again, the higher MW products observed in the 1 week and 4 week samples were not seen in the older stains (data not shown).

A reduction in the higher MW band had been observed for room temperature samples, however it was possible that exposure to various environmental conditions would cause a reduction in MW to be observed in a shorter amount of time. In order to assess the presence of the higher MW band in environmentally compromised samples, bloodstains were stored under UV light, stored outside and covered (exposed to heat, light and humidity, HLH) and stored outside and uncovered (exposed to heat, light, humidity, and rain, HLHR). RNA from these samples was used in the RACE-PAT assay in order to determine if the mRNA was degrading at a faster rate than at room temperature and if there was still a decrease in the higher MW products as the age of the stain increases. A reduction in the higher MW products was observed for the HLH and HLHR samples (data not shown). However, the UV samples showed no reduction in MW amongst the three samples (1 day, 4 weeks, and 3 months).

While the RACE-PAT assay did not provide an indication of whether degradation was occurring at the 3'-end poly(A) tail (as we had hoped), it did provide evidence for a reduction in the MW of the mRNA in the dried bloodstains. Therefore attempts were made to develop assays that would examine possible degradation to the 3' and 5' ends of the mRNA molecules using standard PCR amplification reactions. The first attempt at making such a determination was through the use of a linear amplification. In an

exponential amplification it is possible to miss degradation products if they occur to the ends of the mRNA outside of where the primers bind. A linear amp would allow the ends of the mRNA to be amplified, and if the expected product size was known, any differences in this length would indicate degradation. The PBGD gene was chosen for the linear amplification experiments since it had demonstrated a reduction in MW in the PAT assays. A primer was designed to bind to the middle of the mRNA and then amplify towards the 5' end, keeping the product length ~300bp. Another primer was designed to bind to the middle of the mRNA and then amplify towards the 3' end, again keeping the product length ~300bp. However, the linear amplification was not very efficient and did not produce results that provided any indication of whether degradation was in fact occurring or from at which end it was occurring.

Since no significant results were obtained with the linear amplification, attempts were made to position PCR amplification primers at various locations throughout the mRNA sequence to determine if any changes in amplification would be observed. If primers were designed to hybridize to the extreme 3' and 5'-ends of the mRNA, degradation at either of these ends would result in the loss of a primer binding site and therefore loss of amplification products. Therefore, absence of an amplification product may indicate degradation to one or both ends of the mRNA. GYPB was selected for use in this study as it had demonstrated differences in the higher MW band between fresh and aged stains with the RACE-PAT assay. The original primers for GYPB amplification were located towards the middle of the mRNA and would not necessarily detect degradation occurring at the 3' or 5' ends. Two forward primers were designed to be

located near the 5' end and three different reverse primers were designed to be located at various positions in relation to the 3' end.

Initially samples stored at room temperature were amplified with the R1 and F1 primers, which were the primers located closest to the 5' and 3' ends of the GYPB mRNA. These primers thus detect the presence of relatively, intact non-degraded mRNA. Using these primers, amplification products were observed up to 3 months, with no amplification products obtained with the 6 month sample (data not shown). This experiment was repeated with a second set of samples also stored at room temperature in order to confirm the results. With this set of samples (1 day, 3 days, 1 week, 4 weeks, 3 months, 6 months, 1 year, and 1.5 years), no amplification products were observed in samples 3 months or older (data not shown).

The loss of amplification product with the R1 and F1 primers gave an indication of possible degradation to the ends of the GYPB mRNA in dried bloodstains. Since the R1 and F1 primers are the closest primers to the 5' and 3' ends, moving the primers in away from the ends should recover amplification in some degraded samples that still comprise shorter amplimers. In order to demonstrate this, the R2 and R3 primers were used with the F1 primer. The samples that did not have an amplification product using the F1 primer were amplified using the R2 and R3 primers for this experiment. Amplification products were recovered in each of these samples using both the R2 and R3 primers (data not shown). Therefore, placement of the reverse primer away from the 3'-end of the mRNA resulted in recovery of amplification products. This indicated a possible degradation to the 3'-end of the GYPB mRNA. The products obtained using the R3 primer seemed to be more consistent and R3 was therefore used in future experiments.

The samples used in this initial testing were stored at room temperature. The R1 and R3 primers were tested using bloodstains stored at 37°C to determine if amplification products could again be recovered with the R3 primer that were not obtained with the R1 primer. Additionally for this experiment, the use of random hexamer and oligo dT primed RT reactions was examined to determine if both types of RT primers would produce similar results. Amplification with the R1 primer resulted in no amplification product being obtained for the 1 month samples in either set of samples (data not shown). However, when the R3 primer is used with the same sets of samples, an amplification product is recovered for each sample (data not shown). This result was observed for both the random hexamers and oligo dT primed RT reactions.

While recovery of the amplification products using the R3 primer was visualized on the gel, there was no way to determine quantitatively if there are any differences in the amount of product recovered as the stain age increases or if there are any correlative changes between the R1 and R3 products that may be able to be used to determine stain age. Therefore, attempts were made to move this assay to a real-time PCR assay format. The R1 assay required the amplification and detection of an ~400bp fragment, whereas the R3 assay required the amplification and detection of only an ~200bp fragment. When designing a real time PCR assay, it is recommended to keep the amplification product size to ~150bp to ensure efficiency of the assay. Despite the recommendations, attempts were made to design and optimize detection of this ~400bp fragment. The R3 assay worked efficiently with all samples tested, however the R1 assay did not perform consistently. Numerous attempts were made to improve the efficiency of the R1 assay. Highly processive polymerase enzymes were added to the assay as well as drastically

increasing the length of the extension step (increase from ~30sec to 1 min to ~15min). While limited improvements were observed after incorporating the highly processive enzymes and increasing the extension step, the R1 assay was still very inefficient and inconsistent results were still obtained.

Even though the first attempt at developing a real time assay for the detection of degradation to the GYPB mRNA was unsuccessful, the original gel based assay had given an indication of possible degradation to this mRNA. So a new approach was taken to try to detect this possible GYPB mRNA degradation using a real time PCR format so that quantitative measurements could be made. The new approach to the real-time PCR assay was to develop an assay at both the 3' and 5' ends, rather than keeping one primer for both assays at the 5' end. This would allow for the product size to be within the recommended size range (~150bp). Comparisons of the Ct values for both assays could be made in order to provide an indication if there is perhaps more degradation or a more progressive degradation occurring to one of the ends as the age of the stain increases. The Ct values from each assay were examined to determine if recovery of products was reduced in older stains compared to that of the younger stain. However, after examination of the data, no trend was identified within either assay (data not shown). Ratios of 5' to 3' end Ct values and also 3' to 5' end Ct values were also calculated in order to determine if a relationship could be identified (data not shown). Again, no trend was identified. Slight differences in Ct values for samples within each assay were observed. These differences could due to possible random chain breaks that may be occurring relatively infrequently.

Despite the lack of success of the GYPB real time assays, the results of the gel-based GYPB R1 vs. R3 assay indicated that degradation to mRNA was present and could

be correlated to the age of the stain. Therefore, attempts are underway to develop a capillary electrophoresis-based assay in order to examine differences in recovery of R1 and R3 products in aged bloodstains. A capillary electrophoresis-based system would allow for a larger amplification product to be used and therefore no changes to the primer sequences would need to be made as was necessary for development of the real-time PCR assay. The relative peak heights of the obtained products may provide the means to obtain a quantitative relationship between the R1 and R3 amplification products which may allow for a correlation to time since deposition.

In summary we have so far failed to observe a robust correlation between mRNA degradation and the age of a bloodstain. Additionally, no clear evidence as to the direction of mRNA degradation in aged stains (5'-end or 3'-end) was obtained.

Degradation studies involving DNA were not pursued since our own previous studies indicated that only gross molecular changes (i.e. reduction in average molecular weight) would likely be detected and would, in any case, not provide sufficient resolution for the determination of the time since deposition of biological stains. We had determined that the dehydrated state of dried biological stains affords a degree of protection from potential DNA damage (in particular UVC-mediated damage) [47]. Therefore, it did not seem advantageous to perform additional studies involving an assessment of DNA degradation or damage for possible correlation to time since deposition. Based on the inconsistent results obtained from the mRNA degradation studies, it was also decided that an evaluation of damage to the RNA bases would not be a fruitful avenue to pursue at this stage.

Aim 2: Assessment of UV-Visible Spectrum of Hemoglobin

UV-Visible Spectroscopy of Hemoglobin

Initial studies performed to examine changes in the spectral profiles of hemoglobin involved a small number of room temperature bloodstains of different ages (15 minutes, 6 hours, 24 hours, 1 month, 3 months, 6 months and 1 year). These samples were selected since there was a significant difference in their age and would hopefully provide an indication of whether this method might provide a higher resolution (distinguish between the minutes-hours-days samples) than previously attempted TSD methodologies. Spectral profiles from 200-450nm and 500-600nm were obtained because they contained the characteristic hemoglobin absorption peaks and it was thought these areas might be of interest. From the profiles obtained from these initial samples, several areas of interest were identified (Figure 1). A decrease in the absorption peak at ~410-415nm (Soret band) was observed as the age of the stain increases, as well as a shift in the wavelength where this occurs (Figure 1, top panel). A deterioration of the characteristic $\beta_{541\text{nm}}$ and $\alpha_{576\text{nm}}$ absorption peaks was also observed in these samples (Figure 1, bottom panel). By 6 months the two absorption peaks are almost not visible and by one year they are completely unidentifiable (Figure 1, bottom panel). An examination of the spectral profiles demonstrated that the shape of the β peak (542nm-560nm) may not be the same as the α peak (576nm-560nm). This difference in peak shape was particularly evident in the 1 month and 6 month samples (Figure 1, bottom panel).

As a result of these initial findings, a set of spectral shift parameters were determined that would be further examined (Figure 2). Five spectral parameters were identified and included: 1) changes in the maximum absorbance of the Soret band ($\Delta\text{Abs}_{\text{Soret}}$); 2)

changes in the wavelength of the λ_{max} for the Soret band ($\Delta\lambda_{\text{Soret}}$); 3) changes in the absorbance of the β band at 541nm ($\Delta\text{Abs}_{\beta(541-560)}$); 4) changes in the absorbance of the α band at 576nm ($\Delta\text{Abs}_{\alpha(576-560)}$); 5) the ratio of absorbance changes of the α and β bands ($\Delta\text{Abs}_{\beta(541-560)} / \Delta\text{Abs}_{\alpha(576-560)}$). Early attempts to utilize changes in hemoglobin spectral profiles have utilized changes to the α and β bands. However, based on the results obtained in our studies all four parameters (1, 3, 4 and 5) involving changes in the absorbance of the characteristics peaks in the blood spectra did not provide a reliable correlation with the age of the stain. A possible correlation between the maximum absorbance of the α and β peaks and the age of the stain was observed. Figure 3 depicts the relationship obtained between the age of the stain and the change in absorbance of the $\beta_{(541-560\text{nm})}$ peak. As can be seen from this data, month differences in stain age were required before a possible correlation could be developed. The resulting R^2 values are also ≤ 0.78 with a large standard error for several samples. Similar results were also obtained with the $\alpha_{(576-560\text{nm})}$ peak was examined (data not shown). Previous studies had demonstrated a difference in the morphology of the α and β peaks as the age of the stain increased. Therefore, the final parameter utilizing a change in absorbance that was examined was an evaluation of the ratio of the ΔAbs of the α and β peaks (Figure 4). When a ratio of the two ΔAbs values was used, a stronger correlation with the age of the stain was observed ($R^2 > 0.94$, except for one data set whose $R^2 = 0.87$). However, month differences in stain age were still required.

As a result of the poor resolution of the spectral parameters involving absorbance changes in the three characteristic hemoglobin bands (Soret, α , β), attempts were made to further examine the possible λ_{Soret} shift that was observed during initial testing. This

parameter was of particular interest since, to the best of our knowledge, it has not been reported in any previously published studies. The λ Soret was graphed as a function of stain age for bloodstains that had been stored at room temperature and 37°C samples for 15 minutes to 1 year (Figure 5, top panel). From this data, a strong correlation between the age of the stain and the $\Delta\lambda$ Soret was observed ($R^2 = 0.96$ and 0.84 for the 22°C and 37°C samples, respectively). The 37°C samples show a sharper decrease with early samples indicating a possible affect of temperature on the shift. With the range of samples examined, it was difficult to see the relationship in the early time points. In order to further examine the early time points, only the 15 minutes to 2 days samples were plotted (Figure 5, bottom panel). When this smaller set of samples was examined, a strong correlation between the wavelength shift and the age of the stain was demonstrated (R^2 values of 0.95 and 0.98 for 22°C and 37°C samples, respectively). As a result of the significant correlation observed in the minutes to year and minutes to day intervals, additional time intervals were examined to determine if this method could be utilized to distinguish samples minutes, hours, days, weeks and months different in age. The R^2 values for each of the time intervals (15 min - 2 days, 15 min - 1 week, 15 min – 1 month, and 15 min – 1 year) for both bloodstains stored at both 22°C and 37°C are provided in Table 1. The R^2 value for each time interval for both temperatures (except for the 15 min – 1 year interval for the 37°C samples) was ≥ 0.95 . The slightly lower R^2 value for the 15 min – 1 year 37°C sample set could be a result of exposure to such a high temperature (~98°F) for a long period of time. It is unlikely, except for in extreme cases, that bloodstains would be exposed to this level of constant temperature. However, it does indicate that TSD estimates may be more accurate for younger stains at extreme

temperatures. Overall, this data demonstrated the potential to distinguish bloodstains differing in age by minutes, hours, days, weeks and months..

