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Proof of Concept for the Forensic Analysis of Blood Spots to Distinguish Time Since Deposition and Relative Age of Blood Spot Originator

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Proof of Concept for the Forensic Analysis of Blood Spots to Distinguish Time Since Deposition and Relative Age of Blood Spot Originator

An honors thesis presented to the Department of Chemistry University at Albany, State University of New York

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Abstract:

In the field of forensics, blood is considered a highly useful tool. DNA found in blood is currently viewed as the best source of identifying a suspect; however, DNA analysis is mainly used as a comparative method. DNA analysis can single out a suspect they are physically present in custody or if a match is made through a database of stored profiles, such as the Combined DNA Index System (CODIS).\textsuperscript{1-3} There are new methods of DNA analysis that can identify a person of African/Caribbean decent, hair and eye color, and even the age of a person.\textsuperscript{4-6} However, this DNA analysis is complex, time consuming, and expensive compared to the biocatalytic-analysis of enzyme, protein, or metabolite levels in blood that can be done quickly, while still determining similar attributes of a blood sample originator.\textsuperscript{7} Using spectrophotometric analysis of a newly designed bioaffinity-based assay of the enzyme alkaline phosphatase, the relative age of a blood sample originator can be distinguished with high accuracy. Alkaline phosphatase (AP) is an enzyme that is commonly used in clinical diagnostics because it is essential to bone growth.\textsuperscript{8-9} Due to its role in growing bones, AP is present in higher levels in people who are younger. The difference in AP levels between someone who is growing, or “young”, and someone who is no longer growing, or “old”, can be determined using the enzymatic reaction of AP with p-nitrophenol phosphate. Results achieved in this experiment show that this system can be used, even after real human serum samples were exposed to mimicked crime scene conditions for up to 48 hours. Additionally, the experimentation done proved that the time since deposition (TSD) of a blood sample at a crime scene could also be determined using the newly developed assay. The information extracted from this novel assay could potentially help police narrow down the suspect pool, and with further advancements it could even be used on-site at a crime scene in order to provide quick and accurate distinctions.
Background and Introduction:

DNA analysis is strongly considered to be the gold standard in forensic analysis of blood samples found at crime scenes because of its highly accurate results. If a DNA match is made the chance of it being a false positive or a coincidental match is on the scale of 1 in 34 million. This is due, in part, to how DNA is analyzed. There are generally two different types of DNA that can be analyzed. One type is chromosomal DNA found in the nucleus of a cell that is often referred to as nucDNA. This nucDNA is the most common form that is analyzed to give a comparative profile for identification. It is analyzed using a polymerase chain reaction amplification step called a short tandem repeat (STR). The varying length of the DNA fragments depends on the composition of the STRs, and since no two people have the same composition of their STRs (aside from twins), the nucDNA profile will always be distinct. In a forensic investigation these distinct profiles are entered and logged into the Combined DNA Index System (CODIS) so that a comparison can be made to a person who has been previously arrested. Additionally, if a suspect is held in custody and a nucDNA profile is established the suspects DNA, if not already in CODIS, can be compared to the nucDNA profile. The problem with this method of DNA analysis is that it is only useful to make a comparison, if there is no suspect in custody or no match in the CODIS database then the nucDNA profile is rendered essentially useless for the police. This is where the other form of DNA is proving to be more useful.

The other type of DNA that is commonly analyzed is mitochondrial DNA (mtDNA), which is found in the mitochondria of a cell. This form of DNA is being analyzed more often in recent years is and is even now being used to distinguish hair color, eye color, and even ethnic origin of sample originator. The information that can be gained from DNA of both types is clearly very extensive and could be very useful for identifying a suspect or narrowing a suspect pool in a forensic investigation.

