Development of bioaffinity based assays for forensic applications

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DEVELOPMENT OF BIOAFFINITY BASED ASSAYS FOR FORENSIC APPLICATIONS

By:

Juliana M. Agudelo Cano

A dissertation submitted to
The University at Albany, State University of New York
In partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

College of Arts and Sciences
Department of Chemistry
FALL 2021
This thesis is dedicated to my mother,
Gloria B Cano Montoya.

For her endless love, support, and encouragement throughout all these years.

Esto es para vos, mamá.
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ABSTRACT

In the field of forensic science, body fluids are a major contributor of evidence in investigations. However, body fluids are usually used only for their genetic content, overlooking its unique composition of proteins and low-molecular-weight compounds that often serve as biomarkers in clinical diagnostics. This investigation aimed to develop simple, effective, and economical models that can be used to identify different attributes of the originator. The focus of this research was to develop biocatalytic cascades that could analyze body fluids by using UV-Vis spectroscopy. These models could be implemented in forensic settings, as well as in biometric authentications. The central points were: I) Forensic determination of blood sample age using a bioaffinity-based assay, II) The simultaneous estimation of a blood spot’s time since deposition and age of its originator via a bioaffinity assay, and III) Continuous Tracking Utilizing Amino Acids in Skin Secretions for Active Multi-Factor Biometric Authentication for Cybersecurity.

Genetic material analysis is usually the main use for body fluids in Forensic serology; due to the natural high volume of samples that are collected daily in the country, alternative analytical tools that can quickly assist in forensic investigations are a top priority in developments. Thus, in this research enzymatic activity was measured applying the flexibility of UV-Vis spectroscopy; taking advantage of the proteins properties found in body fluids, and the different enzymatic and amino acid levels of individuals.
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A significant amount of the work reported in this thesis has been published. Copyright permissions for the inclusion of this previously published work, such as that which appears in Chapters 2-4 were acquired. These are appended here in facsimile form (see below). All of the experimental work reported herein, as well as that which is in the referenced publications, was performed by Juliana Agudelo. Substantial contributions to the writing of the featured manuscripts were also made by Juliana Agudelo. The previously published content is included not only because it forms a substantial segment of the body of research that was conducted to fulfill the requirement for the Ph.D., but also because it provides an important contextual framework within which to view the successful development of the proofs of concept described here. A listing of the publications whose content has been integrated in whole or in part into this thesis is enumerated below. This is followed by facsimiles of the permissions from the publishers.


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABO</td>
<td>A, B, AB, and/or O blood type</td>
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<tr>
<td>ABTS</td>
<td>2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5′-diphosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5′-diphosphate</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
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<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>AlaDH</td>
<td>alanine dehydrogenase</td>
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>ALT</td>
<td>alanine transaminase</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5′-triphosphate</td>
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<tr>
<td>ATP</td>
<td>adenosine 5′-triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>CEBR</td>
<td>Capacity Enhancement and Backlog Reduction</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>Crt</td>
<td>creatine</td>
</tr>
<tr>
<td>Crt-P</td>
<td>creatine phosphate</td>
</tr>
<tr>
<td>Crt-P</td>
<td>creatine phosphate</td>
</tr>
<tr>
<td>CODIS</td>
<td>Combined DNA Index System</td>
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<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>Glc-6-P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>KTG</td>
<td>α-Ketoglutaric acid</td>
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<td>L-Amino acid oxidase</td>
</tr>
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<td>Lac</td>
<td>Lactate</td>
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<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
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<td>LDHA</td>
<td>L-Lactate dehydrogenase</td>
</tr>
<tr>
<td>LOx</td>
<td>Lactate oxidase</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted liquid desorption ionization</td>
</tr>
<tr>
<td>MANOVA</td>
<td>Multivariate analysis of variance</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>NAD+</td>
<td>β-Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>β-Nicotinamide adenine dinucleotide reduced</td>
</tr>
<tr>
<td>NADP+</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>NADPH</td>
<td>dihydronicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NIJ</td>
<td>National Institute of Justice</td>
</tr>
<tr>
<td>NIR</td>
<td>near infrared</td>
</tr>
<tr>
<td>PEP</td>
<td>phospho(enol)pyruvic acid</td>
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<td>PheDH</td>
<td>phenylalanine dehydrogenase</td>
</tr>
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<td>PhPyr</td>
<td>phenylpyruvate</td>
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<tr>
<td>PK</td>
<td>pyruvate kinase</td>
</tr>
<tr>
<td>pNP</td>
<td>p-nitrophenol</td>
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<tr>
<td>pNPP</td>
<td>p-nitrophenol phosphate</td>
</tr>
<tr>
<td>Pyr</td>
<td>pyruvate</td>
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<tr>
<td>RMSE</td>
<td>root mean squared error</td>
</tr>
<tr>
<td>RMSEP</td>
<td>root mean square error of prediction</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operating characteristic</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SVM</td>
<td>support vector machine</td>
</tr>
<tr>
<td>SVMDA</td>
<td>SVM in the form of discriminant analysis</td>
</tr>
<tr>
<td>SVMR</td>
<td>support vector machine regression</td>
</tr>
<tr>
<td>TSD</td>
<td>time since deposition</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible (UV-Vis) spectroscopy</td>
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**Figure 3.3 B.** ROC Female

The tradeoff between sensitivity and specificity are shown by presenting data ROC curve. The AUC is 100%, which is the probability for the assay to identify young and old group based on the ALP levels in blood. The optimum cutoff point was chosen with sensitivity of 100% and specificity of 100%. Random choice is denoted by the grey diagonal line.44

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CHAPTER 1: INTRODUCTION

The main focus of this research was to develop biocatalytic cascades that could analyze blood spots directly at the crime scene. These models can provide binary “Yes/No” responses, which can ratify or disregard a suspect’s physical characteristics. The design aimed for economic, efficient, and straightforward detection models that can be used on-site for the analysis of forensic samples by individuals that do not have scientific training. It provides an additional tool for developing forensic investigations prior to samples being taken to a laboratory for DNA analysis; having the potential to speed up investigations and helping to make informed decisions regarding forensic cases. The bioaffinity-based cascades can determine blood originator characteristics, such as ethnicity, sex, and age. Forensic serology relies heavily on genetic material analysis; due to the natural high volume of samples that are collected daily in the country, it is important to develop alternative straightforward analytical tools that can rapidly aid in forensic investigations. In this work enzymatic reactions were quantified by taking advantage of the proteins properties, the different enzymatic levels of individuals and utilizing the versatility of UV-Vis spectroscopy. The two central points were: I) Forensic determination of blood sample age using a bioaffinity-based assay, II) The simultaneous estimation of a blood spot’s time since deposition and age of its originator via a bioaffinity assay.

Biocatalytic cascades can be utilized for biometric authentication by analyzing skin secretions. This new concept that utilizes amino acids found in sweat can be exploited for the establishment of an amino acid profile capable of identifying an individual user of a mobile or wearable device. Active multi-factor biometric authentication for cybersecurity is a new concept, this investigation outlines preliminary results for utilizing forensic biometrics to develop a new
biochemical approach to data collection for continuous active authentication and trace-history information gathering.

1.1 Brief introduction to spectroscopy

The development and use of spectroscopy to understand our surrounding started in 1801 by William Wollaston, when he discovered the existence of dark lines in the solar spectrum. Since then, thanks to the contribution of numerous scientists, spectroscopy techniques have elucidated infinite aspects of the universe. By studying the interaction between light and matter and depending on the resulting absorption and emission of light, spectroscopy provides different properties of the analyte. All analytes have specific wavelengths at which they absorb light, after light absorption there are three main resulting events: photoluminescence, transmission, or scattering.

Some of the main spectroscopy techniques include, but are not limited to: Raman spectroscopy, nuclear magnetic resonance (NMR), nuclear quadrupole resonance (NQR), microwave and gamma-ray spectroscopy, electron paramagnetic resonance (EPR), electron spin resonance (ESR), Infrared (IR) Spectroscopy, and Ultraviolet-Visible (UV/Vis) Spectroscopy.

1.1.1 Ultraviolet-Visible (UV-Vis) Spectroscopy

UV-Vis is a widely used analytical technique due to its versatility and wide range of molecules that can be analyzed. Absorption in the UV (~200–400 nm) and visible (~400–750 nm) ranges occur from electronic transitions between energy states in a chromophore. When the Uv-vis light passes through a sample, the transmittance of the light is measured based on the transmittance (T), the absorbance (A) can be calculated as [A=-log (T)]. The resulting spectrum shows the absorbance of a compound at different wavelengths. This absorbance is due to the
chemical structure of the analyte; chromophores with aromatic structures, such as amino acids, nucleic acid bases, and NADH, absorb in the accessible region of the spectrum.

Uv-vis experiments are generally inexpensive, simple to perform, and very informative. One of the most commonly used applications of this technique is to measure the concentration of proteins, such as NADH, by using Beer’s law, where there is a direct relationship between absorption and concentration of the protein.[1]

\[ A = \varepsilon lc \]

In Beer’s law “A” is the absorbance, “\( \varepsilon \)” is the molar absorptivity, with units \( \text{Liters} \cdot \text{moles}^{-1} \cdot \text{centimeters}^{-1} \); “c” represents the concentration and has the units \( \text{moles} \cdot \text{Liters}^{-1} \); and “l” is the path length (typically reported in centimeters).

For this work, all absorbance measurements were performed using a UV–Vis spectrophotometer/microplate reader, SpectraMax Plus 384 - Molecular Devices (San Jose, CA). This spectrophotometer uses a xenon flash lamp as its light source and possesses a monochromator for wavelength selection. A photodetector converts absorbed light into a measurable electric current. This instrument is a microplate reader where multiple samples can be analyzed in sequence by using 96-well microtiter plates.[2] See scheme of the SpectraMax Plus 384 below.
1.2 BACKGROUND AND SIGNIFICANCE

1.2.1 Current challenges in forensic serology

The crime scene investigations, where victims and suspects need to be identified, require tedious and expensive processes that can take an extensive amount of time (months or years) to complete. The process of identifying victims and suspects consists of sample collection from the crime scene and then transportation to a specialized laboratory for analysis. This process is also documented with a “chain of custody”, in which every single person who has had the evidence in their possession has to be reported in order to make sure the evidence has not been tampered with. The most common technique used for biological samples is DNA analysis, which provides exclusive genetic information from the individual that it originated from. Due to the complex nature of this type of analysis, it cannot be immediately performed at the crime scene in order
to determine its evidentiary value. The major drawback in crime scene investigations, currently, is the lack of techniques and equipment that can analyze biological samples on-site in order to provide fundamental information of the sample originator.

This limitation often costs law enforcement time and money, as they have to wait for relevant information that could be used to help the investigation progress. The research presented here focused on aiding in the development of on-site forensic serology techniques that can maximize the use of bloodstains prior to DNA analysis. Blood is one of the most common bodily fluids found at violent crime scenes; for many years people have only focused on blood spot pattern and DNA evidence, however blood spots can offer more immediate information prior to being taken to a laboratory to be analyzed.