Instrumentation

All previous data had been obtained using the BioTek Synergy 2 microplate reader. Before any further work was conducted, an evaluation of the same sets of bloodstains was performed using the Hitachi U-0080D Spectrophotometer. This was performed in order to ensure that the Soret band shift would be observed on a different instrument and that it was not the result of the microplate reader itself. Table 2 provides a comparison of the λ Soret obtained for bloodstains stored at 22°C for 15 minutes – 1 year using both the microplate reader and the standard spectrophotometer. As can be seen from this data, the same hypsochromic shift in the λ Soret was observed using both instruments. There were differences in the obtained wavelength values with the spectrophotometer values typically 1-1.6 nm higher than what was observed for the microplate reader. The total wavelength shift between the 15 minute and 1 year samples was generally the same between the two instruments for both data sets. The 22°C data set when run on the standard spectrophotometer had a slightly larger overall shift, 7.7nm, compared to the 5nm overall shift that was observed when the same samples were run on the microplate reader (Table 2). However, the 1 year data point on the spectrophotometer had a larger standard error and therefore likely caused a larger shift than what was observed on the microplate reader. When the $\Delta\lambda$ Soret was plotted against the age of the stain, the R^2 for the 22°C data sets (15 minutes to 2 days) on both instruments was greater than 0.9 (Figure 6). The same trend can be seen in the data from both instruments, with the

wavelengths from the spectrophotometer slightly higher than the microplate reader which was indicated in Table 2. From this data it is evident that the observed shift is a genuine occurrence in aged stains and can be observed on different types of spectrophotometers. The data also indicates a need to calibrate any spectrophotometer that will be used for TSD measurements using this method. A series of standards would need to be run prior to any unknown samples. Table 3 provides a summary of the R^2 values obtained on both instruments for the 22°C and 37°C using both a 15 minute – 2 day and a 15 minute – 1 year data set. For all data sets, the R^2 values from the microplate reader were slightly higher than those obtained from the spectrophotometer, although all R^2 values are acceptable. The microplate reader does offer the advantage of being able to run replicates of the same sample at the same time, as well as the ability to run more samples at one time (96-well plate vs. a single cuvette with the spectrophotometer).

Affects of Temperature and Humidity

The previous experiments confirmed that the Soret band hypsochromic shift was a genuine phenomenon and could be observed on multiple instruments, and also demonstrated that temperature affected the rate of the Soret band shift. Therefore the affects of temperature and humidity were further examined. All sample sets used previously had been stored at room temperature (22°C), protected from light or in a 37°C incubator. The average humidity and temperature for the laboratory (measurements taken in the mornings) are approximately 22°C and 50% humidity. However, small changes throughout the day can occur. In order to more accurately control the temperature and humidity to which the samples were exposed, a Cincinnati Sub-Zero MicroClimate®

Humidity Chamber MCH-3 was used (Figure 7). This chamber allows for a range of temperatures from -65°C to 190°C and a range of humidity from 10% to 95% (limited to 85°C) to be used. The chamber is digitally controlled and monitored. Samples are placed inside the chamber and are thus exposed to constant temperatures and humidity levels.

In order to select appropriate temperatures and humidity levels, national average temperatures and humidity levels were examined (Figure 8). Most of the country experiences humidity levels between 56 and 85% for most of the year with western states experiencing lower humidity levels (35-50%) for most of the year. Therefore some states, such as Arizona or New Mexico, humidity levels above 60% might not be that relevant. Additionally, humidity levels below 65% might not be that relevant for states such as Florida. This data simply indicates that different regions of the country will be affected by differing humidity and temperatures and might therefore need to conduct simple experiments using temperatures and humidity levels relevant to their particular region. For experiments to be conducted in the humidity chamber 22°C and 30°C were selected with a range of humidity levels from 50 – 90% (50, 75, 80, 85 and 90%). The data for 30°C is still currently being collected. However, data is available for 50%, 80% and 85% humidity at this temperature.

A graph of the affects of the various humidity levels on the $\Delta\lambda\text{Soret}$ measurements for bloodstains stored at 22°C can be seen in Figure 9. As the humidity level increases, the hypsochromic shift decreases and is not observed with 90% humidity. As a result of the differences observed between different humidity levels, in order to determine the age of an unknown stain the storage conditions to which the sample was exposed would need to be known (or estimated.) The affects of 50, 80 and 85% humidity on samples stored at

30°C can be seen in Figure 10. Again, as the humidity level increases, the hypsochromic shift decreases. At the 85% humidity level with the 22°C data set, the $\Delta\lambda_{\text{Soret}}$ was relatively small. At 30°C, as seen in Figure 10, a distinct shift can still be observed. Additional data is being collected at 30°C using 75% and 90% humidity in order to obtain a more complete assessment of the affects of differing humidity levels at this temperature.

The previous experiments had examined the affects of different humidity levels at a constant temperature. Conversely, the affects of temperature at a constant humidity was also examined. Data for bloodstains stored at -20°C, 4°C, 22°C (one set at room temperature lab storage and one set stored in the humidity chamber), 30°C and 37°C with ~50% humidity was obtained. The λ_{Soret} was plotted for each temperature as a function of stain age (Figure 11). The hypsochromic shift was more significant and occurred at a faster rate with increased temperature. This data again indicates the need to have some knowledge or reasonable estimate of the environmental conditions that any unknown bloodstain sample was subjected to prior to collection and analysis. Of additional interest was the -20°C sample set. As can be seen from Figure 11, almost no change in the λ_{Soret} was observed. This data suggests that bloodstains removed from a crime scene and brought back to the laboratory for future analysis could be stored at -20°C without any further change to the λ_{Soret} . Additional studies would need to be conducted in order to confirm this observation.

Molecular Basis for the Hypsochromic shift

The precise molecular basis for the observed hypsochromic shift is unknown but possible mechanisms can be posited. Thus as the age of the stain increases, protein conformational changes (loss of secondary structure, disruption of hydrogen bonding, etc) may occur occur. The previously protected heme cavity may become more exposed as a result of these structural changes. Water, if allowed entry into the cavity, will successfully compete with the His residue (from Hb) coordinated to Fe(II) in the 6th hexa-coordinate position. The presence of the more electronegative oxygen will then cause the Fe(II) electrons to change from a low spin (paired) state to a high spin (unpaired) state. This causes Fe(II) to be more susceptible to oxidation. Over time, Fe(II) would then be oxidized to Fe(III). As a result of this oxidation, the electron configuration for the Fe molecule will therefore be altered and can interact with the π bonds of the porphyrin ring structure of hemoglobin. This interaction may cause an increase in energy of π to π^* transitions thereby resulting in absorbance at a shorter wavelength or a hypsochromic shift.

It is more difficult to explain the effects of temperature and humidity on the extent of the hypsochromic shift. With regard to temperature its well characterized effect on chemical reaction rates (such as the oxidation of Fe(II) \rightarrow Fe (III)) may explain a greater shift with increasing temperature. With humidity one might have expected that the higher humidity would result in more retained water and the bigger the hypsochromic shift. However the converse is found in that higher humidity results in less of a hypsochromic shift. Perhaps the more water retained with higher humidity leads to the dehydrated protein regaining more of its native structure with a concomitant reduction in the hypsochromic shift.

Sensitivity

All previous data that had been collected involved the use of 7.5 μg of total protein into a 75 μl reaction in an individual well in a 96-well plate. A range of input volumes was tested in order to determine if the Soret band shift could still be observed with smaller amount of input total protein. Visible spectral profiles from 1.1 – 8.8 μg of total protein are shown in Figure 12. It can be seen from these profiles that 2.2 μg of total protein is needed to obtain the characteristic α and β bands needed for the identification of the stain as blood. As little as 1.1 μg (0.5 μl) is needed to analyze the hypsochromic shift to determine the age of the stain (Figure 13). However, the sensitivity of this assay was determined to be 2.2 μg total protein since that amount was needed for a complete hemoglobin spectral profile to be obtained. While this is not an optimal input level, it is still sufficient to allow for a determination of the time since deposition.

The samples used in the previous experiment were simply smaller aliquots of a standard bloodstain extract. In order to further test the sensitivity of the developed method, bloodstains were prepared using a small initial volume of blood. Bloodstains ranging in size from 15 μl down to as small as 0.2 μl were prepared. Figure 14 shows the size of each of these stains. For the sensitivity testing, the 0.2 – 1 μl bloodstains were extracted in only 25 μl of 0.2M Tris-HCl buffer. Various volumes of the 25 μl extract using a 1 μl bloodstain were tested ranging from 0.25 – 3 μl (10-120 nanoliters). The spectral profiles obtained using these input volumes are shown in Figure 15. The Soret band is visible using 0.5 μl (20 nanoliters) or more. However, in order to obtain the α and β bands as well, 1 μl (40 nanoliters) was required (Figure 15).

Even though 40 nanoliters (from a 1 μl stain) was required to obtain the α and β bands, the Soret band was still observed with a smaller volume (Figure 16). Therefore attempts were made to determine if the $\Delta\lambda\text{Soret}$ could be measured with smaller input amounts. As can be seen from Figure 17, when 0.25 μl (input of 10 nanoliters) was used the absorbance of the obtained Soret band was too low and the curve too jagged for an accurate λ_{max} to be obtained. However, when 0.5 and 0.75 μl (20 – 30 nanoliters) was used, the Soret band shift could be observed. When bloodstains smaller than 1 μl were examined, the resulting Soret bands were too broad for accurate measurements to be obtained (Figure 18-19, Table 4). Therefore the sensitivity of this assay was determined to be 40 nanoliters of blood using as small as a 1 μl bloodstain.

Portable “Point-Of-Use” Spectrophotometer

The ability to obtain $\Delta\lambda\text{Soret}$ measurements and characteristic blood spectral peaks from bloodstains as small as 1 μl was demonstrated. However, for this method to be of greater use in forensic casework, the ability to perform such measurements at the scene could be useful. This would allow investigators to identify if a stain was blood and possibly how old the stain was. In order to do this, a portable spectrophotometer would have to be available at the crime scene. Recently, a portable spectrophotometer has become commercially available from Implen called the NanoPhotometerTM. The instrument has no moving parts making it more durable during transport to a crime scene and weighs less than 10 lbs. Concentration measurements (protein, DNA, RNA), full wavelength scans (190-1100nm), standard curves, ratio calculations and kinetic measurements can be performed on the instrument with as little as 0.7 μl of sample. A

close up of the sample area is provided Figure 20. The cuvette is inserted into the holder and the sample is then placed on the top of the cuvette. The mirrored lid is then placed on top of the cuvette and the measurement can be taken. Once a measurement is made, the sample can be collected from the top of the cuvette and the area cleaned between measurements. The wavelength data obtained from the instrument is provided in 1 nm intervals, which is the same resolution that is provided by the microplate reader used in all previous studies. When a sample measurement is taken, the data can be printed directly from a built in printer or can be connected to a computer through a USB or Bluetooth wireless connection. If the data is printed from the built-in printer, a small receipt-like print out is obtained such as those shown in Figure 21 (bottom panel). The print out contains the date and time the measurement was made, the serial number of the instrument is was collected on, and provides a full spectral profile including the wavelength and absorbance of recorded peak maxima. If the data is sent to a computer, the same data is provided in a report generated in the software that accompanies the instrument (Figure 21, top panel). Additionally, the data can be sent directly into an Excel file which creates its own graph of the data which can then be modified.

Initial work was performed with the NanoPhotometer to determine if it would be possible to obtain reliable $\Delta\lambda$ Soret measurements. Bloodstains stored at 22°C and 80% humidity from 15 minutes to 1 week were analyzed using the NanoPhotometer and then compared to the original data obtained using the microplate reader. The results of this comparison can be seen in Figure 22. The R^2 value for the data set on both instruments was above 0.9, however was lower for the NanoPhotometer (0.9089) compared to the microplate reader (0.9861). As was seen with the standard spectrophotometer, the

wavelength for the whole data set using the NanoPhotometer was slightly higher than what was observed with the microplate reader. Again, this indicates the need to calibrate any instrument before measurements with unknown samples can be made.

Despite the slight differences observed in the R^2 values obtained from data collected using the NanoPhotometer, the initial data indicates the potential utility of this instrument (or one like it) for use at crime scenes. Further validation of this instrument may also help improve the results. An optimization of the sample volume and concentration would need to be performed in order to determine the proper amount of sample to use for analysis.