The problem with using DNA of either type to identify a suspect or narrow down a suspect pool is the time, money, and technical skill required to analyze a single blood sample. While expense and skill are very limiting factors with DNA, the time alone that it takes to get results back is a problem that needs a solution. Just to run a fairly straight forward DNA sample will take about 60 hours to complete. This 60 hour time frame broken up into about an 8 hour work day, assuming that only this one sample is worked on, would take 7.5 days, or just over a week to run. This is not even including other work that the laboratory might have to do including testifying,
quality control checks, and other laboratory responsibilities. Additionally, most laboratories have extensive back logs of samples that need to be run before any new samples can even begin to be analyzed. If a laboratory has only 20 samples that need to be run when a new sample is brought in, the best case scenario is that it would take about 21 weeks or just over 5 months to get that new sample’s DNA profile back. An estimate of 5 months could even be considered on the faster time line for a result to be returned, in Connecticut it can take up to two years for a DNA test to be returned for a property crime case. After such a long time spent waiting a case may go cold, or some cases may even be solved before a sample results come in. The long time spent waiting, on top of the extensive skill and money required to actually run a sample, are the reasons that a faster, cheaper, and simpler means of suspect identification is of vital importance to forensic science.

Blood samples also contain various proteins and enzymes that can give suspect identification through their vital roles in numerous biological processes. These proteins and enzymes are often found in characteristic levels, which are used in clinical diagnosis to determine if someone is sick. The markers used for clinical diagnosis have two categories; reliable and unreliable. Reliable markers are levels of a protein or enzyme that are considered to be relatively constant for all ages, biological sexes, and ethnicities. Unreliable markers, on the other hand, vary based on a person’s personal attributes. These unreliable markers, while considered not as useful for clinical diagnostic purposes, can be used in reverse to determine a personal attribute of an assumed healthy individual. Previous research conducted in the Halámek research laboratory or by Dr. Halámek himself before he came to the University at Albany, State University of New York has proved that the biological sex, ethnicity, and age of blood sample can all be determined using multi-enzyme assays. A multi-analyte system works as described in figure 1.

![Multi-analyte system](image)

**Figure 1.** Multi-analyte system where the substrate₁ produces product₁, which is reacted with enzyme₂ to create the final product₂, which would result in the final color change to be monitored using a spectrophotometer.
The system outlined in figure 1 only works if Substrate$_1$ and Enzyme$_1$ are present so the second reaction can occur. Similarly, the entire reaction would show no final color change if Enzyme$_2$ was not present. This type of system was used to conduct research using the markers creatine kinase (CK) and lactate dehydrogenase (LDH), in combination with pyruvate kinase (PK), to distinguish between African American and Caucasian blood sample originators.\textsuperscript{13-14} Both the blood levels of CK and LDH vary in clinical diagnostics for Caucasian and African American populations, so the two markers combined into one assay amplify the generated signal to distinguish between African American and Caucasian samples.\textsuperscript{13-14} In a similar reaction combining CK, PK, LDH, and alanine transaminase (ALT) gender of a blood sample originator was determined.\textsuperscript{14-16} Both CK and ALT levels vary in diagnostics for males and females making the total assay able to distinguish between a male and a female blood sample.\textsuperscript{14-16} In analyzing the use of this assay the samples were allowed to age for up to 120 hours and it was determined that the assay could also be used to determine the time since a sample was deposited.\textsuperscript{14,16,17} All of these attributes are important for forensic investigations and the previous experimentation allowed for an extensive background knowledge when deciding to use a much smaller and simpler single enzyme system to analyze the age of a blood spot originator, instead of the more complex multi-analyte systems used before.