In addition, most of the crime laboratories in the country are backlogged due to the large amount of samples they receive on a daily basis.[3, 4] The government has developed the “Capacity Enhancement and Backlog Reduction Program” (CEBR) [4] in an attempt to alleviate some of this pressure, but it is insufficient due to the high demand of samples that need to be analyzed. However, the biggest downside is that this backlog makes the turnaround time for a DNA sample around 150 days. Even though the program has improved the time table, it still takes more time than law enforcement would like.[3] Since 2004, the Department of Justice (DOJ) has awarded nearly one billion dollars to states and local jurisdictions through the CEBR program to help increase lab capacity and reduce the amount of DNA evidence awaiting analysis at laboratories. Still, although labs are processing more requests, backlogs of crime scene evidence in laboratories continue to increase.[3-5] According to a report from the Government Accountability Office, eliminating the backlog may be “unachievable” because as science
advances, there is a surging demand for new tests. They estimated that for every request that is fulfilled, 1.2 requests are generated.[5]

After the evidence collection from a crime scene, initial desired information includes: the sex, ethnicity and age of the suspect or victim. These questions are rarely addressed by the currently used analyses. However, it is possible to draw conclusions about the general health of the sample originator, along with identifying drugs or medications present in the circulatory system, depending on the type of evidence left behind. Although this information can be gathered from the samples, it takes in average 150 days and overlooks important physical characteristics that can be obtained from the same sample. This time can be crucial to finding the originator of the sample that could be either the victim or the suspect. In addition, the intricate nature of the decomposition of biological samples plays a major role in these periods of waiting, thus, quick analysis techniques should be preferred for immediate information.

Scientific analysis of biological traces left at a crime scene, both persons of interest as well as victims, gives some of the most valuable information investigators can use to elucidate crimes. Drug metabolites and DNA analysis have been major breakthroughs in the field; however, the major drawback remains on the complex instrumentation required and the high demand for these analyses increasing the time and cost of the investigations. Currently, there is also a deficiency in portable equipment for performing on-site analysis of biological samples, which is currently restricted to the discrimination of biological samples, such as fingerprints and blood.[6]

This approach uses bioassays for the analysis of enzymes and other proteins for the application of novel paradigms in forensic science. It will provide crucial personal information about the originator’s phenotype on-site, reduce costs, and expedite investigations. This
investigation focused its efforts to develop bioanalytical assays that eventually could be performed directly at the crime scene by law enforcement personnel to identify if blood samples were relevant to the particular case, prior to DNA laboratory analysis, and to identify certain attributes of the blood spot originator.

1.2.2 Literature review

The forensic science field has revolutionized investigations and complements the procedures and decisions brought to court by providing unbiased facts about crimes. Before the advent of forensic science, a large emphasis was placed on circumstantial evidence and witness testimonies. Now, one of the major scientific supports for criminal investigations and subsequent prosecutions are biological samples left at the crime scene.[7, 8] The analysis of biological evidence is indispensable for the identification of victims and suspects. Forensic serology, a branch of forensic science, analyzes biological fluids such as saliva and blood,[7-10] by using chemical and biochemical techniques.[7, 11] Currently, bloodstains are restricted to provide mainly two types of information, genetic material and blood spatter for the reconstruction of the crime scenes, overlooking major components that blood contains which could contribute immensely to the investigation.

DNA profiling and DNA matching are molecular biology techniques to analyze genetic material from the DNA short tandem repeats (STR-DNA). Combined DNA Index System (CODIS) is a DNA database created by the National Institute of Justice (NIJ) to identify recurring people that commit crimes, or their relatives.[12-14] Most of the STR-DNA is usually located in the noncoding regions of the genome, which is a weakness in the elucidation of the originator’s phenotype. The genetic material found at the crime scene is one of the primary pieces of information that the
legal system use in court to connect suspects to the investigations, however if the sample originator is not in custody or the DNA profile is not saved in CODIS, this information is obsolete for the investigation. Consequently, single nucleotide polymorphisms (SNPs) are currently being studied to determine phenotypes, such as hair color, skin color, ethnicity, and eye color.\textsuperscript{[14-16]} The downside of this technique is that the phenotype can be masked by environmental and nutritional factors,\textsuperscript{[14]} not to mention the sophisticated instrumentation that this requires, causing the laboratories to become backlogged and further increasing the time and cost of the investigation.\textsuperscript{[8, 17]} Crime laboratories around the country are experiencing these backlogs\textsuperscript{[3, 4]} due to the high demand of DNA analysis from the court system, hence why NIJ developed the “Capacity Enhancement and Backlog Reduction Program”,\textsuperscript{[4]} as mentioned in section 1.1, Current challenges in forensic serology.

The most commonly used methods for the analysis of biological traces include, but are not limited to, PCR and electrophoresis.\textsuperscript{[7, 18]} These molecular methods are reliable and accurate, but are not suitable for on-site analysis. Due to the extensive backlogs and the complex nature of the analyses, obtaining crucial information from the sample originator, such as age or sex, is a lengthy and expensive process. The inability to obtain basic information about the sample originator on-site demonstrates the lack of potential technology for on-site characterization and detection of biological samples.

One of the promising developments for on-site technology used for crime scenes is ParaDNA screening units that were developed by LGC Forensics.\textsuperscript{[19]} By combining PCR and fluorescence, ParaDNA determines if human DNA is present in a biological sample. The analysis software within the screening unit detects any changes in fluorescence, which results from the
melting of a fluorescent HyBeacon™ probe[20], – used to identify and monitor the amplification of specific DNA sequences, away from the amplified sequence. Lastly, ParaDNA screening can be used to determine sex based on the presence of Amelogenin X and Y alleles.[21] However, the analysis requires up to 75 minutes and is limited exclusively to the identification of human origin and sex. [22] According to Kulstein and colleagues, at the moment the ParaDNA system cannot meet the challenges of forensic relevant samples, such as mixed bodily fluids, and therefore cannot be recommended for implementation into forensic routine casework.[23]

In clinical diagnostic testing, “point-of-care” systems have become very popular over the last decade and have been used on a daily basis by regular people, not trained clinical staff.[24] The point-of-care techniques analyze different levels of biomarkers found within the body and are used routinely in clinical settings, this same concept can be implemented in crime scene settings. In addition to nucleic acids, blood also contains proteins and low molecular weight compounds that can be used as biological markers. Age of the blood spot can be determined using biocatalytic cascades.[25] Also, sex determination and differentiation between African American and Caucasian have been elucidated using biocatalytic assays as well.[26, 27]

The unique composition of blood has allowed not only for the identification of human origin from other types of blood, but also the identification of different characteristics of the originator. This has been done through several analytical methods. Human blood has been identified using techniques such as high-performance liquid chromatography (HPLC), mass spectrometry (MS), and Raman or infrared spectra,[28-30]. In addition to instrumental techniques, the precipitin test can also be performed.
This work was focused on the creation of innovative human blood bioanalytical assays that can be used directly at the crime scene. The re-programmable bioaffinity-based cascades can determine if the blood is of human origin and the characteristics of the originator such as ethnicity, sex, and age. The main purpose was to design economic, efficient, and straightforward detection models that can be used on-site for the analysis of forensic samples by individuals that do not have scientific training. These groundbreaking models can provide binary “Yes/No” responses, which can ratify or disregard a suspect’s physical characteristics. This can be achieved by creating a sensing chip or lateral flow strip, similar to the ones used for a pregnancy test or glucometer. The sensing chips or lateral flow strips can be incorporated into the equipment of law enforcement members for the purpose of crime scene investigations, reducing the cost and the time of the investigations. Currently, intricate biosensor platforms described throughout this proposal do not require bulky instrumentation such as spectrophotometers, but instead can be connected to small devices, such as smartphones or tablets.[31]

This methodology branches from a new area of biosensing that uses affinity-based biomolecular systems that can be linked together to perform combination processes. Complex, but adaptable biomolecular designs can be pre-programmed to evaluate specific biochemical information from blood samples. This networking system facilitates the processing of complex combinations of biomarkers, where the parallel analysis of different characteristics of the blood sample originator would be much simpler than it is today with the methods currently available.

This proposal has the potential to establish pioneering bioanalytical techniques where the input of biomarkers results in a basic diagnostic conclusion in a binary “Yes/No” format.[32, 33] The first applied model systems were used to analyze biomarkers in medical settings, specifically
for traumatic injuries.[34-41] Furthermore, this system successfully expanded to animal studies for the analysis of a combination of numerous markers of interest in a single analytical test.[39]

It is important to note that these analytical methods provide qualitative information rather than quantitative (“positive/negative”).

Next, the bioassay paradigms that can be successfully applied to forensic analyses will be discussed. The differentiation between sexes[27], as well as the distinction between African American and Caucasian[26] ethnicities from anonymous human serum samples, has led to the motivation to design more bioanalytical assays along these lines. These projects received significant public[42, 43] and scientific[44-46] attention, which gives confidence that the results from this investigation will have a large impact within the academic and law enforcement communities.

The main objective of this work aimed on identifying different originator characteristics from a blood sample by developing sensing systems. It began with the basic single feature-aimed sensor and concluded with the development of a dependable, quick, and precise multiplex-sensing paradigm fully applicable in the area of on-site forensic analysis. Previous studies have shown the potential of the bioanalytical assays applied to real human serum samples as a helpful tool for forensic investigations. These systems were able to discriminate between African American and Caucasian.[26, 47] The determination of sex[27] was also achieved with this methodology, as mentioned above.

Just like blood can provide a large amount of information from the originator, other bodily fluids can be exploited in the same manner. Biochemical processing of metabolites present in skin secretions can be utilized for biometric-based cybersecurity systems. The amino acids found
in sweat can be exploited for the establishment of an amino acid profile capable of identifying an individual user of a mobile or wearable device.[48, 49] Individual and combinations of amino acids processed by biocatalytic cascades yield physical (optical or electronic) signals, providing a time-series of several outputs that, in their entirety, should suffice to authenticate a specific user based on standard statistical criteria.[50]

Biological samples are routinely analyzed to solve investigations, but many aspects of the information that these can provide are overlooked. DNA has played a very important role to solve different investigations; however, there is currently no assay that can be used on site. Biocatalytic cascades are not designed to replace existing technology or processes, but it provides an additional valuable tool for developing investigative intelligence from expert or appropriately trained non-expert operators; having the potential to speed up investigations and helping to make informed decisions regarding forensic cases.

REFERENCES


CHAPTER 2: Forensic determination of blood sample age using a bioaffinity-based assay

2.1 ABSTRACT

A bioaffinity-driven cascade was developed to determine the time elapsed from the point at which a blood sample was left at a crime scene to the point of discovery. The healthy adult physiological concentrations of creatine kinase (CK) and alanine transaminase (ALT), were utilized to determine the time since deposition (TSD) of a blood spot based on their natural denaturation process. This research was conducted using human serum samples that underwent an aging process under three different temperatures to resemble potential environmental conditions that may occur at crime scenes. The CK/ALT biocatalytic cascade was composed of two parallel subsystems, with each of them following the activity of one marker. Both markers have very distinct denaturation rates – one that is too fast and one that is too slow – which does not allow them to be used in a single marker setup. However, by tunable monitoring of both markers simultaneously, it was possible to provide information regarding the TSD of a blood sample with low temporal error for a prolonged period of time. To further support this investigation, these samples were aged under the aforementioned conditions for up to 5 days.

2.2 INTRODUCTION

Forensic investigations were revolutionized upon the introduction of biological samples to the investigation process. This importance stems from their ability to elucidate important facts about the crime scene and the crime itself. Biological traces found at crime scenes represent important leads in identification and the subsequent confirmation of possible suspects. Chemical and biochemical techniques utilized for the analysis of biological traces at crime scenes are the main scientific support of criminal investigations and subsequent prosecutions.[1] Forensic
serology, a branch of forensic science, focuses on the analysis of biological fluids, including but not limited to saliva and blood.[1] Blood is deemed one of the most commonly encountered bodily fluids at violent crime scenes. Blood patterns are analyzed based on shape, size, geometry and location, [2, 3] these characteristics can vary significantly, making crime scene reconstruction analysis difficult.[4-7] Blood is a major source of critical information for investigators in violent crimes cases such as, homicides, or murders, representing a major piece of forensic evidence[8-10].