Aim 3: Assessment of Changes to Enzyme Activity

Enzyme Activity Assays

We evaluated whether the expected decline of enzyme activity as the age of a stain increases could be used to estimate the age of the stain. Our laboratory is equipped with a microplate reader that is capable of performing enzyme kinetic assays. The instrument functions in a 96-well plate format, allowing multiple enzymes to be evaluated at one time. Depending on the stability of the enzymes examined, it may be theoretically possible to develop enzyme kinetic assays specific for “younger” or “older” stains that could produce high resolution TSD estimates. For example, if certain enzymes were completely inactive after one month and show a linear decrease from immediate to one month of stain age, then they could be utilized to provide finer resolution of the “younger” stains.

Candidate Selection

Numerous candidate enzymes were selected for initial screening (Table 5). These candidates have been selected from the classical serological and human biochemical genetics literature where they were typically analyzed by gel electrophoresis. There are several detection methods available for use with these candidates. Initially, both fluorescence and colorimetric detection methods were evaluated. Many of the candidates utilized a NAD or NADP fluorescence detection scheme, which could also be coupled to a MTT colorimetric detection scheme. Several 4-methylumbellifry-labeled assays were also evaluated which result in the production of a fluorescent product without the use of NAD or NADP. Additionally, there were also numerous enzyme candidates that use a direct MTT, NBT, or other colorimetric detection scheme.

For each candidate being examined, one-half of a 60 μ l dried bloodstain was extracted in the same buffer to be used in the enzyme reaction. One and a half micrograms of total protein were used in each assay. To initially evaluate the reaction to determine if the reaction is working, 100 μ l of this extract was tested with the reaction mixture. A 'no sample' blank was also run to ensure that any reaction obtained was due to the enzyme itself and not enzyme-independent substrate conversion. If enzyme activity was detected, the sample input was varied from 5-100 μ l (5, 10, 20, 40, 60, 80, and 100 μ l) and each volume run in triplicate. The linearity of sample input versus V_{max} (as determined by the instrument software) for fluorescence-based assays and colorimetric-based assays was evaluated. Adjustments to sample and reagent concentrations were made to develop an optimized reaction.

Numerous candidate enzymes were rejected for a variety of reasons (inefficiency of assay to detect enzymes, lack of solubility/compatibility of reagents with recommended buffers). The remaining candidates could be classified into three assay types: Colorimetric-1 (detection at 565nm, for MTT-formazan reactions), Colorimetric-2 (detection at 630nm, for an indophenol reaction), and Fluorescence-1 (detection at 360nm/460nm, for NAD, NADH, and 4-methylumbelliferyl reactions).

Evaluation of Enzyme Activity Assays with Aged Bloodstains

Two sets of room temperature bloodstains, ranging in age from 1 month to 1 year (1 month, 3 months, 6 months and 1 year) were tested using each assay. The data was then evaluated to determine if a loss of enzyme activity over time was observed. Through these trials it was determined that the fluorescence based assays and the colorimetric-2 assay did not perform optimally with inconsistent data being obtained. For each assay, two different samples for every time point were tested, and each sample was run in triplicate. The triplicate data for each sample was averaged, and then the average for the two samples was averaged as well to give an overall MaxV for each time point. The average MaxV was then plotted against the age of the stain in order to determine if any trends could be identified (Figure 23). It can be seen from Figure 23 that a decrease in enzyme activity occurred as the age of the stain increases. Enzymes exhibited varying rates of enzyme activity loss. This initial data indicated that it might be possible to use the amount of residual enzyme activity detected in a dried bloodstain in order to determine the time of deposition of that stain.

The data was also examined in order to determine the amount of time required for a complete loss of activity to be observed for each enzyme. If the enzymes lost activity at various times, it could be possible to determine an approximate age of an unknown stain based on which enzymes were still active and which enzymes were lost assuming that the protein input was normalized. The amount of time required for each enzyme to totally lose its activity (by direct observation or by extrapolation from the activity-time graph) is listed in Table 6. Glycerate dehydrogenase, lactate dehydrogenase, and isocitrate dehydrogenase lost activity in slightly less than one year (10.1, 11.4 and 11.8 months respectively). Alcohol dehydrogenase, phosphogluconate dehydrogenase, and malate dehydrogenase lose activity between one and two years (14.4, 16.0, and 20.7 months respectively). L-xyulose reductase and 3-hydroxybutyrate dehydrogenase lose activity after two years or more (28.7 and 144.7 months respectively). Interestingly, phosphoglucomutase (PGM), glucose-6-phosphate, and gluconate dehydrogenase can be considered relatively stable enzymes which may be useful in future experiments for use as potential normalizers or positive controls.

While this initial data requires month differences in age in order for TSD estimates to possibly be made, it does provide an indication that enzyme activity measurements may be useful in estimating the age of the dried bloodstain. Only a small number of enzymes have been evaluated and therefore additional enzymes may require smaller time differences for relationships to be seen and therefore provide better resolution. This method could also be applied to other biological fluids in addition to blood whereas the previous Soret band hypsochromic shift method is limited to bloodstains.

CHAPTER 4: CONCLUSIONS

We have developed two novel approaches to the determination of the time since deposition of dried bloodstains. The first approach utilizes a hypsochromic shift of the Soret band of hemoglobin. A significant correlation between the age of the stain and the $\Delta\lambda_{\text{Soret}}$ (hypsochromic shift) was demonstrated ($R^2 > 0.9$ in most cases). The hypsochromic shift measurements allow for a distinction of bloodstains differing in age by minutes, days, weeks and months. This is the highest resolution of any previously developed TSD method. The sensitivity of this assay has been demonstrated with the ability to analyze the hypsochromic shift with as little as 1 μg of total input protein or with as little as 40 nanoliters of blood from a bloodstain as small as 1 μl . The potential to perform this analysis “on-site” at crime scenes was also demonstrated through the use of a portable “point-of-use” spectrophotometer (NanoPhotometerTM). With this instrument, it would be possible to identify the presence of a bloodstain (through a characteristic UV-VIS spectral profile) and also determine the time since deposition of that bloodstain using microliter or sub-microliter volumes of blood. The second approach to the determination of the time since deposition of dried bloodstains involved an examination of the progressive loss of enzyme activity over time. A linear correlation between the age of the stain and the loss of enzyme activity was observed for six enzyme candidates (lactate dehydrogenase, malate dehydrogenase, alcohol dehydrogenase, isocitrate dehydrogenase, phosphogluconate dehydrogenase, and glycerate dehydrogenase). While month differences in stain age were needed, the resolution of this method could be improved with the identification of additional enzyme candidates. Additionally, this method could

be used to determine the time since deposition of other biological fluids in addition to blood.

In summary, we report the development of two novel approaches to the determination of the time since deposition of dried bloodstains. The use of a newly identified Soret band hypsochromic shift shows particular promise for use in forensic casework providing the highest resolution (minutes, hours, days, weeks, months) and sensitivity (nanoliter volumes from 1 μ l bloodstains). With more work, his approach could therefore provide investigators with novel probative information from a crime scene stain, namely a reasonably accurate estimate of the time since deposition of a bloodstain.