While the results of Dr. Halámek’s showed promising and useful results the multi-analyte systems are more complex than a simple one enzyme system. The multi-analyte system has a greater room for error when it is being developed because there is potential that the product of one enzyme may inhibit the activity of another. Similarly, for an enzyme to produce enough product to be later used by many other enzymes and still show signal, the concentration of the starting substrate must be rather high. The higher the concentration of the substrate that needs to be input in the system to start the reaction the more money is spent per run of the assay on chemicals for the system. Also the more enzymes involved the more expensive the assay becomes to run, though still not nearly as expensive as running DNA analysis.\textsuperscript{10} For these reasons it was decided that the next system be utilized should be a one enzyme assay in order to reduce costs and the potential for negative influence by other enzymes or their products. The one enzyme biocatalytic cascade that was chosen to be analyzed is simple, inexpensive, and it is useful for forensic investigations because it can distinguish the age of a blood sample originator.
One personal attribute that is rather important for a forensic investigation to identify about a suspect is their age. 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. Crimes committed by people under the age of 18 are considered juvenile crimes and are thus tried, prosecuted, and sentenced differently than adults. Knowing this distinction between a juvenile and adult suspect can be advantageous for criminal investigators in how they begin to build their case. Additionally, having the ability to differentiate between a suspect’s age at the 18 year mark is necessary because the age range that most property crime is committed, is between 15 and 25 years old. If investigators can determine if a blood sample is from someone younger or older than 18 they can effectively eliminate half of the suspect pool for a property crime. The mean crime age for all types of property crime varies between 20 and 34 years old, for crimes against a person (i.e. assault, murder, weapons, etc) the mean age is between 27 and 31 years old, for substance abuse it is between 24 and 32 years old, and for public order crimes it is between 25 and 26 years old. With the total mean age for committing any crime being at 26 years old, knowing if a suspect is under the age of 18 would drastically eliminate a large portion of suspects. While DNA evidence from blood can determine the age of a suspect, the turnaround time is so long that the information gathered from the DNA is often rendered useless. This is why a quick and accurate analysis of enzyme levels to determine age is of particular interest for forensic investigations.

The enzyme alkaline phosphatase (AP; E.C. 3.1.3.1) is found to be an important enzyme in the function of bone growth. AP is a metalloenzyme with Mg$^{2+}$ and two Zn$^{2+}$ present at each active site and it functions through the formation of a covalent phosphoseryl intermediate. As a phosphatase AP functions by removing a phosphate group from a protein to help modulate the activities of the numerous proteins found in a cell, usually in response to external stimuli on the cell. In the body AP specifically hydrolyzes pyrophosphate (P$_2$O$_7^{4-}$) at a high pH in order to provide the inorganic phosphate that is needed to promote the process of mineralization, which is the early stage of bone formation. While a person is growing, usually through the ages of 0-18,
he or she has high levels of AP to facilitate the formation and growth of bones. However, as a person stops growing their AP levels begin to decrease linearly over time. Due to the AP’s key function in bone growth and development its characteristic levels at different age ranges are known and used to diagnosis medical conditions. If a patient is in the growing stage of life (0-18) and has low levels of AP it could be a warning sign of a growth defect. Conversely, if an older person who is no longer in the growing stage of life has high levels of AP it is a sign for osteomalacia, or a softening of the bones that often occurs in the elderly. Assuming that a person is healthy, however, the linear decrease in AP levels in blood as someone ages can be used forensically to predict the age of a blood sample originator through a simple enzymatic assay. According to published values the mean AP values for young males (age 0-18) is 343.9312 U/L, young females (age 0-17) is 326.9212 U/L, older males (19-60+) is 111.2277 U/L, and older females (18-60+) is 100.0700 U/L. Using these published values of AP levels for varying age ranges and a simple catalytic assay it was established that a distinction can be made between a younger (juvenile) suspect and an older one quickly and accurately.
Experimental Model:

The biocatalytic assay that was used to distinguish between “old” and “young” AP levels is represented in figure 3. The substrate, para-Nitrophenol phosphate (pNPP), is colorless in solution, but once it is hydrolyzed it becomes a characteristic yellow color. The change in color from colorless to yellow can be monitored at 405 nm using a spectrophotometer. In the biocatalytic assay, the more substrate present in the system, with the enzyme concentration kept constant, the more yellow color change there will be. Similarly, more enzyme present, with the substrate concentration kept constant, will result in a more notable yellow color. This is quantitated through a larger absorbance value at the yellow wavelength of 405 nm.

![Figure 3. Hydrolysis of para-Nitrophenol phosphate (pNPP) to para-Nitrophenol (pNP) and H$_3$PO$_4$.]