In addition to its commonality at crime scenes, blood is also one of the most common sources of DNA. Because of this, blood samples are often extracted for DNA, specifically short tandem repeat (STR)-DNA, using a variety of molecular biology techniques. Once DNA is obtained, it can be used for DNA profiling and DNA matching to determine the identification of the originator. [11, 12]

However, blood is not only a valuable source of information because of its visibility or possession of genetic material. It is also valuable because of the unique composition of proteins and low molecular compounds present in the circulatory system. Specifically, the use of these compounds can be used for determining the age of a blood sample left at a crime scene. This information has the potential to be incredibly valuable in several situations. For instance, multiple blood spots or spatters could be present at a crime scene and there would be no guarantee that every sample is relevant to the case. Having the ability to estimate the age of a blood spot would be significantly important in identifying blood that is relevant to a crime investigation and ruling out that which is not. Furthermore, many persons-of-interest often claim they were present at a location before the time at which the crime was committed. Some spots could have been left
days, weeks, or even months ago and to separately match every sample to an individual in order to establish a timeline or alibi would be costly and time consuming. Currently, there is only a technique available for distinguishing two individuals from overlapping bloodstains,[1, 13] but no technique to identify how old the samples are, and therefore, they may be completely irrelevant. Being able to estimate the age of the blood samples would allow investigators to exclude samples too old to be relevant and reduce the amount of DNA work needed, therefore aiding in the effort to improve turnaround times.

Due to the degree of denaturation and other changes, blood samples have the potential to provide the time that has elapsed since the blood left the originator's body. Numerous techniques have been suggested for this purpose over the past decades, but due to the lack of practicality they have never been practically applied.[14] In 1930, the pioneer in this area, Schwarzacher, attempted to find the relationship between the solubility of blood in water and its age.[15] Spectrophotometry analysis was first applied in 1960 by Patterson[16], where he determined that the change in color of a bloodstain is dependent on environmental conditions by recording the bloodstain's reflectance. Later, in 1983, Tsutsumi studied changes in the individual proteins present in bloodstains.[17] More recent methods for blood analysis at a crime scene include electron paramagnetic resonance (EPR)[18], high performance liquid chromatography (HPLC)[19], oxygen electrodes[20], RNA degradation[21], near infrared (NIR) spectroscopy[22], atomic force microscopy (AFM)[23] and Raman spectroscopy[24]. Unfortunately, the vast majority of these techniques require sample preparation and need to be performed in a laboratory setting, preventing the possibility to perform direct analysis at the crime scene which adds time and possible error to investigation. In addition to these
requirements, these techniques have yet to report any environmental influences that play a key factor in the decomposition of the sample, such as humidity, temperature, or exposure to light.[23]

In the research presented here, a novel tunable parallel assay was developed for the analysis biomarkers for the TSD of blood samples left at crime scenes. Two protein markers, the creatine kinase (CK; E. C. 2.7.3.2) and alanine transaminase (ALT; E. C. 2.6.1.2), were used in a concerted manner to determine the age of bloodstain samples.[25-28] The mean concentrations of markers, CK (100 mU/mL) and ALT (20 mU/mL), were based on their physiological levels present in a healthy adult.[25-28] Previously, the elevation of these markers, individually, were used as an indication of various illnesses and injuries.[29-33] In addition, the combination of these two markers has been used to distinguish the ethnic origins and sex of their donors.[34, 35] However, the combination of markers was used to follow the increase in response to the increase in the presence of the enzymes individually, whereas the methodology here follows the denaturation rate of the enzymes in parallel.

Here, a biocatalytic cascade was designed and optimized where the activity of both markers was followed in two parallel branches/subsystems, each following the enzymatic activity decay of one marker. Both subsystems represent the tunable elements; by modulation of their respective performances, the time for which the assay can follow the sample age with low temporal error and increased reproducibility can be flexibly prolonged or shortened.
2.3 MATERIALS AND METHODS

Scheme 2.1. The biocatalytic multi-marker cascade in which (A) represents the CK pathway, (B) represents the ALT pathway, and (A) and (B) together represent the entire biocatalytic CK/ALT cascade. The abbreviations used in the scheme are: CK (creatine kinase), PK (pyruvate kinase), LDH (lactate dehydrogenase), ALT (alanine transaminase), Crt (creatine), Crt-P (creatine phosphate), ATP (adenosine 5′-triphosphate), ADP (adenosine 5′-diphosphate), NAD⁺ (β-nicotinamide adenine dinucleotide), NADH (β-nicotinamide adenine dinucleotide reduced), PEP (phospho(enol)pyruvic acid), Pyr (pyruvate), Lac (lactate), Ala (alanine), KTG (α-ketoglutaric acid), and Glu (glutamic acid).

Scheme 2.1 represents the CK/ALT biocatalytic cascade, which follows the enzymatic activities of both blood markers. In the proposed cascade, performed in 50 mM glycyl-glycine buffer solution at pH 7.95, the biocatalytic reaction of the CK marker was coupled with the consequent reaction catalyzed by pyruvate kinase (PK; E.C. 2.7.1.40) to produce adenosine triphosphate (ATP) and pyruvate. In a parallel manner, the ALT marker also produced pyruvate via the deamination of alanine (Ala) with α-ketoglutaric acid (KTG) acting as a co-substrate. The last biocatalytic step is the reduction of pyruvate into lactate by lactate dehydrogenase (LDH, E.C. 1.1.1.27).
1.1.1.27), with the simultaneous consumption of NADH which can be spectrophotometrically followed at 340 nm (Scheme 2.1).

Segments A and B of scheme 2.1, respectively, indicate each subsystem of the biocatalytic assay. In this case, segment A represents the CK pathway, while segment B is composed of the ALT pathway. In the first sets of experiments each pathway was analyzed and optimized independently, where the cascade was following only one marker, CK or ALT, at a time. This allowed for the specific evaluation of the performance of each independent marker.

2.3.1 Chemicals and reagents used

All enzymes and substrates were purchased from Sigma-Aldrich and used with no further treatment: Creatine Phosphokinase from rabbit muscle, Type I (CK; E.C. 2.7.3.2), Glutamic-Pyruvic Transaminase from porcine heart (ALT; E. C. 2.6.1.2), Pyruvate Kinase from rabbit muscle, Type II (PK; E.C. 2.7.1.40), L-Lactic Dehydrogenase from rabbit muscle Type II (LDH, E.C. 1.1.1.27), serum from a human male (Type AB), creatine anhydrous (Crt), adenosine 5’-triphosphate disodium salt hydrate (ATP), phospho(enol)pyruvic acid monopotassium salt (PEP), L-alanine (Ala), α-Ketoglutaric acid disodium salt dihydrate (KTG), and β-Nicotinamide adenine dinucleotide reduced dipotassium salt (NADH). The water used for all experimental procedures was ultrapure water (18.2 MΩ•cm), obtained from PURELAB flex, ELGA water purification system.

2.3.2 Instrumentation and measurements

A temperature-controlled 96-well plate reader (SpectraMax Plus 384, Molecular Devices, CA) was used to take optical measurements of the samples at $\lambda = 340$ nm at 37° ± 0.1 °C. A microtiter polystyrene (PS, Thermo Scientific) plate was utilized. The samples were incubated in MyTempTM Mini Digital Incubator (VWR).
2.3.3 Composition and operation of the model systems

The first step in determining the TSD of a blood samples was to utilize human serum under mimicked conditions. To accomplish this, human serum samples spiked with the appropriate concentrations (CK: 100 mU/mL and ALT: 20 mU/mL) were based on their physiological levels present in a healthy adult males.[25-28] Markers were placed on a glass surface and allowed to age for variable periods of time (0, 6, 24, 48, 96 and 120 hours) at three different temperatures 18 °C, 25 °C and 40 °C. Time zero corresponds to the analysis of the freshly prepared sample.

Following the aging process, the dried samples were re-suspended with water just before being used for analysis. The biocatalytic cascade was activated by mixing the samples with the necessary enzymatic cascade substrates, co-substrates and auxiliary enzymes. The concentrations of the substrates were as follows: Crt 15 mM, ATP 10 mM, PEP 6 mM, KTG 1 mM and NADH 0.25 mM. The concentration of PK and LDH were 2 U/mL and 150 mU/mL, respectively. All reagents were prepared in 50 mM glycyl-glycine buffer (pH 7.95). Afterwards, the samples were immediately subjected to continuous optical measurement at \( \lambda = 340 \) nm in order to monitor the consumption of NADH. For each measurement taken, a set of three (n=3) human serum samples containing the markers was analyzed.

2.4 RESULTS AND DISCUSSION

As previously indicated, the aging process was followed at a rather high temperature (40 °C), which is not expected at most crime scenes, but allows us to enhance the aging effect on the particular markers. Figure 2.1, part A, shows the real time response (oxidation of NADH, as shown in Scheme 2.1) of the biocatalytic cascade subsystem following the CK marker as a function of the analysis time.
The inset bar diagram (Figure 2.1 B) illustrates the decay in response a function of blood sample age. Figure 2.1 shows the rather fast decay of the marker activity where after 6 hours of the aging period, the CK activity is reduced to approximately 20 % of its original level. This shows that the CK branch of the sensing cascade provides a low error of blood sample age determination, but its overall performance decreases drastically for samples older than 6 hours due to the lack of marker activity. To address this problem and simultaneously improve the tunability of the presented bioanalytical paradigm, ALT was also evaluated as a marker.

Figure 2.1. (A) Change in absorbance ($\lambda = 340$ nm) corresponding to the consumption of NADH upon operation of the CK-subsystem. These traces correspond to samples ($n = 3$) that mimic bloodstains, incubated at 40 °C from 0 hours to 120 hours, as indicated in the legend. (B) Inset bar diagram represents the change in absorbance at $\lambda = 340$ nm, after 30 minutes of assay completion.
In Figure 2.2, Part A shows the real time response of the samples analyzed via branch B – following only the ALT activity – under the same experimental conditions as the CK branch. Part B of Figure 2.2, shows that the enzymatic activity of this particular marker undergoes a constant decay within the aging process, where more than 50% of the enzymatic activity still remains after 120 hours. This would allow for the determination of the blood sample age even beyond the 120 hours. On the other hand, slow decay of the response in combination with a low signal change overall, resulted in a high temporal error of the blood sample age determination. This can be a substantial drawback, especially in shorter aging times.

Figure 2.2. (A) Change in absorbance (\( \lambda = 340 \) nm) corresponding to the consumption of NADH upon operation of the ALT-subsystem. These traces correspond to samples (\( n = 3 \)) that mimic bloodstains, incubated at 40 °C from 0 hours to 120 hours, as indicated in the legend. (B) Inset bar diagram represents the change in absorbance at \( \lambda = 340 \) nm, after 30 minutes of assay completion.
Following the analysis of the individual pathways, the next step was assess the functionality of the combined pathways. Figure 2.3A shows an example of real time responses in which both paths of the cascade are active and plotted as a function of analysis time. Figure 2.3B (inset bar diagram) illustrates the overall signal decay as a function of sample age. The resulting response forms a compromise within the system; for shorter times, it offers a significantly better response than a single (ALT) marker output, while still being able to assess samples at prolonged time points than that of CK alone. In the fully assembled cascade, the CK marker provides the

Figure 2.3. (A) Change in absorbance (λ = 340 nm) corresponding to the consumption of NADH upon operation of the CK/ALT representing the whole parallel bioaffinity cascade. These traces correspond to samples (n = 3) that mimic bloodstains, incubated at 40 °C from 0 hours to 120 hours, as indicated in the legend. (B) Inset bar diagram represents the change in absorbance at λ = 340 nm, after 30 minutes of assay completion.
majority of the output when the sample is rather young (as we know this marker undergoes a fast denaturation), while the ALT marker is responsible for the signal in longer aging periods due to its slower denaturation. However, the combination of these systems offers a parallel analysis of both markers’ activity within the aging process and balances both branches to afford a compromise for the performance between low error and a prolonged age determination.