APPENDIX A: FIGURES

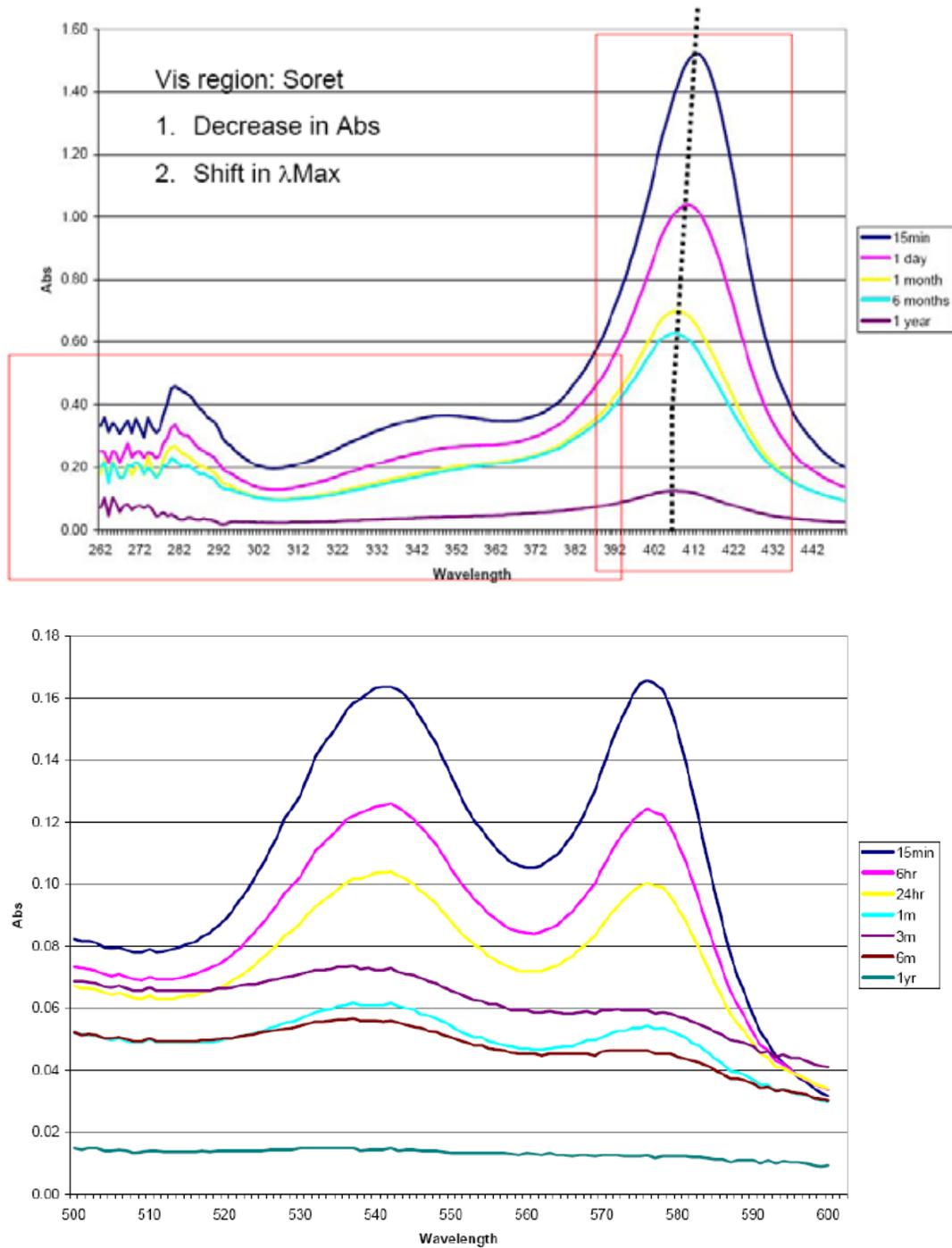


Figure 1. Hb UV-VIS Spectral Profiles From Aged Bloodstains Stored at 22°C

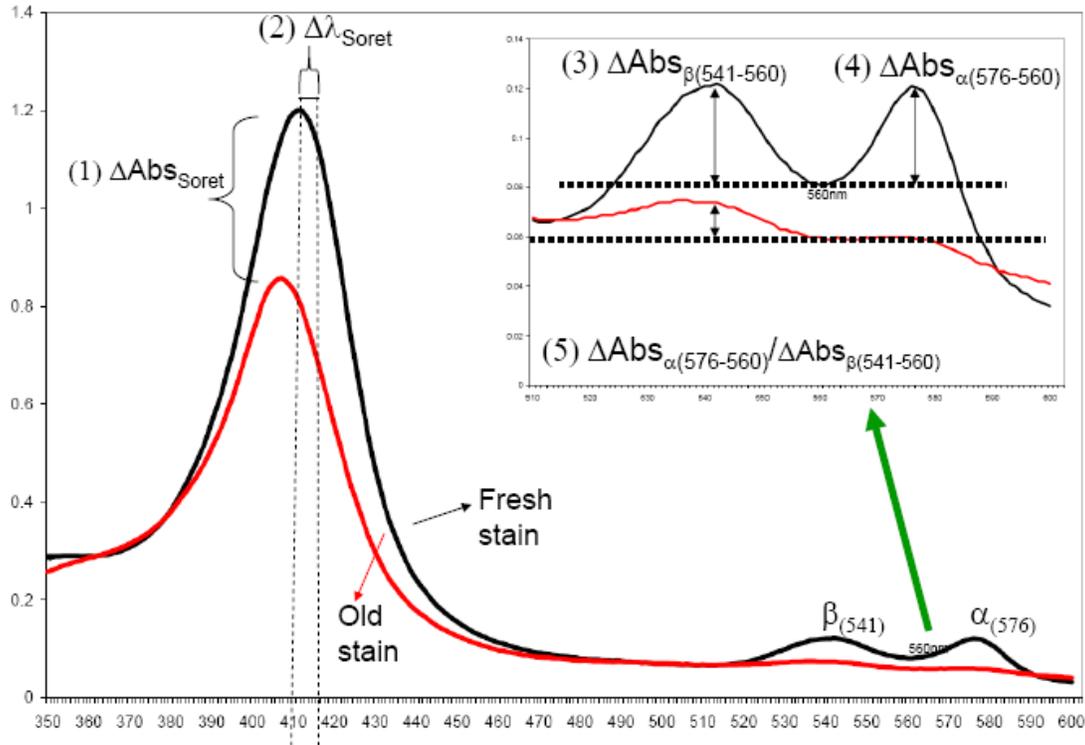


Figure 2. Hemoglobin Spectral Shift Parameters

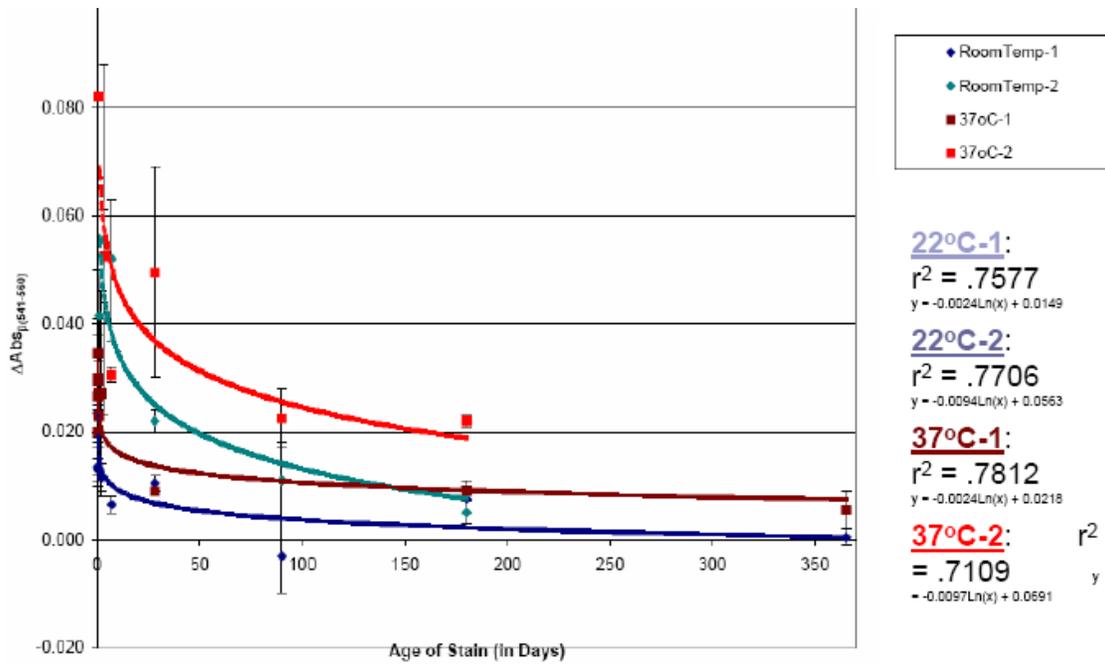


Figure 3. $\Delta\text{Abs}\beta_{(576-560)}$ in Aged Bloodstains (22°C, 37°C)

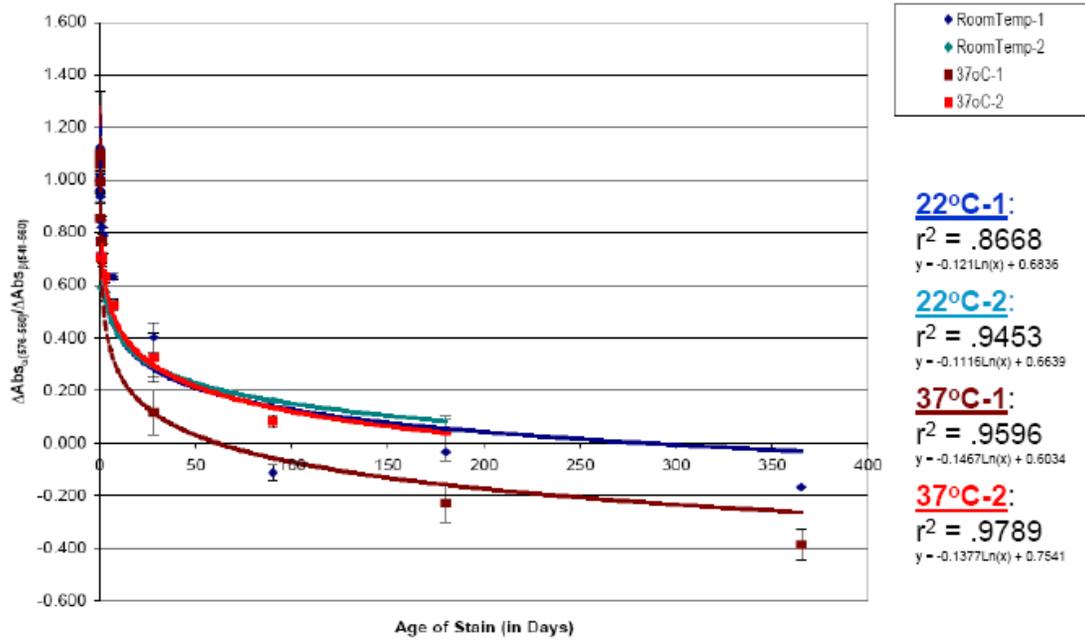


Figure 4. $\Delta\text{Abs}\alpha_{(541-560)}/\Delta\text{Abs}\beta_{(576-560)}$ in Aged Bloodstains (22°C, 37°C)

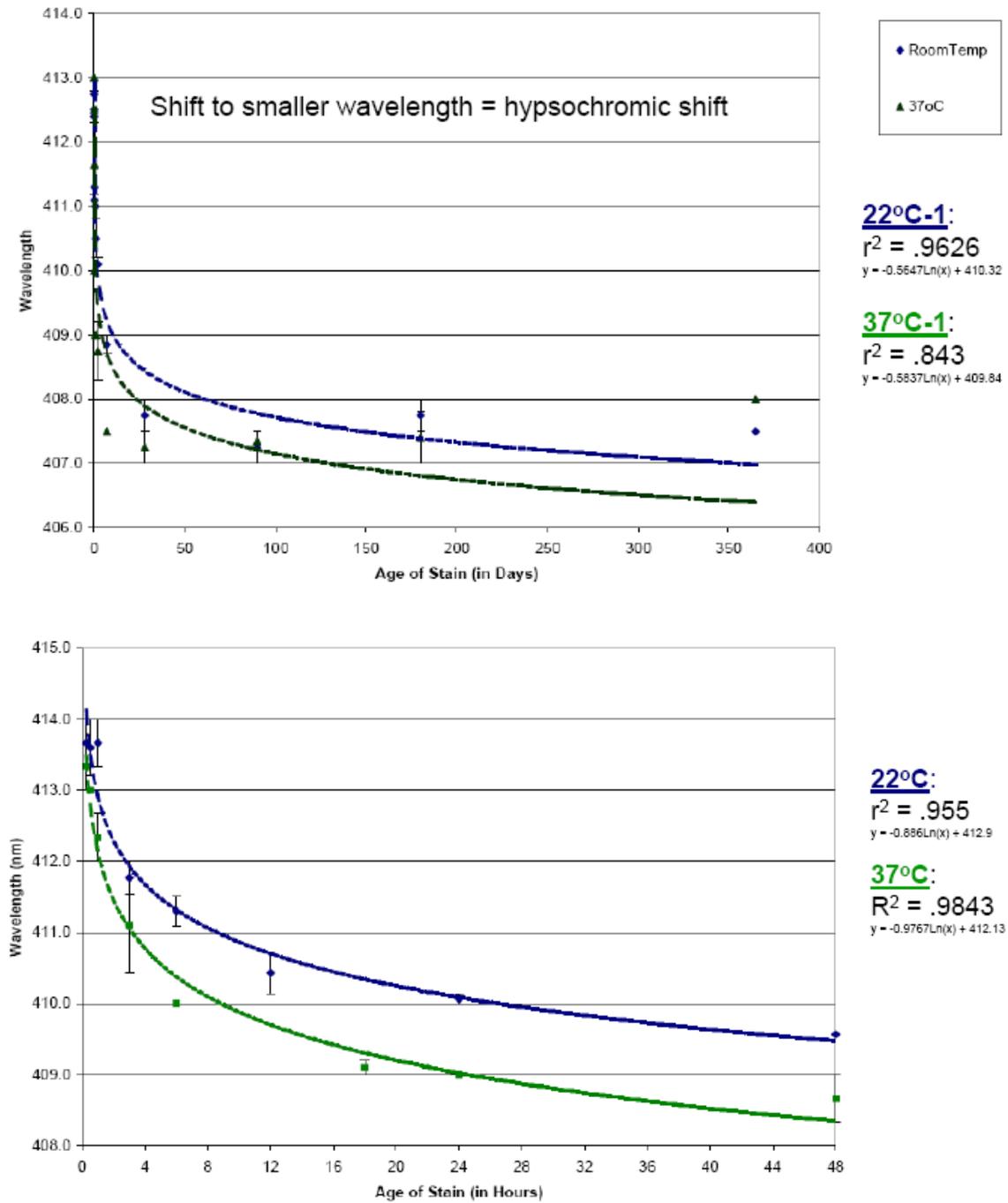


Figure 5. $\Delta\lambda$ Soret in Aged Bloodstains (22°C, 37°C)

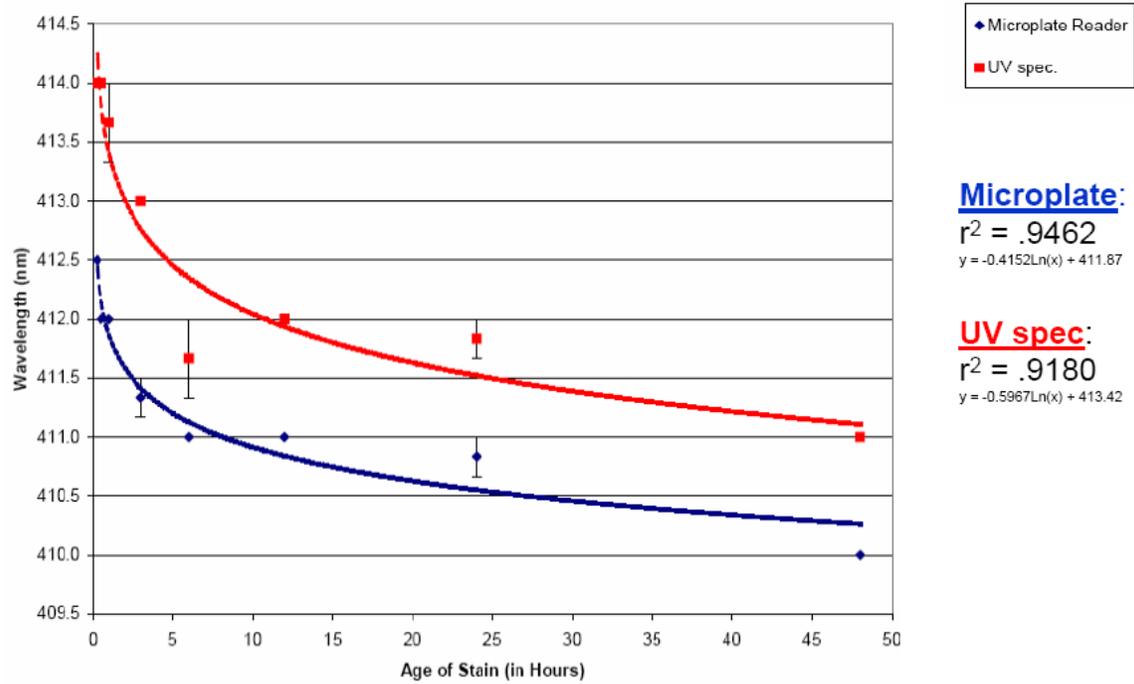


Figure 6. Comparison of $\Delta\lambda$ Soret Measurements Using Different Instruments