All spectrophotometric analyses were completed using a Molecular Devices Spectramax NanoDrop 384 spectrophotometer and a Tecan Infinite F200 Pro microplate reader. All reactions were carried out in 0.1 M carbonate buffer at a pH of 9.0. Additionally, the absorbance spectrum was monitored at 37°C using microtiter polystyrene (PS, Thermo Scientific) plates for 900 seconds. The AP purchased was phosphatase, alkaline from bovine intestinal mucosa (AP; E.C. 3.1.3.1) and was purchased from Sigma-Aldrich. The pNPP used was also purchased from Sigma-Aldrich and it came in the form of para-nitrophenyl phosphate disodium salt hexahydrate, which is dissolved in 0.1 M carbonate buffer of pH 9.0. The concentration of pNPP was kept constant at 2.5 mM while the concentration of AP in units per liter was varied based upon the known values for young and old males and females. All water that was used in this experiment was ultrapure deionized water (18.2 MΩ•cm). All samples were run in real human serum (type AB; Sigma-Aldrich) and were spiked with varying AP levels.
Results and Discussion:

Calibration Plot

To first determine if AP would actually show a linear change in absorbance as the level of AP was varied a calibration plot was established using the assay conditions outlined in the experimental model section. This can be seen in Figure 4.

![Calibration Plot](image)

**Figure 4.** Calibration plot representing the increasing absorbance with increasing AP levels and decreasing age. Each concentration was run in triplets (n=3) for 300 seconds, and each point is determined as the absorbance at time zero subtracted from the maximum absorbance reached at 300s.

In figure 4 the red star represents the corresponding absorbance value for the average “old” female value of AP. The blue diamond is the corresponding absorbance value for the average “young” female value of AP. This calibration curve shows the linear increase of absorbance as age decreases and AP level increases. Although this calibration plot cannot be used to determine the precise age of person, it does show that absorbance is linearly dependent on AP level. This proved that AP has potential to be used to distinguish between the known values of a “young” and “old” male and female.
Male vs Female and Old vs Young

After it was proven that the absorbance of AP would increase linearly with increasing AP level the challenge was to determine if the difference in “young” and “old” could actually be observed. Human serum samples were spiked with the known AP values for a young female, young male, old female, and old male in U/L to see if there was a distinct difference between “young” and “old”, or if the biological sex would alter the results. The value of pNPP was set as explained in the experimental model section, and the resulting color change was monitored over the course of 900 seconds.

![Graph showing absorbance over time for different groups](image)

**Figure 5.** Hydrolysis of pNPP to pNP by AP in real human serum samples monitored at $\lambda=405\text{nm}$.

The results shown in Figure 5 confirmed that a clear distinction can be made between the average levels of AP for young and old females and males. Additionally, the results confirmed that whether the sample came from a person who is biologically male or female it does not influence the ability to distinguish “young” and “old”. This result determined that biological sex will not
inhibit the ability of the assay to distinguish between the “young” and “old” blood sample originators. While this result could not be used to differentiate between a young male and a young female, the biological sex of the sample originator can be determined using other sample analysis that are not the focus of this research.13-17

Population Statistics

The results in figure 5 proved that the determination of a blood sample’s age group could be determined, so the next step was determining how accurate and precise the biocatalytic-assay was. To do so computer generated randomized population distributions of “young” and “old” samples for both male and female were created and analyzed.

\[ \text{Figure 6A.} \quad \text{Black lines represent young male, while red lines represent old male randomized AP levels and the corresponding absorbance as it was measured at } \lambda=405\text{nm through the hydrolysis of pNPP to pNP.} \]
Based on the literature values for AP levels of young males, young females, old males, and old females the distribution of these levels is best represented by a log-normal population distribution.\textsuperscript{8} Using this log-normal population distribution for AP levels 25 randomly distributed AP levels were calculated for young and old males and females that would fit the log-normal model. Then, these randomly distributed AP values were analyzed with the assay conditions outlined in the experimental model section to generate the resulting absorbance curves for all 100 samples of young and old male and females. These absorbance curves for the hydrolysis of pNPP to pNP by a population distribution of AP levels are plotted in figures 6A and 6B for young and old males and females, respectively.