The performance of each of the subsystems and the combined paths was compared, as can be seen in Figure 2.4. This figure illustrates the difference in absorbance in the different time intervals where the two pathways and the entire system where evaluated. The combination of both subsystems allowed “tuning” of the sensing cascade, where both reaction branches can be optimized for longer/shorter aging intervals. For the proposed CK/ALT tunable multi-marker cascade, the effect of temperature on the aging process was also examined. Human serum samples spiked with markers underwent an aging process at an exaggerated temperature of 40 ºC to create an initial database of optical outputs of blood samples submitted to different degradation times. The samples were subsequently incubated at 18 ºC and 25 ºC (room temperature) to mimic different scenarios that could be encountered in a forensic investigation. Just as with the initial experiment, the samples were also analyzed for up to 120 hours (5 days).

The samples that were incubated at 18 ºC, the lowest temperature, expectably show the lowest decomposition decay, while an elevated temperature, such as 40 ºC, caused the faster denaturation (Figure 2.5). The difference in optical responses among samples incubated at the same temperature, and among samples that vary in temperature incubation, makes this biocatalytic assay a perfect fit for the forensic investigation setting, because it could be done by using a portable spectrophotometer. These results show the potential of the proposed pre-
calibrated cascade approach for a wide range of temperatures. Consequently, when an unknown blood sample is found at a crime scene where the environmental conditions are known, the output signal can be attributed to a particular time that translates to when the blood sample was deposited.

**Figure 2.4.** Absorbance change at $\lambda = 340$ nm, corresponding to the consumption of NADH after the analysis of the two different paths in the proposed biocatalytic assay, as well as the entire biocatalytic assay. Time zero corresponds to the analysis of the freshly prepared sample ($n = 3$). The rest of the samples were re-suspended in water after they underwent aging at 40 °C. The black square represents the CK path. The red circle represents the ALT path and the blue triangle represents the entire biocatalytic cascade, CK/ALT. The zero time intervals correspond to the freshly prepared samples without drying.
2.5 CONCLUSIONS

This investigation showed that the combination of multiple enzyme markers, such as CK and ALT, can be used as a tool to determine blood spot ages at a crime scene. This study also shows the advantage of having a parallel marker sensing cascade, over a single marker cascade. This is because the combination of two markers provides an improved response from the sample compared to a single marker. Due to the differences in the denaturation rates of various markers present in body fluid, a single marker assay may not allow for a reliable determination of blood sample age. For instance, markers with high stabilities would continue to provide a signal for long periods of time but would also cause a high percentage of error in the analysis. On the other
hand, other markers that have a rapid denaturation, such as CK, cannot be used for a prolonged period of time, but would provide a lower chance of error. In our parallel assay, we use a combination of both types of markers to provide a “compromised” response. By using the high sensitivity of markers with short half-lives together with the stability of markers with longer lives, the response proved more reliable. This, apart from a prolonged time horizon, also allowed for “tuning” of the sensing cascade by optimizing both reaction branches for longer/shorter aging intervals.

These experiments confirm the applicability of the multi-marker CK/ALT biocatalytic assay for the analysis of the age of blood samples. In a real crime scene, these biomarkers will be found in bloodstains; therefore, these experiments were performed in human serum solutions dried on a common glass surface, to closely mimic real crime scene samples. The environmental conditions, such as temperature, that the samples were exposed to, affect the enzymatic activity of the markers. Enzymatic activity varies drastically with time and temperature; therefore, the samples in this investigation were incubated at different temperatures. Furthermore, because of its simplicity and robustness, this methodology aims to be adapted as a component of a forensic field kit; moreover, it can potentially be used by personnel, whom are not trained scientifically, at crime scenes.

Further development in this area will be oriented around the incorporation of this and similar approaches into portable lateral flow strip-like devices. The research into these approaches is currently under development in our laboratory.
REFERENCES


CHAPTER 3: The simultaneous estimation of a blood spot’s time since deposition and age of its originator via a bioaffinity assay

3.1 ABSTRACT

In the field of forensic science, blood is a major contributor of evidence in investigations involving violent crimes. However, blood is usually used only for its genetic content, overlooking its unique composition of proteins and low-molecular-weight compounds that often serve as biomarkers in clinical diagnostics. A biocatalytic assay has been developed for on-site forensic investigations to identify the age range of the blood sample originator and the time since deposition (TSD) of the sample, simultaneously. Alkaline phosphatase (ALP), a biomarker commonly used in clinical diagnostics for bone growth and disease, was chosen for this assay due to its age-dependent and enzymatic nature. ALP levels corresponding to old and young originators using authentic human serum were monitored after deposition for 48 hours in order to mimic potential crime scene conditions. It was determined that the stability of ALP in serum allows for the differentiation between old and young originators up to two days after the sample was left under these mimicked crime scene conditions. This system has the potential to become part of a forensic field kit and to be utilized by all personnel in law enforcement.

3.2 INTRODUCTION

Forensic investigations were revolutionized with the incorporation of science. The newly emerging field of forensic science has contributed immensely to criminal prosecutions in regard to providing unbiased facts about crimes. Biological samples such as blood,[1, 2] saliva, and sweat belong to forensic serology, a branch of forensic science that utilizes biochemical techniques for
analysis.[3, 4] DNA obtained from these biological samples is one of the major contributors to crime investigations. With the use of molecular biology techniques, scientists have created databases, such as the Combined DNA Index System (CODIS), that allow for the identification of the sample originator.[5-7] However, if the suspect’s or victim’s DNA is not in the database, DNA information cannot be used to determine the identity of the originator.[8] Furthermore, the current techniques implemented for the analysis of biological samples in criminal investigations depend on sophisticated instrumentation, and a complex chain of custody that often increase investigation times and have the potential to introduce unnecessary error.[9]

Besides genetic material, blood contains proteins and low molecular weight compounds that can be used as biological markers. Our previous study has shown that the TSD of a blood spot can be determined using bio-catalytic cascades.[10] However, this has not yet been utilized outside of a research setting. Characteristics such as biological sex and ethnicity of a sample originator have been elucidated using a similar technique.[11, 12] However, one attribute that is missing is the age of the originator. While there has been research into this topic, there is currently no technology being used at crime scenes to determine the age of the originator. Here, a biocatalytic assay that has been modified to characterize not only the age of the originator— who may potentially be a person of interest in the crime—from biological samples left at a crime scene, but also the TSD of the particular blood spot.

In the past, genetic methods were investigated to determine the age of a person based on the shortening of telomeres or the accumulation of mitochondrial DNA deletions. Unfortunately, these methods showed low accuracy, and therefore were deemed inappropriate for forensic applications.[13] Aspartic racemization (AAR) is another method typically used to
estimate the age of remains, which involves a destructive technique to examine bones, teeth, and ligaments. This approach is also extremely sensitive to temperature, thus limiting its forensic application. Another technique to estimate age of the originator is advanced glycation end products (AGEs). This consists of the analysis of reduced sugars and other a-carbonylic compounds formed by non-enzymatic reactions, such as oxidative and non-oxidative reactions, with amino groups on proteins, lipids, and nucleic acids. However, this technique is limited to bodies aged up to 45 years, and the sample can only be obtained from a corpse or from long-lived proteins. One of the most recent investigations used T-cell DNA rearrangements to estimate human age. This technique is promising but still requires a laboratory setting and involves different biomolecular techniques. One of the most recent techniques used to identify age based on blood samples was Raman spectroscopy, this technique was able to discern between newborns, adolescents and adults.

As for the ability to determine the relative time since deposition (TSD), numerous techniques have been developed as well, but none of them have been successfully applied in an on-site setting. In 1930, there was an attempt to find the relationship between the solubility of blood in water and its TSD. Later, applied spectrophotometric analysis was done for the first time.

**Scheme 3.1.** Conversion of p-Nitrophenol phosphate (pNPP) by ALP into p-Nitrophenol.
time in 1960.[17] In 1983, the changes in individual proteins present in bloodstains were studied.[18] The latest methods include electron paramagnetic resonance (EPR),[19] high performance liquid chromatography (HPLC)[20], oxygen electrodes,[21] RNA degradation,[22] near infrared (NIR) spectroscopy,[23] and atomic force microscopy (AFM)[24] and Raman spectroscopy[25]. Similar to the techniques developed for the determination of the age of the blood sample originator, these techniques require sample preparation and complex instrumentation, preventing the possibility to perform direct analysis at the crime scene.

The research presented here developed a biocatalytic assay utilizing Alkaline phosphatase (ALP; E.C. 3.1.3.1) for the simultaneous determination of the age of the originator and the TSD of the blood sample. ALP is a membrane bound enzyme that hydrolyzes monophosphates at a high pH and is a blood marker that is routinely used in clinical settings to identify bone diseases and hepatobiliary disorder.[26, 27] There are three main sources that produce ALP in the body: liver, kidney and bone.[28, 29] ALP was chosen for this investigation because of its age-dependent nature, as it is released in the body predominantly during active bone growth.[30-32] In females, the highest production of ALP is between 0-17 years old and in males, the highest production of ALP is between the ages of 0-18 years old.[33-40] Other studies have successfully shown the application of clinical markers in forensic and biosensing cascades.[41-45]

The identification of a suspect’s or victim’s age from biological traces, specifically blood, along with the TSD of a blood spot at crime scenes can be a tremendous help to forensic investigations in order to reduce the pool of suspects. In support of this work, knowing a suspect’s age, particularly if they are younger or older than the age of 18, is pertinent to distinguishing between crimes committed by a minor or an adult. Criminal offenders under the age of 18 are
considered juveniles,[46] and are tried, prosecuted, and sentenced differently than adult offenders who are 18 years and older.[47-49]

3.3 MATERIALS AND METHODS

3.3.1 Chemicals and reagents used

All enzymes and substrates were purchased from Sigma-Aldrich and used with no further treatment: Phosphatase, Alkaline from bovine intestinal mucosa (ALP; E.C. 3.1.3.1), p-Nitrophenyl phosphate disodium salt hexahydrate (pNPP), and human serum (Type AB; Sigma-Aldrich). The water used for all experimental procedures was ultrapure water (18.2 MΩ•cm), obtained from PURELAB flex, ELGA water purification system.

3.3.2 Instrumentation and measurements

The Molecular Devices UV/vis spectrophotometer/plate reader (SpectraMax NanoDrop 384), and an Infinite F200 PRO (Tecan) microplate reader were used to take optical measurements of the samples at λ = 405 nm. A microtiter polystyrene (PS, Thermo Scientific) plates were utilized. Additionally, all measurements were recorded kinetically at 37 °C.

3.3.3 Assay Conditions

In this biocatalytic assay, ALP converts p-Nitrophenol phosphate (pNPP) to p-Nitrophenol, which absorbs at λ = 405 nm.[27, 50] The absorbance generated by this conversion is proportional to the concentration/activity of the enzyme in the sample. The biocatalytic cascade was performed in 0.1 M carbonate buffer solution at pH 9.00 and the pNPP concentration was 2.5 mM. All measurements were taken at 37 °C in order to optimize the activity of the enzyme.
3.3.4 Establishment of Model Systems for the Determination of the Age of the Originator

As depicted in Scheme 3.1,[51] alkaline phosphatase (ALP) was used in a concerted manner to determine the age group of the originator, as well as the time since deposition (TSD) of the sample.[52-55] The concentrations of ALP used for identifying the age group of the originator, as reported in the literature, were the following: young female (0-17 years) 326.9 U/L, old female (18-60 years) 100.1 U/L, young male (0-18 years) 343.9 U/L, and old male (19-60 years) 111.3 U/L.[33] These concentrations correspond to ALP levels from healthy individuals. The age ranges for the two different groups, old and young, were determined by the values that were reported in the literature and routinely used in clinical settings.[33, 50] R-project software was used to generate randomized concentrations of ALP based on the aforementioned average concentrations for each of the four age groups in order to create 100 samples, each with a different concentration of ALP. Ultimately, 25 samples represented each of the four age classifications. The generated ALP concentration values were used in conjunction with the biocatalytic system for the analysis of samples mimicking the real distribution of this marker’s concentration in blood.