Figure 7. MicroClimate® Humidity Chamber MCH-3

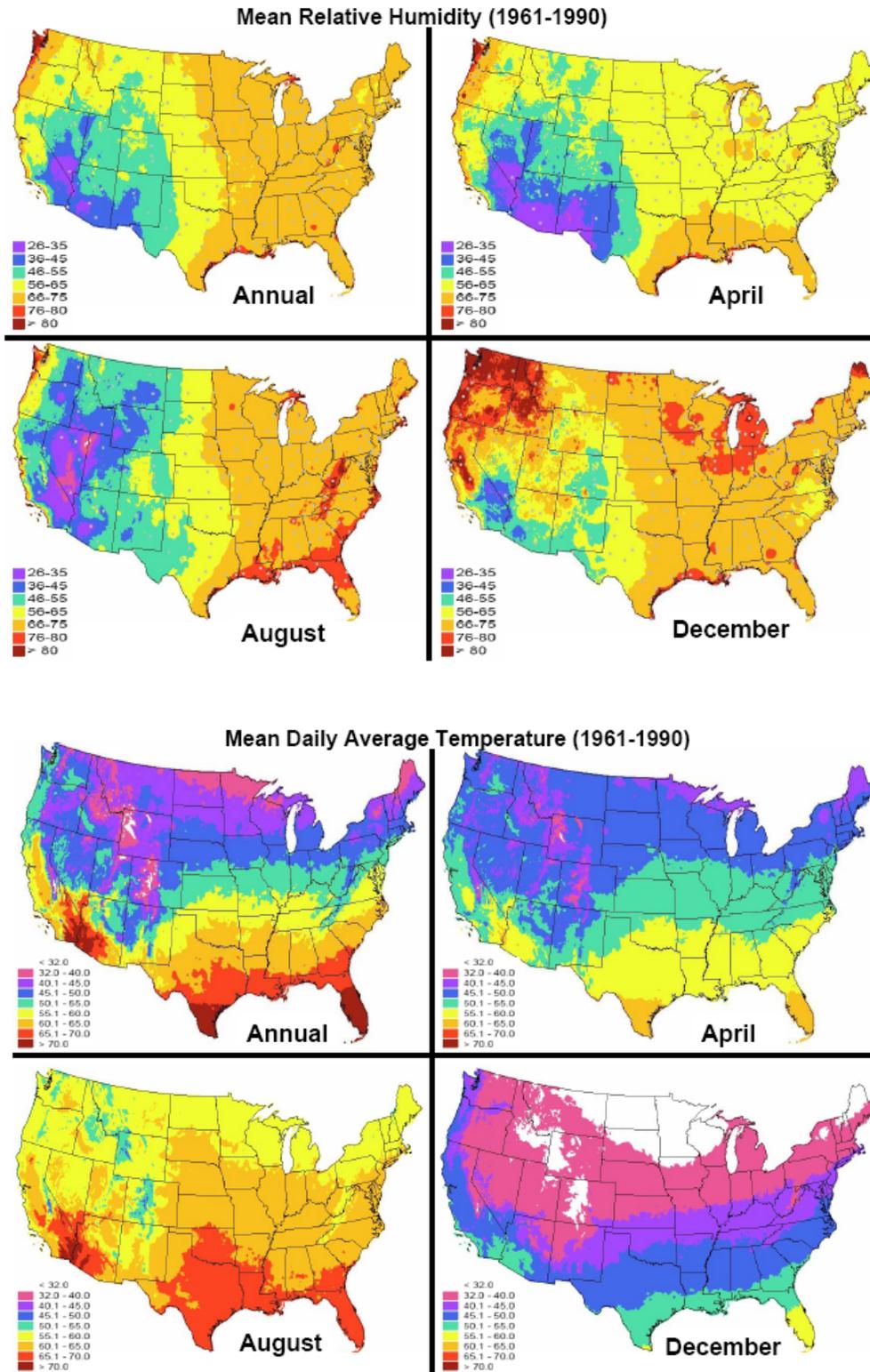


Figure 8. National Average Humidity and Temperatures (1961-1990)

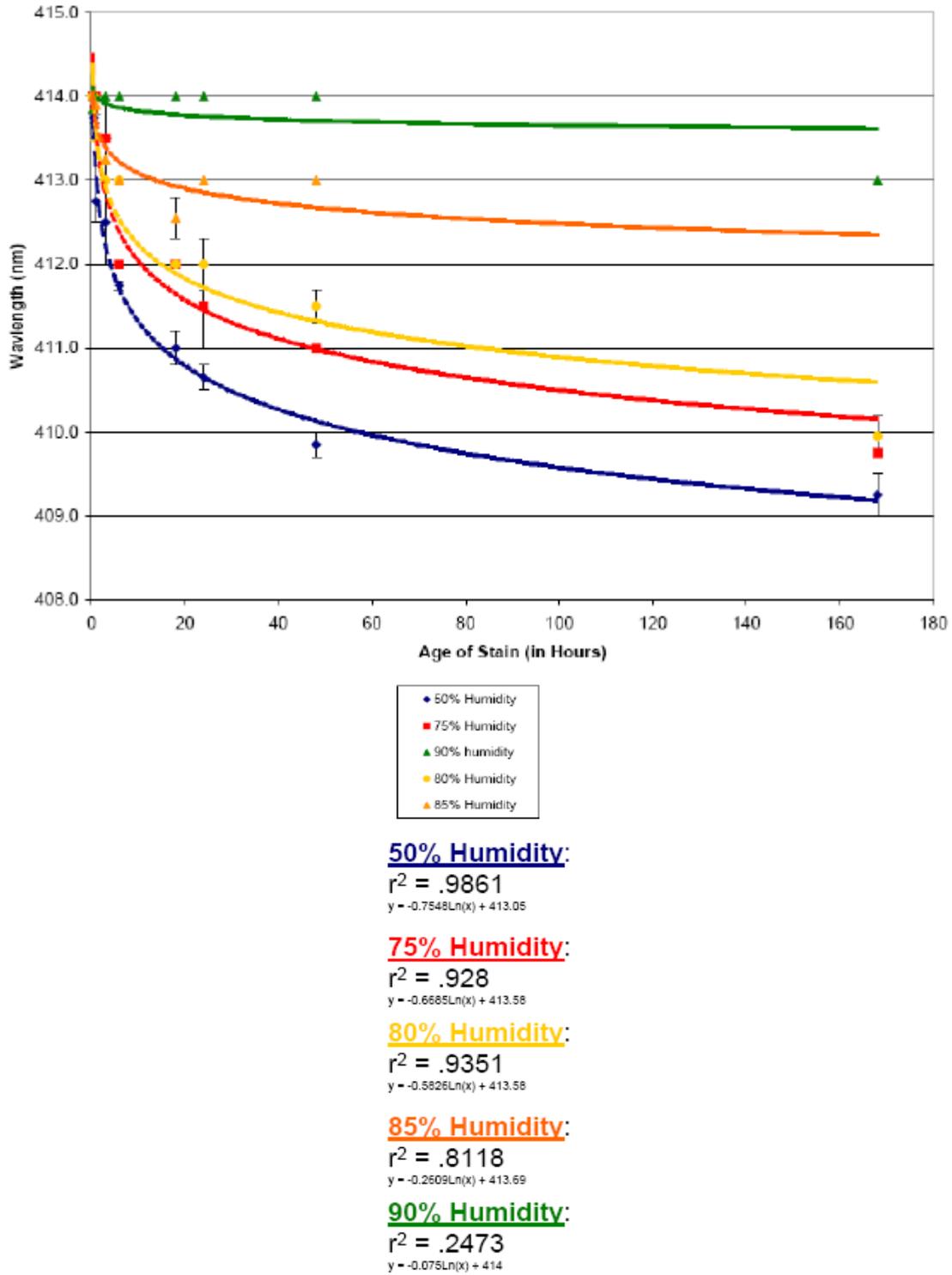
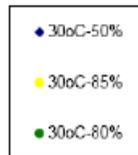
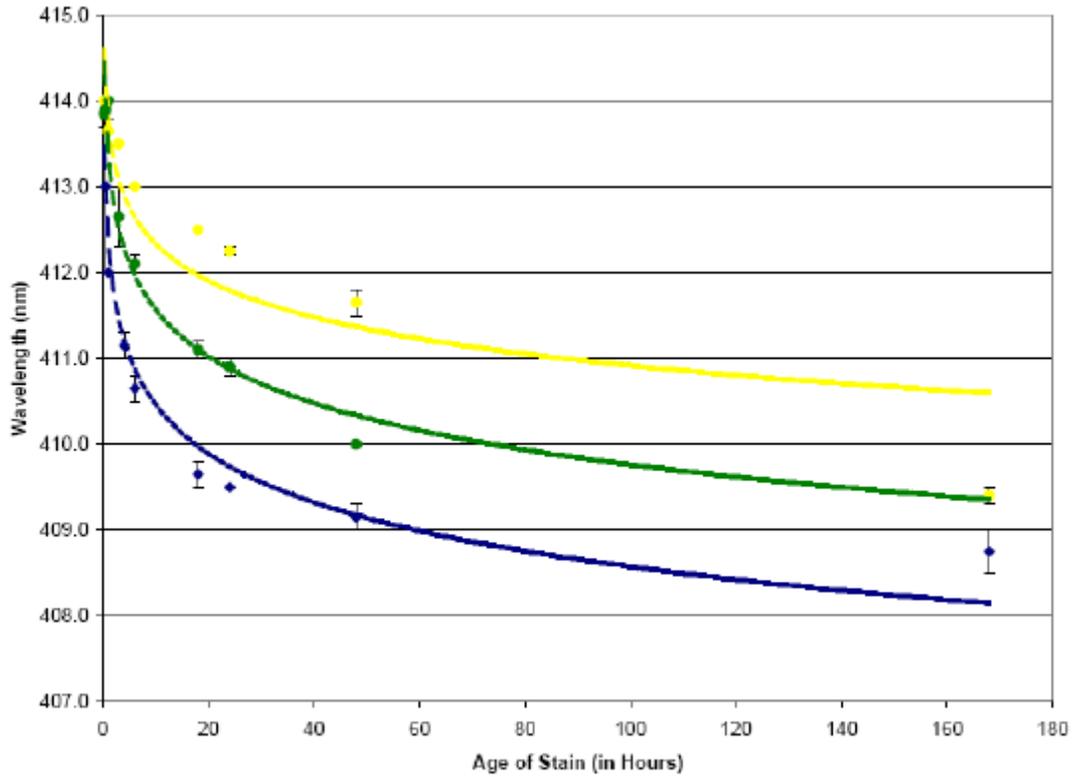


Figure 9. Affects of Humidity on $\Delta\lambda$ Soret For Bloodstains Stored at 22°C



50% Humidity:

$r^2 = .9631$

$y = -0.8162 \ln(x) + 412.34$

80% Humidity:

$r^2 = .9452$

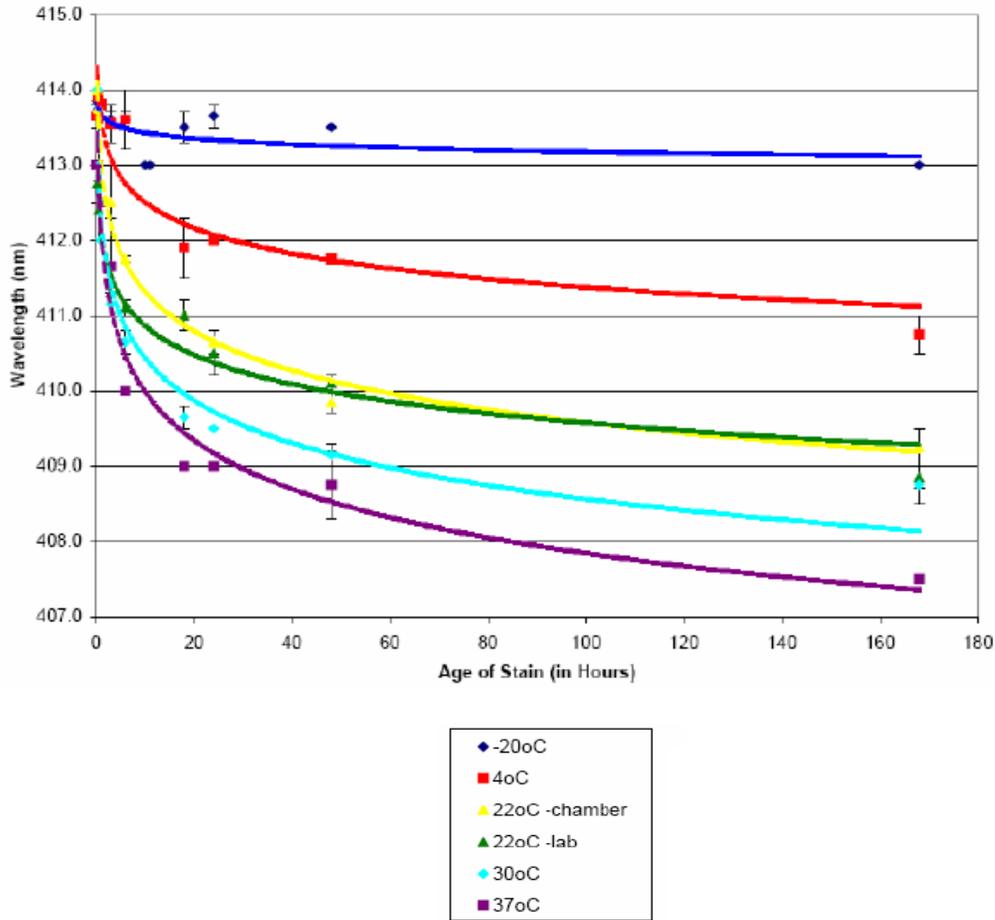
$y = -0.7821 \ln(x) + 413.36$

85% Humidity:

$r^2 = .8428$

$y = -0.6141 \ln(x) + 413.74$

Figure 10. Affects of Humidity on $\Delta\lambda$ Soret For Bloodstains Stored at 37°C



-20°C:

$r^2 = .4533$

$y = -0.111\text{Ln}(x) + 413.68$

4°C:

$r^2 = .8495$

$y = -0.4899\text{Ln}(x) + 413.63$

22°C-Chamber:

$r^2 = .9861$

$y = -0.7548\text{Ln}(x) + 413.05$

22°C-Lab:

$r^2 = .948$

$y = -0.5614\text{Ln}(x) + 412.16$

30°C:

$r^2 = .9602$

$y = -0.8137\text{Ln}(x) + 412.31$

37°C:

$r^2 = .9664$

$y = -0.9322\text{Ln}(x) + 412.13$

Figure 11. Affects of Temperature on $\Delta\lambda$ Soret Measurements

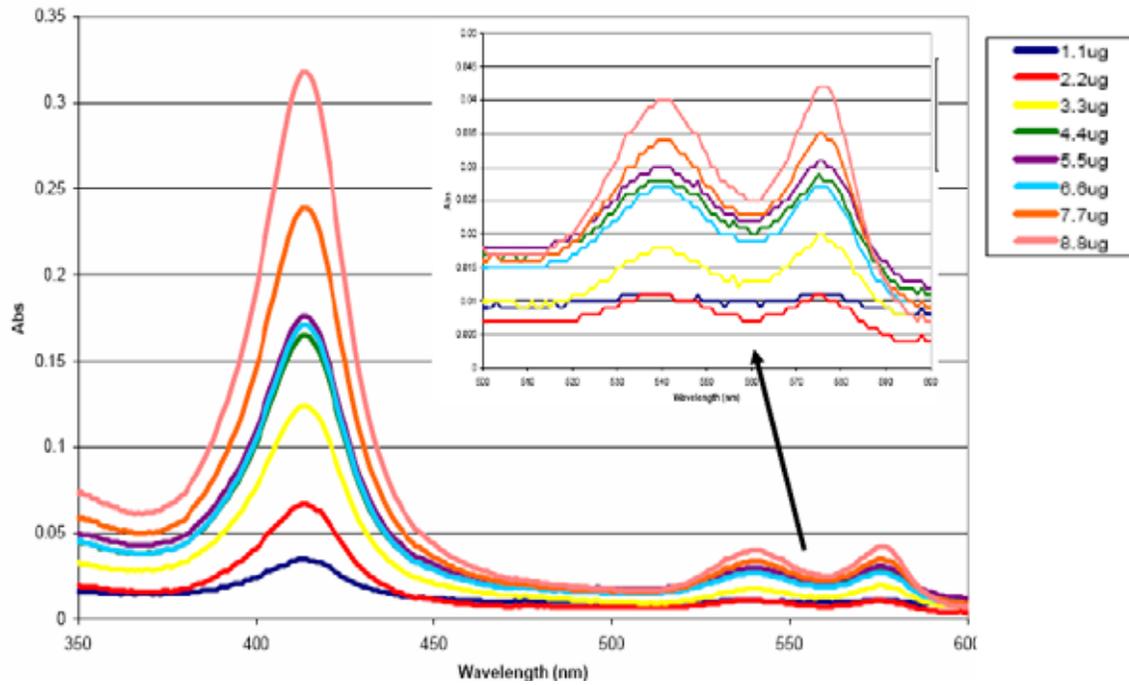


Figure 12. Hb UV-VIS Spectral Profiles using Reduced Total Protein Input

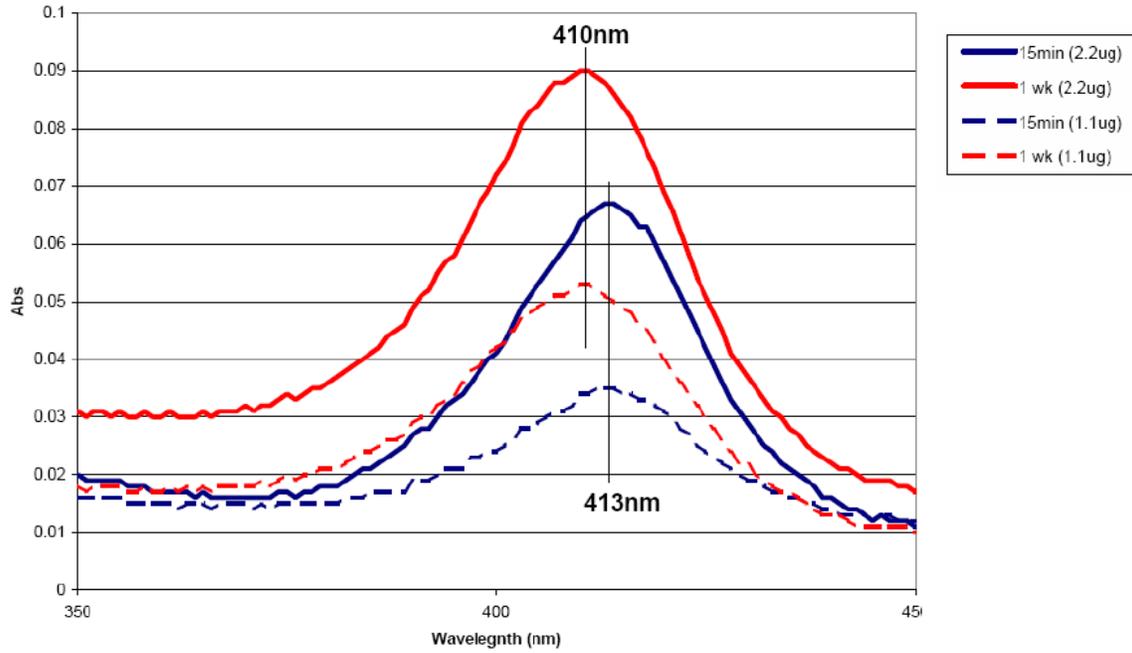


Figure 13. $\Delta\lambda$ Soret Sensitivity – Total Protein Input

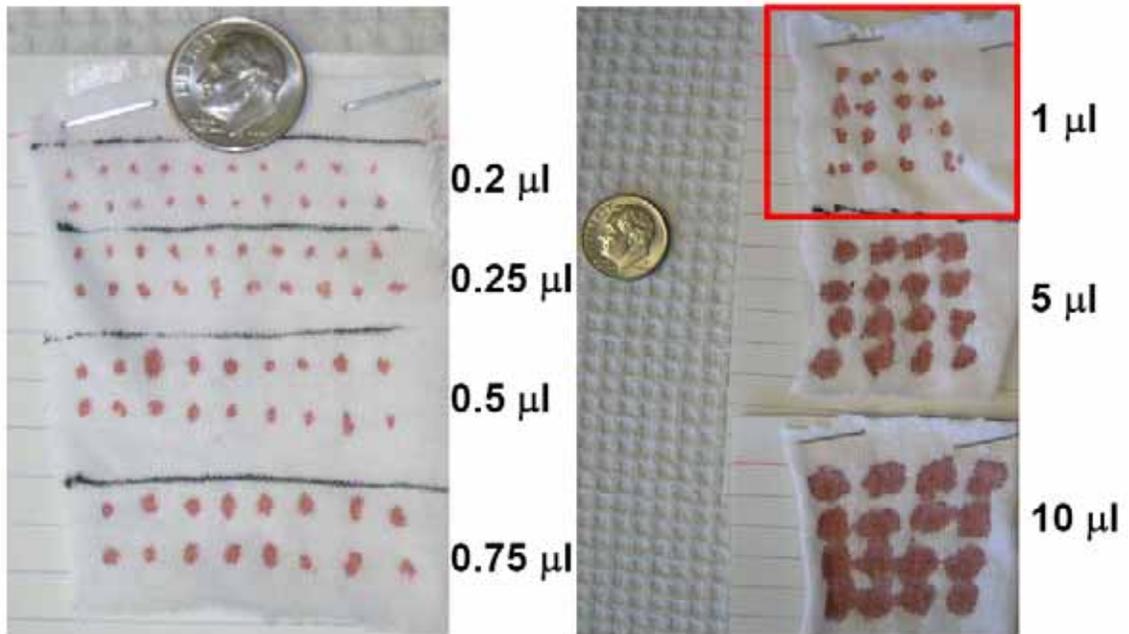


Figure 14. 0.2 μl to 10 μl Bloodstains Used for Sensitivity Studies

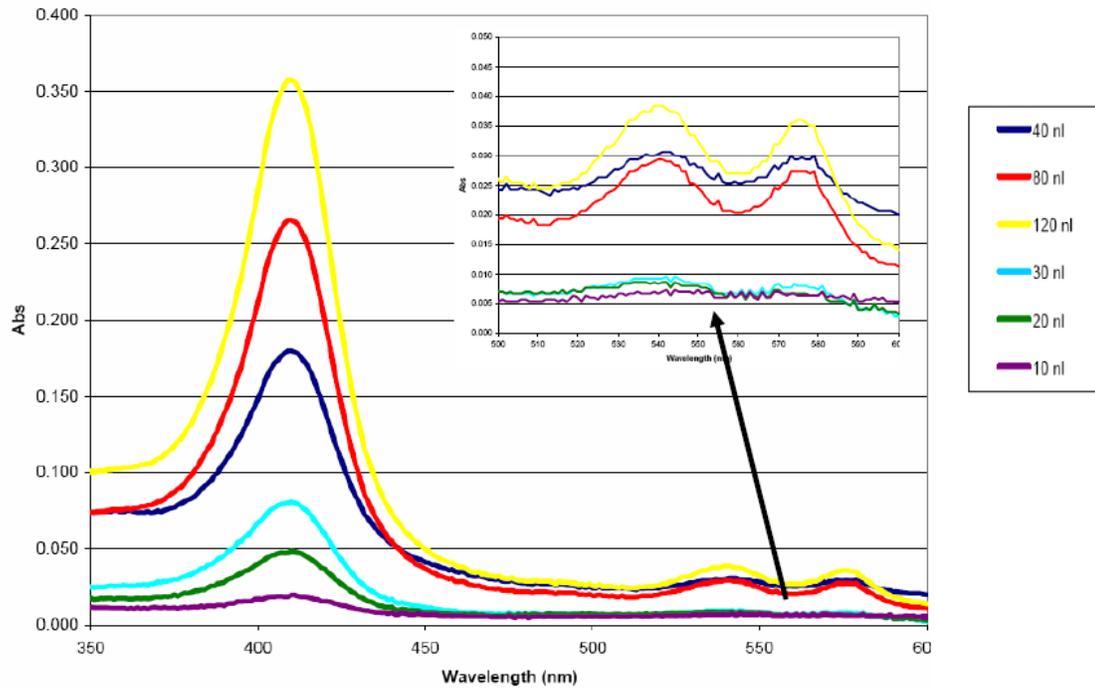


Figure 15. Hb UV-VIS Spectral Profiles using 1 µl Bloodstains

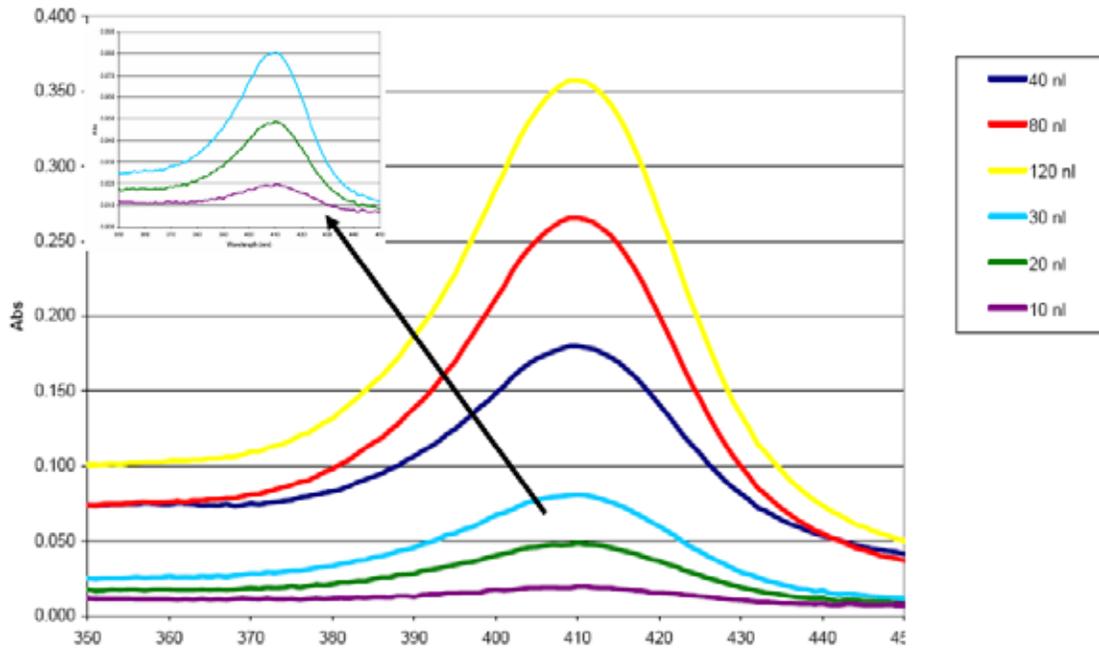


Figure 16. Soret Band Intensity Using Nanoliter Volumes from 1 μ l Bloodstains