The fact that we only performed the following experiments and statistical analyses for the ALP values of healthy individuals presents a minor challenge in terms of being able to apply this assay directly on-site. This is because there is a small percentage of the population with bone diseases that alter the ALP levels regardless of the age of the originator. This would mean that a blood sample from an originator with a certain bone disease would not be correctly categorized as old or young if the biocatalytic assay is conducted. However, given that this is the first use of this biocatalytic assay for a forensic application, it was important to base the study on what is
“the majority” of the population. This way, a strong understanding of the platform was able to be ascertained before honing in on the technicalities.

3.3.5 Blood spot analysis for determination of the time since deposition

To mimic authentic blood samples, human serum (type AB; Sigma-Aldrich) was spiked with levels of ALP relevant for healthy females both young (0-17 years old) and old (18-60 years old), 326.9 U/L and 100.1 U/L, respectively. The mimicked samples were left on of a glass surface on the work bench and were naturally dried by the environment (approximate temperature 25 °C ± 3 °C). The time intervals after which the samples were analyzed were: 0, 3, 6, 12, 24, 30, 36, and 48 hours. Following incubation (aging process) the samples were resuspended in water and mixed with pNPP, which activated the biocatalytic assay, and subjected to continuous optical measurement at λ = 405 nm, in order to monitor the conversion of pNPP to p-Nitrophenol.

3.3.6. Statistical Analysis

For data generated by the age of the originator experiment, receiver operating characteristic (ROC) analyses were performed in order to assess the discriminatory power of this biocatalytic assay for the distinction between young and old populations for both biological sexes, separately. The ROC curve was plotted as a function of sensitivity (true positive rate) verses specificity (true negative rate) for varying thresholds of class assignment. The area under the ROC curve,[56] also known as AUC, was estimated by the trapezoidal integration method, and the corresponding 95% (Confidence Interval) CI was estimated using the method described by DeLong et al.[57] The AUC indicates how well the model ranks samples according to the change in absorbance assigned to the positive class. Using the ROC analysis, the best thresholds (above which the change in absorbance
is assigned to the positive class) for a ranking biocatalytic assay to separate young and old populations were identified. AUC ranges from 0 to 1, where an AUC of 0.5 represents a random classifier and an AUC of 1 indicates a perfect test. ROC analyses were carried out with package pROC.[58]

With respect to the investigation into the determination of the age of the originator while simultaneously determining the TSD, support vector machine regression (SVMR) analyses were conducted. SVM constructs a hyperplane in a multidimensional space, which is used for regression. The training data set is marked as values describing the TSD and an SVM training algorithm assigns the new validation data by the predicted value for the TSD. For this purpose, recorded data was divided into two datasets, calibration and validation, by moving each 5th absorbance spectrum into a validation data set. Remaining measurement spectra were used for calibration. SVM in the regression form to build quantitative model to predict the TSD of the dried blood spots of the young and old groups. The SVMR is used with the radial basis function (RBF) as a kernel function and it is optimized by a combined approach of 2-fold cross validation (two samples out) and a systematic grid search of the parameters. The quality of the predictions is expressed by predicted-versus-measured plots of the calibration dataset and prediction models based on absorbance changes for young and old groups. Additionally, an internal cross-validation step in the calibration process to determine the number of latent variables was used. The effectiveness of SVMRs was then calculated using the Root Mean Squared Error (RMSE) and the coefficient of determination ($R^2$).
3.4 RESULTS AND DISCUSSION

3.4.1 Viability of ALP as a marker for age of the originator

Here, the ALP biocatalytic assay was utilized for forensic age of the originator determination. This assay was first employed for the analysis of a male group and a female group. Additionally, this approach is also capable of determining the TSD simultaneously. The performance of the biocatalytic assay was evaluated by ROC analysis. This novel approach is non-invasive and can be performed directly on-site at a crime scene, just like point-of-care devices that routinely used in clinical settings. This duality of our sensing system is especially important for the efficiency of the system, potentiating the possible utilization of our enzymatic cascade in the field. A single sensor capable of simultaneously measuring two parameters would be much more cost and time effective, enabling broader use and application of enzyme-based sensor technology.

Prior to starting this investigation, the viability of ALP as a marker was tested. To accomplish this, the dependence of the biocatalytic conversion of pNPP to p-Nitrophenol using different concentrations of enzyme (ALP) while keeping the substrate concentration constant was measured. The results of this experiment are shown in Figure 3.1; where a concentration dependence indicates the applicability of ALP as a marker for the biocatalytic assay to determine the age range of the originator. In figure 3.1 The star represents the old female concentration, 100.1 U/L, whereas the diamond represents the young female concentration, 326.9 U/L. Experimental section includes the exact composition of reactant solutions used. The male concentrations will performed very similar to the female ones in an ALP biocatalytic cascade. Thus, this experiment was not
performed for the male concentrations. ALP is a marker that can be used for age analyses as well as for TSD analyses, thanks to the nature of the enzyme, however the production of ALP between females and males does not vary significantly to distinguish between sexes.

3.4.2 Enzymatic and statistical analysis of model ALP solutions for age of the originator

Given the positive results of this proof of concept experiment in female concentrations, a detailed biochemical assay to mimic an authentic crime scene scenario was performed. ALP distribution in blood has been previously studied, therefore, its

![Standard curve of the changes in absorbance (λ = 405 nm) corresponding to the biocatalytic conversion of pNPP to p-Nitrophenol via ALP. These points correspond to samples measured at different concentrations (n = 3). The star represents the old female concentration, whereas the diamond represents the young female concentration. Experimental section includes the exact composition of reactant solutions used.](image)

**Figure 3.1** Standard curve of the changes in absorbance (λ = 405 nm) corresponding to the biocatalytic conversion of pNPP to p-Nitrophenol via ALP. These points correspond to samples measured at different concentrations (n = 3). The star represents the old female concentration, whereas the diamond represents the young female concentration. Experimental section includes the exact composition of reactant solutions used.
variability and correlation to age has been reported.[30] Since the study reported significantly different levels of ALP for different age groups, we used these parameters to imitate the concentration of the enzyme in real human serum for the four age groups previously described. The presented values deviated from a normal distribution and were consistent with a log normal distribution. We followed the published study and subdivided the age groups into smaller groups according to biological sex and conducted the measurements separately.

ALP can be implemented in biocatalytic assays that can be performed on-site, in order to help build a profile of the originator’s age range and provide the TSD. Using spiked human serum samples, an authentic crime scene scenario was mimicked and the potential for this assay to distinguish between the four age groups (young female, old female, young male, old male) was determined. Advanced statistical analysis was performed using MATLAB (MathWorks, Inc., version R2013b) and R-project software, ver. 3.0.0. in order to generate 25 statistically generated ALP values per age group according to the parameters given by the previously reported distribution of ALP,[33] resulting in 100 different samples, each containing a different concentration of ALP, that were used to the overall distribution of ALP in a healthy originator population. As mentioned above, the samples were prepared by spiking human serum samples with the corresponding 25 randomly distributed ALP concentration values for the respective age group.[31, 59] The use of 25 concentration values per group is deemed adequate for statistical calculations as they are sufficient enough to resemble a log normal distribution reported in the study.[56]
Again, ALP converts $p$-Nitrophenol phosphate (pNPP) to $p$-Nitrophenol, which is monitored at 405 nm, and corresponds to the concentration of ALP in the sample.[27, 59] The correlation between the response of the assay and the amount of ALP present makes it possible to determine if the originator is in an age range of young or old. The real time response from male and female samples using the ALP biocatalytic assay are shown, respectively, in Figure 3.2A and 3.2B. Each figure shows the response for young and old ALP concentrations for each biological sex, which was generated from a reference range calculated according to the procedure described above. The output signals measured for females do not overlap between the young and old populations; however, there is a small overlap with the outputs measured for the young and old concentrations in males.

**Figure 3.2A.** Absorbance at $\lambda = 405$ nm, corresponding to the conversion of pNPP to $p$-Nitrophenol by ALP. Black traces correspond to randomized young male enzyme concentrations, whereas the red traces correspond to randomized enzyme concentrations for old males. The exact information of chemicals used is giving in the experimental section.

**Figure 3.2B.** Absorbance at $\lambda = 405$ nm, corresponding to the conversion of pNPP to $p$-Nitrophenol by ALP. Black traces correspond to randomized young female enzyme concentrations, whereas the red traces correspond to randomized enzyme concentrations for old females. The exact information of chemicals used is giving in the experimental section.
ROC analyses were performed in order to assess the discriminatory power of our biocatalytic assay for the distinction between young and old populations for both biological sexes separately. The ROC curve was plotted as a function of sensitivity (true positive rate) verses specificity (true negative rate) for varying thresholds of class assignment. The area under the ROC curve,\[56] also known as AUC, was estimated by the trapezoidal integration method, and the corresponding 95% (Confidence Interval) CI was estimated using the method described by DeLong et al.\[57] The AUC indicates how well the model ranks samples according to the change in absorbance assigned to the positive class. Using the ROC analysis, we identified the best thresholds (above which the change in absorbance is assigned to the positive class) for a ranking biocatalytic assay to separate young and old populations. AUC ranges from 0 to 1, where an AUC of 0.5 represents a random classifier and an AUC of 1 indicates a perfect test. ROC analyses were carried out with package pROC.\[58]

Following the collection of the real-time (kinetic) data, statistical analyses were conducted on the data generate from both sexes, individually. The AUC of ROC curves was estimated as 0.99 (95% CI: 0.98–1.00) and 1.00 (95% CI: 1.00-1.00) for male and female groups, respectively (Figure 3.3 A and B). This means that the biocatalytic assay has a 99% and 100% probability of correctly differentiating between older and younger groups within the respective sexes. Note that the ROC curves were generated from absorbance changes and the best absorbance threshold of 0.640 and 0.699 for male and female groups, respectively. These thresholds balanced the trade-off that exists between
sensitivity and specificity. These absorbance changes are the most accurate cutoff points for discrimination between older and younger populations. As shown, ROC analysis has proven the potential of this assay to differentiate between samples from older and younger groups.

The similar thresholds suggest no significant difference between male and female groups with respect to the enzymatic activity of ALP. As a result, the subsequent TSD analysis was performed for female ALP levels only.

**Figure 3.3 A.** ROC Male
The tradeoff between sensitivity and specificity are shown by presenting data ROC curve. The AUC is 99%, which is the probability for the assay to identify young and old group based on the ALP levels in blood. The optimum cutoff point was chosen with sensitivity of 100% and specificity of 84%. Random choice is denoted by the grey diagonal line.

**Figure 3.3 B.** ROC Female
The tradeoff between sensitivity and specificity are shown by presenting data ROC curve. The AUC is 100%, which is the probability for the assay to identify young and old group based on the ALP levels in blood. The optimum cutoff point was chosen with sensitivity of 100% and specificity of 100%. Random choice is denoted by the grey diagonal line.
3.4.3 Enzymatic and statistical analysis of model female ALP samples for time since deposition

In the next step, the use of the ALP biocatalytic assay to estimate the TSD of dried bloodstains was investigated. Since our previous experiment did not show a significant difference in ALP activity between biological sexes, TSD experiments were performed using only healthy female ALP levels. In addition, the mimicked female samples do not overlap between young and old groups, further supporting the use of only the average levels of ALP that are characteristic of females, young female (0-17 years) 326.9 U/L, old female (18-60 years) 100.1 U/L, for simplicity. The ALP spiked human serum samples (both young and old groups, separately) were placed on a glass surface and underwent an aging process for 2 days at 25 °C +/- 3 °C (from 0 to 48 hours). These samples containing average levels of young and old female ALP levels were naturally dried by the environment; they were not covered or placed under any special conditions. The samples were left on the laboratory bench, near the window to have sunlight during the day, and darkness at night, in order to mimic a crime scene scenario as closely as possible. Then, the samples were re-suspended with distilled water just before measurements were taken. The ALP concentration was determined by measuring the absorbance change corresponding to the conversion of 2.5 mM pNPP to \( p \)-Nitrophenol, absorbance at \( \lambda = 405 \text{nm} \). Each measurement contained a set of 10 samples (\( n = 10 \)). After conducting these experiments, it was determined that ALP has the ability to determine the age of the originator from a blood sample left at a crime scene.
Figure 3.4 demonstrates the results of our assay for TSD estimation. Even after 48 hours of the biological samples being left at the mimicked crime scene conditions, it can be determined whether the sample came from a young or an old originator along with the TSD, by monitoring the activity and biological levels of ALP. The output signals measured for the aged samples show a distinguishable difference for both young and old populations. The difference between the two populations did not change much during the aging process and so the output signal ratio for the young and old populations was distinct.