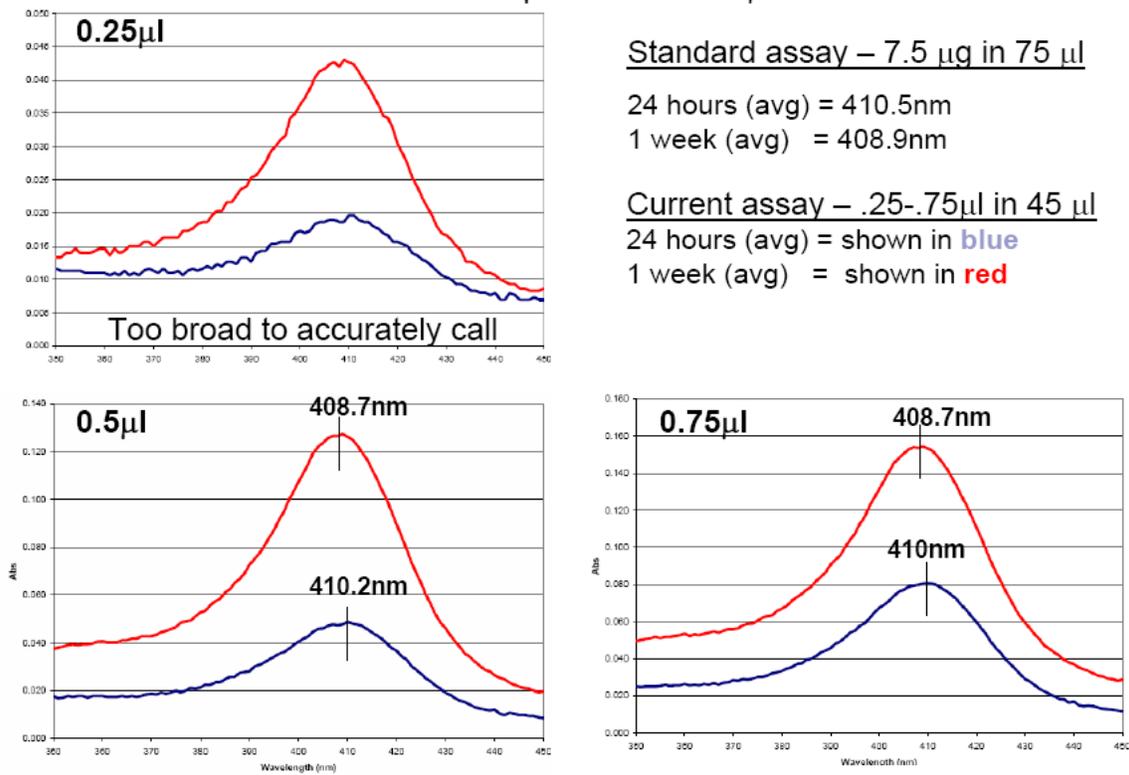


Figure 17. $\Delta\lambda$ Soret Using Nanoliter Volumes of 1 μ l Bloodstain Extracts

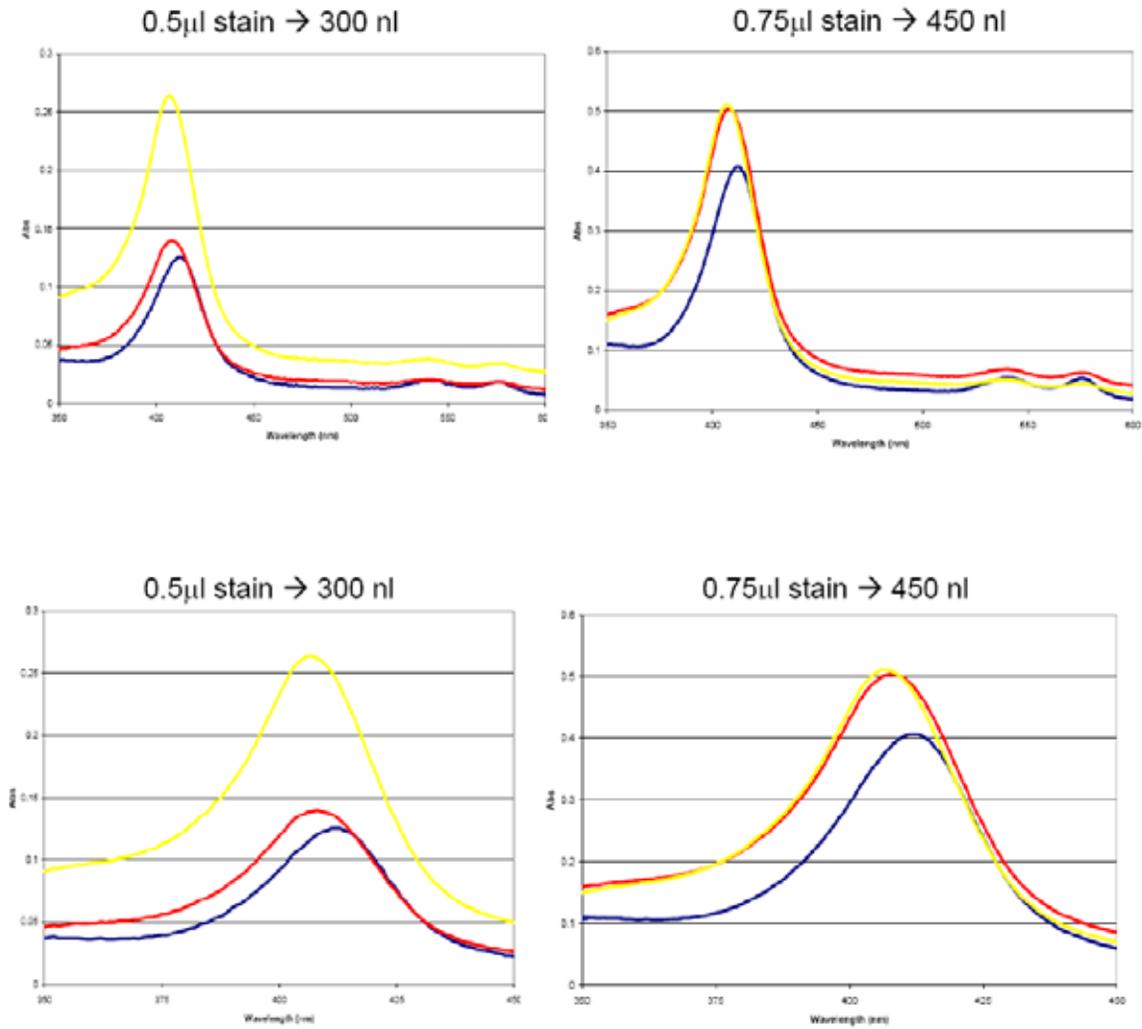
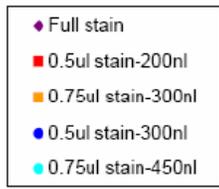
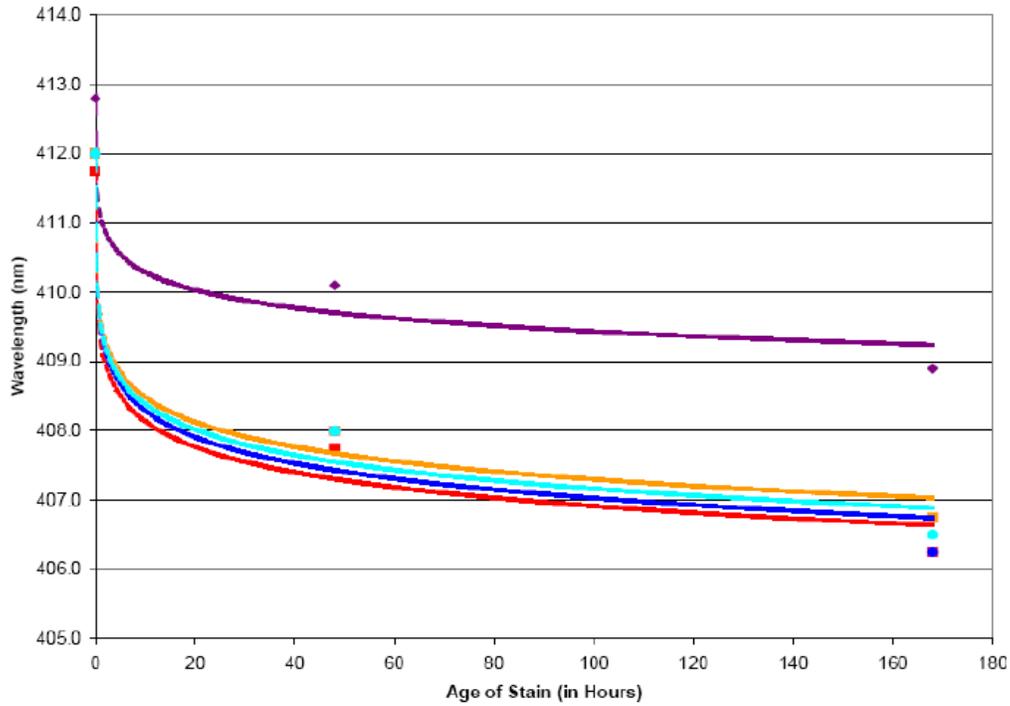


Figure 18. Recovery of Spectral Profiles from <1µl Bloodstains



Full stain:

$r^2 = .9656$
 $y = -0.3709\ln(x) + 411.14$

0.5 μ l – 200nl:

$r^2 = .9782$
 $y = -0.5313\ln(x) + 409.36$

0.5 μ l – 300nl:

$r^2 = .967$
 $y = -0.5476\ln(x) + 409.55$

0.75 μ l – 300nl:

$r^2 = .9877$
 $y = -0.5149\ln(x) + 409.67$

0.75 μ l – 450nl:

$r^2 = .9782$
 $y = -0.5313\ln(x) + 409.61$

Figure 19. Accuracy of $\Delta\lambda$ Soret Measurements using $<1\mu$ l Bloodstains

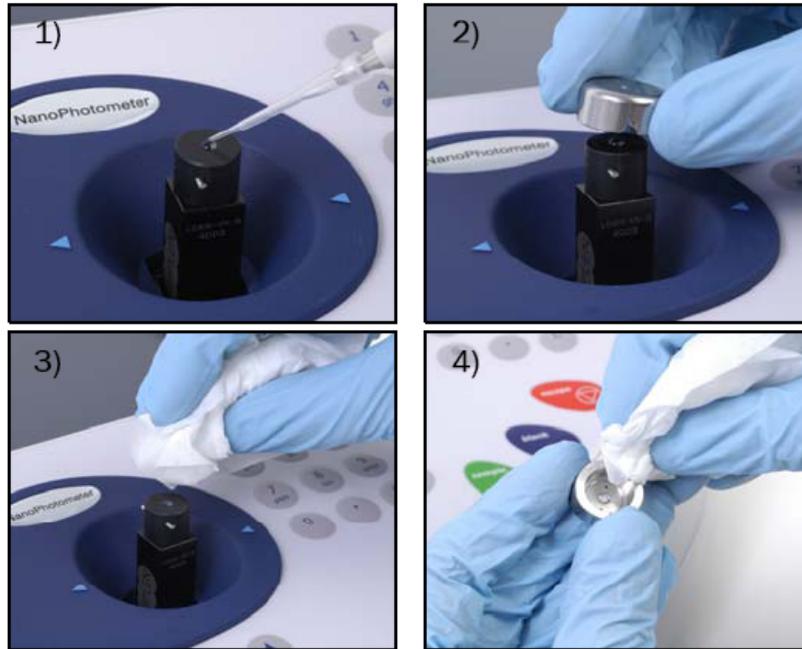


Figure 20. Sample Input Using the NanoPhotometer™ (Implen, Inc.)