**Figure 3.4.** Absorbance change at $\lambda = 405$ nm, corresponding to the conversion of pNPP to p-nitrophenol by ALP. Samples ($n = 10$) were resuspended after undergoing the aging process for up to 48 h. The zero time interval corresponds to the freshly prepared samples without drying. The young samples are represented by red circles, and the old samples are represented by black squares. The error bars represent the relative standard error from the change in absorbance from 10 samples. The exact information on chemicals used is given in the Experimental Section.
even after 48 hours. It is important to reiterate that this was done only for the female group. The outcome will be very similar for both biological sexes, because biological sex did not show an effect on the outcome of the age group of the originator.

Once the correlation between the absorbance of the assay and the TSD was made, SVMR was applied in order to predict the TSD of the dried bloodstains from 0 to 48 hours. Our model was tested with an external data set with absorbance spectra that were excluded from training datasets at the beginning of statistical analysis. As a result, the TSD of the blood spots using the SVM regression models using independent validation sets was predicted. First, the SVM in the form of discriminant analysis (SVMDA) was applied to test if we could differentiate the two age populations within the 48 hour deposition period. Twenty-nine absorbance spectra were chosen for testing and the remaining 119 spectra were used for calibration. The calibration model correctly classified all 29 spectra, 100% discrimination, with no misclassification. This shows the ability of the assay and SVMDA method to correctly discriminate between young and old groups. Next, the SVMR was applied to determine the TSD of the dried bloodstains for each group separately. SVMR was applied to provide statistical models to relate the absorbance spectra to the particular stage of the deposition time frame. SVMR models were created for both groups (young and old population) using four latent variables (LVs), where the red line represents the prediction, and the green line is perfect agreement between measured and predicted values. The models were trained with the training data sets of absorbance spectra (in the same manner as for SVMDA) and calibration models were then used to predict the TSD of dried bloodstains. The estimated ages for all time points were plotted against the actual
age of the dried blood spots. The predictive performance of the model shows a sufficient correlation between the age predicted by the catalytic assay and actual age established by the assay.

As can be seen from Figure 3.5A, the plot shows a wider scatter of predicted age data points for the young population than for the old population, Figure 3.5B. Moreover, it is evident that the best fit for the regression of predicted versus measured values for young population does not intersect with the origin, which suggests greater bias of the model for this case. The reason for the larger spread and greater model bias for the younger population can be explained by the greater changes occurring in the blood due to the higher rate of the enzymatic reaction, therefore causing a naturally higher variability in the responses. However, the spread of data points expressed by $R^2 = 89\%$ and $\text{RMSEP} = 6.20$ hours is sufficiently small enough for the TSD of a bloodstain to be correctly predicted with high accuracy. The SVM algorithm predicted the TSD of the dried bloodspot in the old population more accurately with an $\text{RMSEP}$ of 6.41 hours and $R^2$ of 91%. The dual attribute aspect of this biocatalytic assay is a key advantage for forensic purposes since it is possible to identify the age group of the originator from a discovered dried bloodstain and the sample’s TSD at the same time. To the best of our knowledge this is the first report of the TSD of a bloodstain determination using an enzymatic cascade that is accompanied by further validation of prediction ability.

Our validation data set was separated from the training data set at the beginning of the statistical analysis. As a result, we predicted the TSD of the blood spots using the SVM regression models using independent validation sets.
Figure 3.5. SVM regression plot for samples of the (A) young and (B) old group up to 48 h showing measured TSD values versus predicted TSD values of calibration (black stars) and test (red circles) data sets. The red line demonstrates the actual fit, and the green line is the ideal fit (1:1).
3.5 CONCLUSIONS

Using ALP as a biomarker, experiments and statistical analyses have been performed that corroborate the assay’s viability at crime scenes. As a result, it was concluded that the ALP biocatalytic assay has the potential to be a highly beneficial tool for forensic investigations. Although the differentiation has been made between age groups of male and female sexes, the main focus of this research was not on the biological sex of the originator. The primary focus was to being the process of constructing methodologies that can aid law enforcement in establishing a profile of a person on interest directly at a crime scene using biological markers. Specifically, the research here focuses on utilizing a biocatalytic assay for the analysis of a known biomarker for the determination of a person’s age range from a blood sample. In addition, the assay used here has the capability to simultaneously determine the TSD of the blood sample itself.

At crime scenes, the ALP samples will be found in blood spots which may be subjected to a variety of environmental factors. Because of this, the research presented here utilized authentic human serum samples spiked with the anticipated ALP concentrations of the particular age groups that were subjected to environmental conditions for a period of 2 days. The results generated after this time showed identifiable activity between young and old originators even after the two days. Due to the durability and flexibility of the biocatalytic assay, it has the potential to be incorporated into a novel forensic serology field that that is still under development.

This system has the potential to be part of a forensic field kit and be utilized by all law enforcement personnel. If this method is made portable and able to be brought directly to the crime scene, blood samples can be rapidly analyzed directly at the crime scene in order to obtain
preliminary information about the person or persons of interest in the crime. Ultimately, increasing the turnaround time on a suspect’s physical characteristics can help avoid wasting time looking into someone who does not fit within the identified age range. Additional forensic serology assays that can identify other originator characteristics, and ultimately aid in criminal investigations, are currently under development in our laboratory.

REFERENCES


51. SIGMA-ALDRICH, *SIGMAFAST™ p-Nitrophenyl phosphate Tablets*.


CHAPTER 4: Promises and Challenges in Continuous Tracking Utilizing Amino Acids in Skin Secretions for Active Multi-Factor Biometric Authentication for Cybersecurity

4.1 ABSTRACT

Biometric-based cybersecurity systems for active authentication by continuous tracking is a new concept that utilizes biochemical processing of metabolites present in skin secretions. Skin secretions contain a large number of metabolites and small molecules that can be targeted for analysis. Amino acids found in sweat can be exploited for the establishment of an amino acid profile capable of identifying an individual user of a mobile or wearable device. Individual and combinations of amino acids processed by biocatalytic cascades yield physical (optical or electronic) signals, providing a time-series of several outputs that, in their entirety, should suffice to authenticate a specific user based on standard statistical criteria. Initial results, motivated by biometrics, indicate that single amino acid levels can provide analog signals that vary according to the individual, although with limited resolution versus noise. However, some such assays offer digital separation (into well-defined ranges of values) according to groups such as age, biological sex, race, and physiological state of the individual. Multi-input biocatalytic cascades that handle several amino acid signals to yield a single digital-type output, as well as continuous-tracking time-series data rather than a single-instance sample, should enable active authentication at the level of an individual.

4.2 INTRODUCTION

4.2.1 Overview

The security of electronic devices such as smartphones or smart watches, which are often constantly connected to applications involving sensitive and personal information, is based on
reliable authentication of the actual user/owner of the particular device. However, no single authentication methodology is foolproof. While theoretically, only the owner should know the passcode — be it a phrase or a numerical combination — passwords can be duplicated, e.g., by spying on the owner while unlocking the device. Pattern-based authentication can also be bypassed. For example, fingerprint molds can be “lifted” from fingerprints left on various surfaces, which allows unauthorized users to “trick” a fingerprint reader.[1] Another aspect of the problem has been the reluctance of the users to input their password/passcode too frequently. Both of these issues have led to the advent of active authentication approaches.[2, 3] These typically include trace histories[2, 4] that can be based on web-use (such as browsing history or application usage), digital (facial recognition or speech analysis), physical (user’s gait, touch/swipe dynamics), and/or global/location (tracking the user’s routine, GPS) tracking. These approaches use data collection and analyses to determine that the user is an authorized person, whose additional verification with password will otherwise be requested on each access to any sensitive application. Active authentication requires various continuous authentication and tracking methodologies[2-5] that potentially involve not only the recording of various “histories,” but also continuous monitoring of the user’s patterns of behavior.

This chapter reviews a new concept and outlines preliminary results for utilizing forensic biometrics to develop a new biochemical approach to data collection for continuous active authentication and trace-history information gathering. The methodology is not based on the electronic (digital) or pattern (physical/optical) inputs, but rather on biochemical inputs: metabolites in the user’s skin secretions. The approach is autonomous and can be used by all individuals who own or have access to technology that holds personal information. Specifically,
we address a continuous and unobtrusive user authentication and physiological state monitoring approach using metabolites secreted by the skin. The latter capability: to monitor the physiological state of the user, is unique to such a “biochemical” approach as compared to other “electronic” or “physical” continuous tracking methods, which are more useful for monitoring “lifestyle/habits” information.

Analytes that can be used as “input signals” for monitoring, include, but are not limited to, various amino acids — which we focus on here — as well as pyruvate, and other metabolites. These compounds are substrates, intermediates, and products of many metabolic reactions and processes. As a result, humans secrete different levels of these substances through eccrine glands as components of sweat.[6] We survey studies[6-11] and illustrate results, originally motivated by forensic applications, which suggest that biochemical metabolic profiles, when recorded as a sufficiently detailed, continuously tracked time-series, are different from individual to individual. Larger, more noticeable differences are caused by variations in physical/physiological attributes[11] such as biological sex, ethnicity, and age, but there are smaller differences found between individuals even with similar physical attributes.

Sweat is a biological sample that is generally easy to collect in a non-invasive and unobtrusive manner by having a small sensor at typical point(s) of skin contact with a device, making it an ideal target for new biometric-based security approaches. Ultimately, the proposed approach should enable the development of a new methodology to allow the transitioning from and/or supplementing passcode-, data-, physical- and image-based security with biochemical tracking that includes security measures based on the unique metabolite levels’ trace histories of the device owner/user.
4.2.2 Statement of the Problem

As described in the overview, most approaches and technologies for secure authentication of device users are not hack-proof. Therefore, a new/additional authentication methodology based on biochemical analytes as “signals” will be very useful to reinforce and improve the security of personal electronic devices. This methodology involves targeting analytes present in sweat as “chemical inputs” for unobtrusive, continuous tracking and active authentication. A challenge lies in identifying the multi-analyte sets to probe (measured signals) and establishing the feasibility of user authentication based on the collected data, as well as developing biomolecular processing of the collected data for the latter purposes.

The analytes, numbering \( N \), provide a time series \( x_{n=1,...,N}(t_0 + k\tau) \) of chemical concentrations \( x_{n=1,...,N} \) probed at certain time intervals \( \tau \), starting with the initial time \( t_0 \). Biochemical processing in a patch/sensor then converts this set of the input analytes \( x_{n=1,...,N} \) into a set of \( S \) output chemical and ultimately physical (electronic, optical) signals, \( y_{s=1,...,S} \). Usually, due to the nature of such processing, the conversion is “for the same time step”: The output signals \( y_{s=1,...,S}(t_0 + k\tau + t_g) \) are obtained from \( x_{n=1,...,N}(t_0 + k\tau) \) probed at each specific time step, \( k = 0, 1, 2, ..., \) with usually some delay “gate time” (measurement time) \( t_g > 0 \), usually smaller than or comparable to \( \tau \), which is required for the biochemical processing of the inputs into the outputs.