Wavescan 19/06/2008 14:22:09

Implen NanoPhotometer 1375
19/06/2008 14:22:09

Product NanoPhotometer
Version 7122 V1.6.1
Serial Number 1375
Date 19 June 2008
Time 14:22:09

Instrument Calibration Pass
19 June 2008 13:43:55
Start Wavelength 350 nm
End Wavelength 650 nm
Mode Absorbance
Pathlength 0.2 mm

Sample 37

Peak Detection
Minimum Height 0.01 A
Minimum Width 5 nm
Peaks Sorted By Wavelength

Peak nm	Absorbance A
412	0.582
540	0.162

Cursor nm	Absorbance A
500	0.119

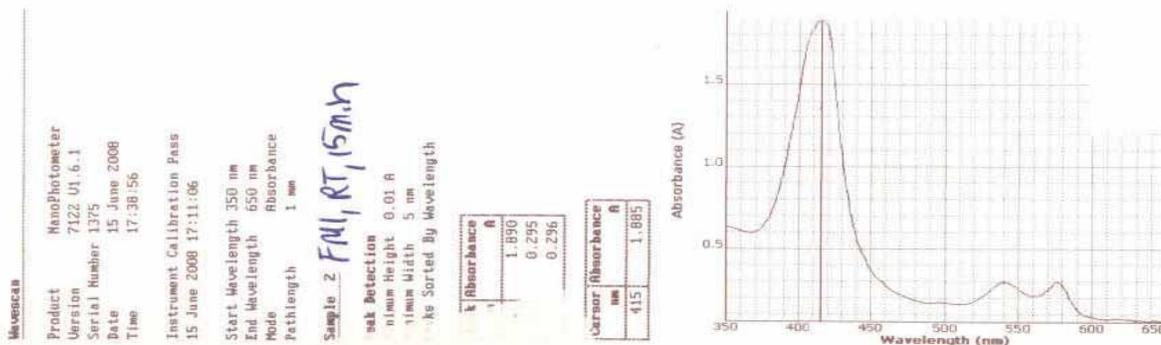


Figure 21. Output Displays from the NanoPhotometer™

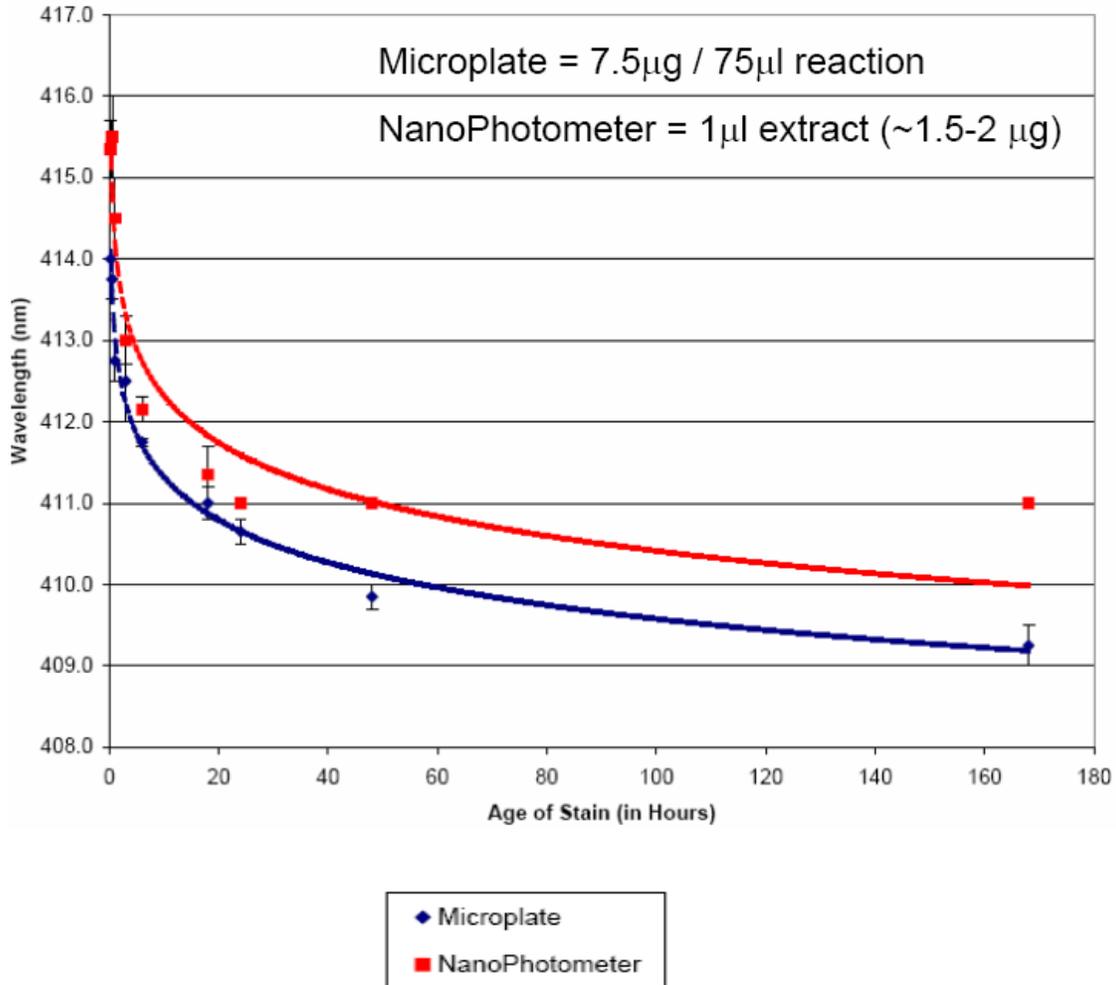


Figure 22. Accuracy of $\Delta\lambda$ Soret Measurements using the NanoPhotometerTM

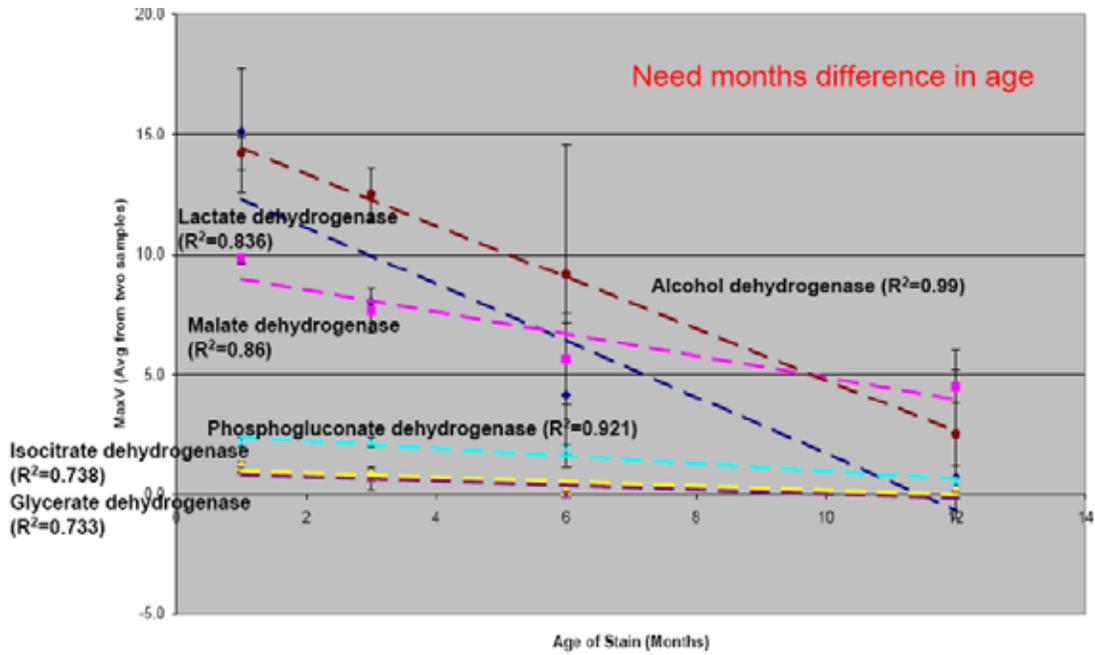


Figure 23. Loss of Enzyme Activity in Aged Bloodstains

APPENDIX B: TABLES

Table 1. Evaluation of the correlation between age of the stain and the Soret band hypsochromic shift using various age intervals

Sample	R^2 value			
	15min-2days	15min-1 week	15min-1 month	15 min-1 year
	“Hours”	“Days”	“Weeks”	“Months”
22°C	0.9550	0.9481	0.9566	0.9626
37°C	0.9843	0.9667	0.9556	0.8430

Table 2. Comparison of the $\Delta\lambda_{\text{Soret}}$ For Bloodstains Stored at 22°C (15 min–1 year) Using Two Different Spectrophotometers

Sample	λ_{Soret} – Microplate Reader	λ_{Soret} – UV Spectrophotometer	Difference
15 min	412.5	414.0	1.5
30 min	412.0	414.0	2.0
1 hour	412.0	413.7	1.7
3 hours	411.3	413.0	1.7
6 hours	411.0	411.7	0.7
18 hours	411.0	412.0	1.0
24 hours	410.8	411.8	1.0
48 hours	410.0	411.0	1.0
1 week	408.7	410.3	1.6
1 month	408.0	409.2	1.2
3 months	407.5	408.7	1.2
6 months	408.0	408.7	0.7
1 year	407.5	406.3	-1.2
	Total shift = 5nm	Total shift = 7.7nm	Average = 1.1nm

Table 3. Comparison of R^2 Values for Correlation of Hypsochromic Shift and Age of the Stain Using Two Different Spectrophotometers

	22°C		37°C	
	15min-48hrs	15min-1yr	15min-48hrs	15min-1yr
MicroplateReader	0.9462	0.9592	0.9858	0.8250
UV- Spec	0.9180	0.9577	0.9534	0.8130

Table 4. Comparison of λ Soret for Standard Size Bloodstains Extracts (60 μ l) and Bloodstains < 1 μ l

	Average λSoret		
	15 minutes	48 hour	1 week
Full stain (60μl)	412.8	410.1	408.9
0.75 μl stain	412.0	408.0	406.0
0.5 μl stain	412.0	408.0	406.0

Table 5. List of Enzyme Candidates For Use in TSD Determinations

Enzyme	Detection	Secondary Detection
Lactate dehydrogenase	NAD	MTT-formazan
Malate dehydrogenase	NAD	MTT-formazan
UDPglucose dehydrogenase	NAD	MTT-formazan
Glycerate dehydrogenase	NAD	MTT-formazan
Galactose dehydrogenase	NAD	MTT-formazan
Octanol dehydrogenase	NAD	MTT-formazan
Aldehyde dehydrogenase	NAD	MTT-formazan
Fumarate hydratase	NAD	MTT-formazan
Dihydrooroate dehydrogenase	NAD	MTT-formazan
L-glutamate dehydrogenase	NADP	MTT-formazan
Isocitrate dehydrogenase	NADP	MTT-formazan
Phosphogluconate dehydrogenase	NADP	MTT-formazan
Glucose-6-phosphate dehydrogenase	NADP	MTT-formazan
Aconitase	NADP	MTT-formazan
Phosphoglucomutase	NADP	MTT-formazan
L-xyulose reductase	NADP	MTT-formazan
Glycerol dehydrogenase	NADP	MTT-formazan
Carbonic anhydrase	4-MU	
Glycerol -3-phosphate dehydrogenase	MTT-formazan	
Alcohol dehydrogenase	MTT-formazan	
3-Hydroxybutyrate dehydrogenase	MTT-formazan	
Gluconate dehydrogenase	MTT-formazan	
Guanine deaminase	MTT-formazan	
Succinate dehydrogenase	MTT-formazan	
Purine nucleoside phosphorylase	MTT-formazan	
Aldehyde oxidase	NBT-formazan	
L-amino acid oxidase	NBT-formazan	
Sarcosine Oxidase	NBT-formazan	
Uricase	NBT-formazan	
Cytochrome-C oxidase	Indophenol blue (vis)	
Cholinesterase	Fast Red RT	
Acid phosphatase	Fast Blue BB	

Table 6. Determination of the Time Required For Complete Loss of Enzyme Activity in Aged Bloodstains

Enzyme	Months to Reach 0 Activity
Glycerate dehydrogenase	10.1
Lactate dehydrogenase	11.4
Isocitrate dehydrogenase	11.8
Alcohol dehydrogenase	14.4
Phosphogluconate dehydrogenase	16.0
Malate dehydrogenase	20.7
L-xyulose reductase	28.7
3-hydroxybutyrate dehydrogenase	144.7
Phosphoglucomutase	Stable
Glucose-6-phosphate dehydrogenase	Stable
Gluconate dehydrogenase	Stable

APPENDIX C: PRESENTATIONS AND PUBLICATIONS

PUBLICATIONS

A manuscript identifying the Hemoglobin spectral shift parameters and their use to determine the time of deposition of forensic bloodstains will be submitted for publication upon completion of the humidity experiments. (Currently in preparation; Expected submission to the Journal of Forensic Science in August 2008).

PRESENTATIONS

- 2007 Hanson, E., Ballantyne, J. "Determination of the Age (Time Since Deposition) of a Biological Stain." NIJ Grantees Meeting. July 23-25, Crystal City, VA.
- 2008 Ballantyne, J., Hanson, E. "Hypsochromic spectral shifts of the hemoglobin Soret band correlate with the time since deposition (TSD) of dried bloodstains." Presented at Round Table Discussion meeting, SWGDAM, July 14-16.
- 2008 Hanson, E., Ballantyne, J. "Hypsochromic spectral shifts of the hemoglobin Soret band correlate with the time since deposition (TSD) of dried bloodstains." NIJ Grantees Meeting. July 23-25, Crystal City, VA.

2008 Hanson, E., Ballantyne, J. "Hypsochromic spectral shifts of the hemoglobin Soret band correlate with the time since deposition (TSD) of dried bloodstains."

**Abstract accepted for the 19th International Symposium on Human Identification, Hollywood, CA; October 13-16, 2008.

2009 Hanson, E., Ballantyne, J. "Hypsochromic spectral shifts of the hemoglobin Soret band correlate with the time since deposition (TSD) of dried bloodstains."

**Abstract submitted for the 61st Annual AAFS Meeting, Denver, CO; February 16-21, 2009.

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