The “vector” (of \( S \) values) time series of the output signals can then be analyzed for authentication. The role of statistical analysis[2-5] is to establish that the signal time-series \( y_{s=1,...,S}(t_0 + k\tau + t_g) \) is sufficient for providing an ongoing user authentication/verification and tracking/physiological-state monitoring for \( k > k_{reg} \), after the initial
“registration/identification” over the first several time steps $k = 0, 1, ..., k_{\text{reg}}$ as the device is initially accessed by that user. The associated biochemical challenge is to identify the optimal sets of analytes, $x_{n=1,...,N}$, as well as devise biochemical processing to yield suitable outputs $y_{s=1,...,S}$.

We point out that the simplest approach of having $S = N$ and converting each analog chemical data value $x_j$ to analog output signal $y_j$, might not be adequate. Indeed, biochemical data of physiological origins are known to have a significant level of random noise. In most situations where such data are the inputs, few- to multi-input biocatalytic and other chemical processes (as well as processes that yield optical or electronic outputs) have to be utilized to “filter away” the noise and produce “digital” (limited to a couple of specific values or more realistically to narrow ranges of values) outputs. Thus, input analytes are “consolidated” into $S < N$ groups, Equation (1), to yield “digital” outputs:

$$x_{1,...,n_1} \to y_1, \ x_{n_1,...,n_2} \to y_2, ..., \ x_{n_{S-1},...,n_S} \to y_S. \quad (1)$$

Substantial work designing such multi-input processes to experimentally realize and theoretically optimize their setup for high-quality “digitized” outputs[12-17] has been carried out in the field of biomolecular computing. This is accomplished by developing biocatalytic (typically enzymatic) assays supplemented with additional biocatalytic or simple chemical-reaction processes, the latter involving either inputs $x_j$ or outputs $y_j$, which act as “filters” to substantially minimize analog noise and variation in the responses.[12-29]

The concept of multi-((bio)marker monitoring has originally emerged in the medical area, specifically, point-of-care techniques.[30] Physiological (body) fluids such as blood, sweat, or
saliva can and have been exploited to extract biomarkers that can be utilized for forensic identification. The latter area is rather recent, with novel approaches being developed that implement “combinatorial” monitoring of two biomarkers commonly present in blood, in order to identify characteristics such as sex and ethnicity of a blood sample originator.[7, 8] Another combination of two markers, with different temporal profiles, was developed for the determination of the time elapsed since the deposition (TSD) and the originator’s age, of a blood sample found at a crime scene as shown in Chapters 2 and 3.[9, 10]

Specifically for sweat, biochemical assays have been developed that are capable of determining biological sex from samples obtained from fingerprints.[6, 11] Some general overviews of the application of biomarkers are available.[31-33] Body fluids can be utilized to reveal information about a particular individual not only via genetic material, but also proteins, low molecular weight compounds, and varying amounts of metabolites that are produced by the body as a result of multiple processes related to metabolism. Metabolism, a process that is regulated by a combination of hormone-based controls[34] is indicative of personal characteristics including, but not limited to, biological sex, age, ethnicity, or health.

Wearable and mobile electronics, as well as most other input devices (keyboards, touch screens) are in permanent or frequent contact with skin, the top layer of which — the epidermis — contains glands that are constantly secreting sweat. The concentrations of various components in sweat are controlled by a complex set of reactions regulated by hormones.[34] Due to the fact that hormone levels significantly vary as a result of age, sex, ethnicity, and lifestyle (e.g., diet and fitness), it is concluded that no two individuals have the same hormone levels as functions of time.[35, 36] Thus, the time-series of concentrations of chemical components in an
individual’s sweat should be specific to that individual. We expect that, the time-dependent concentrations of suitably chosen subsets of the 23 amino acids\cite{37} found in human sweat should suffice to differentiate between individuals.

In this chapter the differentiation (authentication) that relies on the analytes in sweat is addressed. Nevertheless, it is highlighted that certain other aspects of the technology are rapidly becoming available.\cite{38} Specifically, not only are sensors undergoing rapid miniaturization,\cite{39} but a wearable wristband technology that electronically detects certain chemicals in sweat has already been demonstrated\cite{39, 40} in a different context, and other platforms are being investigated as well.

4.3 MATERIALS AND METHODS

4.3.1 Amino acid assays and Instrumentation

To investigate the amino acid assays potentially suitable for such goals, gas chromatography (GC) coupled with mass spectrometry (MS) has been utilized for pre-screening. Measurements were performed on an Agilent 5977E GC-MS equipped with ChemStation and MassHunter data analysis software. Given that GC-MS requires volatile components, the amino acids in the samples extracted from sweat are derivatized using MSTFA (N–methyl–N–(trimethylsilyl)trifluoroacetamide) and acetonitrile according to an established procedure described by Thermo Fisher Scientific.\cite{41} In addition, the following protocol\cite{42} is used for analysis of the derivatized amino acid samples: isothermal at 150 °C for 2 minutes, then 150 to 250 °C at a rate of 8 °C/minute, followed by 250 to 310 °C at a rate of 6 °C/minute, and ending with isothermal at 310 °C for 10 minutes. The samples were injected in the splitless mode with a solvent delay of 4 minutes and helium is used as the carrier gas with a constant flow of
1 mL/minute. The injector temperature was held at 250 ºC. For MS detection, the masses of the derivatized amino acids were scanned in the quadrupole from m/z 10 to 420.

Such experiments revealed the presence of various amino acids in the sample, as well as their corresponding concentrations. The characterization of the samples from several originators is crucial to support data analysis and, specifically, seek correlations that allow active tracking of lifestyle for various groups of individuals and ultimately, with a large enough pool of samples, for specific individuals. The results also provide information for designing new bioassays targeting a single analyte or a pool of analytes and their time dependence, related to a specific trait or behavioral habits. The concentration distributions for the same individual or a group of similar trait individuals under similar conditions can also be studied.

4.3.2 Bioaffinity System for Amino Acid Tracking

Various endogenous and exogenous compounds are present in the composition of sweat; amino acids are one of the main components.[43-46] Here, for illustration we describe assays for targeting (converting to a measurable signal) glutamate (Glu), alanine (Ala), and phenylalanine (Phe). Experimental results for the first two of these amino acids are reported below. Glutamate can be analyzed using an enzyme system containing glutamate dehydrogenase (GIDH; E.C 1.4.1.3). GIDH will consume Glu and NAD⁺ (β-nicotinamide adenine dinucleotide) as substrates, to produce the corresponding amounts of 2-oxoglutarate (KTG), ammonia (NH₃), and NADH. As shown in Scheme 4.1, amino acids (glutamate, in this case) can be analyzed in two ways. Pathway A utilizes the UV properties of NADH alone, which can be observed[47] at 340 nm. Pathway B implements phenazine methosulfate (PMS) and Nitroblue tetrazolium (NBT). Here, PMS mediates the reaction of NADH reducing NBT to a colored product, formazan, which can be
measured spectrophotometrically at 580 nm. This system can be adapted for the detection of alanine and phenylalanine by changing the starting enzyme and other chemicals as appropriate. Alanine can be analyzed using an enzyme system containing alanine dehydrogenase (AlaDH; E. C. 1.4.1.1). AlaDH consumes alanine and NAD\(^+\) to produce pyruvate (Pyr), NH\(_3\), and NADH. Phenylalanine dehydrogenase (PheDH; E. C. 1.4.1.20) produces phenylpyruvate (PhPyr), NH\(_3\), and NADH in the presence of phenylalanine and NAD\(^+\). These two systems can be interpreted similarly to the glutamate cascade, with either pathway A, direct observation of NADH at 340 nm,[47] or pathway B, conversion of NADH to formazan via NBT/PMS which is observable at 580 nm.[48]

4.4. DATA COLLECTION AND ANALYSIS

![Scheme 4.1](image)

**Scheme 4.1.** Biocatalytic cascade for optical detection of three amino acids, using two detection pathways: (A) measurement of NADH at 340 nm, and (B) conversion of NADH to a visible color (formazan) via NBT/PMS which is observable at 580 nm.
In the nomenclature of Equation (1), each assay described in the section 4.3.2 corresponds to the conversion of a single group of chemical inputs into an output. Bioaffinity-based cascades can be tested and optimized for such assays using the buffer-based samples prepared to mimic the amino acid distributions known to be present in sweat, but ultimately authentic sweat samples should be used. For the successful completion of this phase, we estimate[6-9, 11] that the population used must contain a minimum of 25 volunteers per characteristic being studied. Statistical analysis, specifically ROC and AUC analyses[49] have been performed on such data.

These analyses have been reported to distinguish group characteristics such as biological sex and ethnicity[6-11] for forensics. Scaling this approach up to the authentication of individuals should be possible with the use of time-series data sets (of $S$ outputs), instead of single-sample

![Figure 4.1](image)

**Figure 4.1.** Absorbance decrease ($\lambda = 340$ nm) corresponding to the conversion of NADH to NAD$^+$ via the ALT/LDH assay using mimicked sweat samples. The red traces indicate female samples; the blue traces indicate male samples.
(one time) data. As already mentioned, this will likely require the use of several-input processes for each output, Equation (1), to minimize the analog noise in the typical physiological data and produce “digitized” (to narrow output ranges) signals by techniques developed for biomolecular computing.[8-28]

A large amount of work on “digitalization” in signal processing of noisy data gathered by measurements of signals resulting from multi-step biomolecular processes, such as those that are enzyme-catalyzed, has been carried out.[8-29] Indeed, data in the present case are rather noisy, as exemplified in Figure 4.1 Once the results of biochemical preprocessing yield the physical and ultimately electronic signals obtained from the time series of the outputs $y_{s=1,...,S}(t_0 + k\tau + t_g)$, statistical analysis for determining the feasibility of the authentication mechanism at the level of an individual can be carried out using techniques for time-series/trace-history analysis.[2-5] Continuous monitoring/trace-history recording and analysis of the person’s habits and routine behavior is required for active authentication approach.

The approaches considered here can also offer user-group (rather than individual) authentication, e.g., providing age-sensitive access restrictions. Continuously or regularly (but unobtrusively) collected data can be fed into a fully software-based neural-network type system[5] that can then be trained to follow the user’s identity continuously.

4.5. RESULTS AND DISCUSSION

4.5.1 ALT/LDH System

The biocatalytic system (cascade of processes) shown in Scheme 4.2, illustrates detection, with results shown in Figure 4.1, of a single amino acid. Note that in Figure 4.1 and below, the shown measurement times are the probe-times, $\tau$, discussed in connection with Equation (1).
These measurements were for “mimicked” samples (measurements with authentic sweat samples are exemplified in section 4.5.2). The parameters for the amino acid concentration distribution were taken from eccrine sweat and utilized to generate 50 different concentrations for each of the 23 amino acids previously determined to be in sweat from females and males. These 25 concentrations capture a plausible range of amino acid concentrations that can be present in sweat. The concentrations were then arbitrarily grouped to create 25 samples representing males and 25 samples representing females containing varying quantities of all 23 amino acids. These sets of mimicked sweat samples were prepared in Tris-HCl buffer (pH 7.5) and analyzed using the newly designed and optimized dual-enzyme biocatalytic assay that follows only one specific amino acid, alanine.

This single analyte biocatalytic assay, Scheme 4.2, is based on the combined reactions of two enzymes: alanine transaminase (ALT; E. C. 2.6.1.2.) and lactate dehydrogenase (LDH; E.C. 1.1.1.27). The catalytic process converts alanine and KTG into pyruvate and glutamate, respectively, using ALT. The formed pyruvate then undergoes a reaction that is biocatalyzed by

\[
\begin{align*}
\text{ALa} & \rightarrow \text{Pyruvate} \\
\text{GLU} & \rightarrow \text{Lactate}
\end{align*}
\]

\[\lambda = 340 \text{ nm}\]
LDH to convert the optically active NADH to NAD\(^+\). The NADH generates an optical signal at 340 nm, which decreases as LDH convert [7, 8] this compound into NAD\(^+\).

The robustness of this assay was assessed in the context of clinical diagnostics, for the detection of traumatic injury markers and even for the detection of real injuries in a pig model system.[50] The specificity of the ALT enzyme guarantees that the cascade will only respond to the level of alanine, without any significant interference from the other amino acids present in the samples. The results for this single-analyte system show that the monitoring of one particular amino acid can be achieved even when its concentrations are minute.

4.5.2 ALT/POx/HRP System

A somewhat more complicated cascade than that in the preceding subsection, depicted in Scheme 4.3, demonstrates an unambiguous biological sex identification system on the time scale of a couple of minutes. This cascade involves the detection of alanine via the concerted action of three enzymes: ALT, pyruvate oxidase (POx, E.C. 1.2.3.3), and horseradish hprooxidase (HRP, 1.11.1.7). ALT converts L-alanine to pyruvate, which is then subsequently consumed by POx to produce H\(_2\)O\(_2\). H\(_2\)O\(_2\) then drives the oxidation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6- sulphonic acid)) via HRP to provide a visible (optical) signal.

This system has been utilized for the analysis of authentic sweat samples. The success of detecting amino acids in the samples, as well as this single analyte bioaffinity assay’s performance, are demonstrated in Figure 4.2. The results are definitive for the determination of biological sex, but it can be seen that there is also a discernable difference in signal between different individuals. However, as emphasized in the earlier discussion, multi-analyte analysis, as
well as a time-series of measurements for continuous monitoring will be required for a definitive identification of individuals.

**Scheme 4.3.** The ALT/POx/HRP cascade.

**Figure 4.2.** Data obtained from authentic sweat samples from males and females, showing averages and spread (as error bars).
4.5.3 GlDH System

The GlDH system targets glutamate, it demonstrates that differences between individuals can be observed on time scales, \( \tau \), of two minutes and potentially less than that, as shown in Figure 4.3. The graphic representation of this system is shown in Scheme 4.4. Glutamate dehydrogenase (GlDH; E.C 1.4.1.3) consumes glutamate and NAD\(^+\) as substrates, in order to produce corresponding amounts of KTG, NH\(_3\), and NADH. As shown in Scheme 4.4, glutamate can be analyzed in three ways. Pathways A and B were previously described in connection with the Scheme 4.1. In Pathway C, NADH\(_{ox}\) consumes NADH to produce H\(_2\)O\(_2\) in the presence of NADH and oxygen. H\(_2\)O\(_2\) is then consumed by another enzyme, HRP, in order to generate a luminescent response at from 425-445 nm via Luminol.[51]

![Graph](image)

**Figure 4.3.** Results for the GlDH assay using authentic female sweat samples obtained from fingertips.
Note that the latter pathway yields H$_2$O$_2$, which is directly convertible to electronic signals[52, 53] and are frequently used in sensor devices. While optical characterization techniques are convenient for basic-science studies, conversion to electrical signals may be advantageous in applications.

![Biocatalytic cascade](image)

**Scheme 4.4.** The biocatalytic cascade used for the detection of glutamate, along with the three detection pathways: (A) direct measurement of NADH at 340 nm, (B) conversion of NADH to a visible color (formazan) via NBT/PMS which is observable at 580 nm, and (C) oxidation of NADH to produce hydrogen peroxide which is further used to oxidize Luminol via HRP to produce bioluminescence at 425 nm.

### 4.6. FUTURE DIRECTIONS - MULTI-INPUT SYSTEMS

As described previously, it may be advantageous to use several-input processes before converting the output into a physical and ultimately electronic form. The design of such cascades is outline here, in which three amino acids are combined, alanine (Ala), glutamate (Glu), and aspartate (Asp), two of which were encountered in the illustrative single-input studies reported in section 4.5.
Scheme 4.5 considers two inputs, Ala and Glu. In this system ALT enzyme converts alanine to pyruvate (Pyr) which then drives the consumption of KTG as the second substrate for this enzyme, to produce glutamate. The amount of glutamate produced with ALT is added to the glutamate that is present in the sample as an input for the second portion of the cascade.

The enzyme glutamate oxidase (GlOx) consumes the Glu to drive the production of $\text{H}_2\text{O}_2$. $\text{H}_2\text{O}_2$ is then converted to an electronic signal or it can be consumed by HRP, as shown in Scheme 4.5, to drive the oxidation of ABTS which provides a visible signal at the wavelength of 405 nm. This system effectively adds the amounts of the two inputs to produce one output, so it is not of the “digital” nature. However, if needed it can be made “digital” in its response, by adding additional “filtering” chemical or biocatalytic processes.

Scheme 4.5. Ala/Glu Assay.

Scheme 4.6. Asp/Glu Assay.
Another two-input design is the Asp/Glu system, where Asp denotes aspartate. This system is similar to the Ala/Glu system just considered, but here, (Scheme 4.6) aspartate transaminase (AST) is used for targeting aspartate, where, OAC denotes oxaloacetate.

A combination of the two abovementioned systems is possible, to yield a three-input biocatalytic cascade, as shown in Scheme 4.7, involving all four enzymes. Alternative versions of these systems are also possible, such as the use of the GlDH enzyme, with which Glu would be consumed to drive the production of NADH instead of H$_2$O$_2$. This does not provide a visible signal but does produce a readable signal in the ultraviolet range (340 nm).

![Scheme 4.7. Ala/Asp/Glu Assay.](image)

### 4.7. CONCLUSION

The chemical signals obtained with processes described in the preceding sections, some already demonstrated, as well as other multi-input systems that require not only future studies
but also optimization for digital outputs, can be used directly, converted to optical (absorbance, as in Figures 4.2 and 4.3, or fluorescence) outputs for studies of time-series of signal values obtained. However, for digital devices the priority should be given to systems that also allow output in the form of electronic signals. In the considered cascades, the designs that yield H$_2$O$_2$ as the output are thus preferable because the consumption of H$_2$O$_2$ biocatalyzed by the enzyme HRP can yield electronic signals.

An electrode platform can be based on commercial screen-printed technology. Screen-printed gold electrodes (Au-SPEs) can be utilized in small volume cells for optimization of the assays and their performance. The electrodes are modified with the necessary enzymatic machinery for the specific detection cascades in order to retain enzymatic activity, increase the electroactive area, and diminish undesired adsorption.[55, 56] This concept utilizes the detection of H$_2$O$_2$ by biocatalytic action of HRP directly on the electrode surface, allowing for quantitative detection of H$_2$O$_2$. Thus, the considered technology is suitable for the future development of actual chips (sensors) and wearable devices. Research in this area has already demonstrated that human sweat can be analyzed using a wearable device for continuous monitoring of an individual’s state of health.[40]

It is important to note that in 2018 the Halamek lab demonstrated that compounds present in sweat, like amino acids have the ability to differentiate individuals based on person-specific metabolic concentrations over an extended time period.[57, 58] Authentic fingerprints were analyzed using a reliable sample extraction protocol to remove the necessary substrate, alanine. The ROC/AUC analysis of the data from the authentic fingerprint samples further demonstrated the ability of the enzymatic assay to differentiate between authentic male and
female fingerprint samples as it reported a 99.8% chance of correctly identifying the biological sex of the originator.[57]

In summary, a novel emerging concept in utilizing biocatalytic cascades for detection of amino acids in sweat has been reviewed, with promise of applications for active authentication and continuous device-user tracking for cybersecurity of mobiles, wearable, and similar devices has been reviewed. The emphasis lays on the biochemistry aspects of this approach, with some of the considered systems already demonstrated in the preliminary studies, whereas other, more complex systems are still in design stages.

REFERENCES


47. BioTek, *Determination of NADH Concentrations with the Synergy™ 2 Multi-Detection Microplate Reader using Fluorescence or Absorbance 2006*.

48. SIGMA-ALDRICH, NBT.


54. SIGMA-ALDRICH, ABTS™.


CHAPTER 5: CONCLUSIONS

Bioaffinity-based cascades developed in this research have been focused specifically for forensic and biometric authentication used. Well-characterized biomarkers are exclusive to group of individuals, which is why they are widely used in medical settings to diagnose disease states. Enzymatic activity can be classified as a type of biomarker, and is unique to a group of individuals or to certain body fluids, such as blood, hence the versatility of UV-Vis spectroscopy can be applied to explore these characteristics. Herein, we present UV-Vis spectroscopic methodologies that can aid forensic investigations and revolutionize currently employed biometric applications.

Two limiting factors that play enormous roles in forensic investigations are time and money; any method that allows investigators the ability to analyze blood samples directly at the crime scene will circumvent these two factors, thus facilitating faster more economical forensic practices. Described within the first two projects described in this dissertation, characteristics such as TSD of bloodstains and age group of the originator can be determined. TSD of bloodstains can help scrutinize which blood samples are relevant to the investigation to be taken to forensic laboratories for further analyses, such as DNA analysis. The second project, aimed to not only determined the TSD of the blood sample, but to also identify the age group of the originator. This second characteristic determined by the bioaffinity assay, belongs to the specific originator, giving the possibility to investigators to have a narrow pool of suspects and/or victims. Historically forensic serology has relied heavily in genetic analysis, in this research we present protein(enzyme)-forward alternatives that are reliable, economic and effective, that can be performed directly at crime scenes.
Enzymes can serve as biomarkers or as machinery to develop biosensing models for low molecular compounds found in body fluids such as sweat. The low molecular compounds found in sweat are distinctive to an individual, thus they can be used for active authentication by technological devices. Amino acid levels of individuals can serve to track individuals’ health, and to protect their technological devices as shown in this research.

All biosensing models presented here are promising and demonstrate the versatility that enzyme cascades can provide in different settings. Further development of these models is being conducted in the Halamek laboratory.
I. List of Publications by the Author


II. List of Presentations by the Author

ORAL PRESENTATIONS

1. “Ages at the crime scene: a simultaneous estimation of the time since deposition and age of the originator of a blood spot left at a crime scene”
   North Eastern Association of Forensic Scientist 42nd Annual Meeting, Atlantic City, NJ. 2016

2. “The simultaneous estimation of a blood spot’s time since deposition and age of its originator at a crime scene”
   Bridge to the Ph.D. Program Annual Symposium, Columbia University, New York, NY. 2016

POSTER PRESENTATIONS

1. “Single Analyte bioaffinity-based assays for body fluid analysis”
   69th Annual American academy of Forensic Science Meeting, New Orleans, LA. 2017

2. “Simultaneous estimation of the time since deposition and age of the originator of a blood spot at a crime scene”
   American Chemical Society Northeast Regional Meeting (NERM), Binghamton, NY. 2016

3. “Simultaneous estimation of the time since deposition and age of the originator of a blood spot at a crime scene”
   252nd American Chemical Society national meeting, Philadelphia, PA. 2016

4. “Blood markers to identify personal characteristics through biocatalytic cascades”
   North Eastern Association of Forensic Scientist 41st Annual Meeting, Hyannis, MA. 2015

5. “Biocatalytic cascades for the forensic determination of personal properties based on blood markers”
   250th American Chemical Society national meeting, Boston, M.A. 2015

6. “Forensic bio-affinity based assay to determine blood sample age”

7. “Forensic determination of blood sample age using a bioaffinity-based assay”
   Life Sciences Research Symposium VI, University at Albany, SUNY, Albany, NY. 2014
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Forensic determination of blood sample age using a bioaffinity-based assay

J. Agudelo, C. Huynh and J. Halámek, Analyst, 2015, 140, 1411
DOI: 10.1039/C4AN02269F

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