Using these absorbance curves that are representative of the log-normal population distribution of AP levels, a receiver operating characteristic (ROC) analysis was performed using package pROC.\textsuperscript{22} The ROC analysis was done in order to determine both the precision and accuracy of the developed biocatalytic assay. Sensitivity (true positive rate) versus specificity (true negative rate) was plotted for the ROC curve for differing thresholds of class assignment.\textsuperscript{24} The AUC, or Area Under the Curve, for the ROC curve was estimated using the trapezoidal integration method, and the resulting 95 \% confidence interval (CI) was estimated using the method described by DeLong et al.\textsuperscript{23} The results of the ROC analysis can be seen in Figures 7A and 7B.
The AUC specifies how well the assay separates samples according to the change in absorbance assigned to the positive class. The AUC value for the male is 99% and the female is 100%. This means that when a randomly chosen value is placed into the predictive model – which is generated by the 25 old and 25 young samples – it has a 99% chance of correctly determining a young versus an old male and 100% chance of correctly determining a young versus an old female. The lighter diagonal line represents where the AUC, or the predictive power of the model, would be 50%, – the point at which the assay would have a random chance of correctly categorizing a sample as “old” or “young”. The ROC curves show that, as a test to distinguish between a “young” and “old” originator of a blood sample the biocatalytic assay for AP has a 99% and 100% probability of correctly differentiating between the two categories for males and females, respectively.

**Figure 7A.** ROC for males. The blue line represents with what sensitivity and specificity the assay can distinguish between old and young. The AUC of 99% represents the likelihood that the assay would correctly identify an old or a young randomly distributed male sample.

**Figure 7B.** ROC for females. The blue line represents with what sensitivity and specificity the assay can distinguish between old and young. The AUC of 100% represents the likelihood that the assay would correctly identify an old or a young randomly distributed female sample.
The accuracy and precision with which the two categories can be distinguished is very useful in forensic investigations, even if the precise age of a person cannot yet be determined using this assay. Especially if it is done at such a high degree of accuracy, the differentiation between someone below the age of 18 and someone over 18 is of particular interest for forensic investigators due to the fact that a suspect under the age of 18 is a juvenile.\(^{18}\) Since juveniles are processed through the criminal justice system entirely different than adults, knowing with 99 or 100% accuracy if the suspect is a juvenile can assist the prosecution in determining what charges they will be filling and how best to build their case for a juvenile hearing. Additionally, with the propensity to commit crime being so high between the teenage years and 30 years old, the ability to determine if a suspect is under the age of 18 with a 99 or 100% probability of accurately determining the age range, will drastically eliminate a large portion of suspects.\(^{18}\) While this is an excellent result, there is still a question of how the assay would hold up if the blood sample was not spiked and run immediately after, such is often the case when a crime scene is discovered and the blood is not usually freshly deposited.

**Crime Scene Conditions and TSD**

To address the problem of crime scene conditions and delay in finding evidence an aging process was done on samples to determine if the hydrolysis of pNPP to pNP by AP could still distinguish between “old” and “young” samples. Since the difference in the literature AP values for male and female are statistically insignificant, only female spiked human serum samples were aged. The process of aging the samples followed the same experimental model as for the regular spiked serum samples. A total of 160 samples were set up, two groups (young and old) of eight sets (to be used for the eight different time intervals) consisting of 10 serum samples each, were spiked with the old and young AP values for females and left to sit uncovered on the laboratory bench at 25 ± 3°C. The samples were put in mimicked crime scene conditions to age for 0, 3, 6, 12, 24, 30, 36, and 48 hours. All of the samples were rehydrated with water (except for time 0), and the exact same volume of each sample was extracted then analyzed in the assay in the same conditions as explained in the experimental model section. Each marker on the graph represents the average and standard error of the 10 samples run for each time and age group.
According to Figure 8, the difference between an old and a young female can still be determined even after up to 2 days of being left out in crime scene mimicked conditions. Even after 48 hours the lowest young sample still gave a higher absorbance than the fresh old sample. This not only means that this biocatalytic assay has application to real crime scenes even after the sample has been left for up to two days to distinguish the age of the blood sample originator, but it can also be used in reverse to determine the time since deposition (TSD) of the sample. The time at which a sample is deposited can be determined using Figure 8 as a calibration plot, so long as the relative age of the suspect is known. This can be done by simply rehydrating the blood sample, taking the volume used to run the assay, finding the resulting absorbance on the graph, followed by using the person’s age to distinguish between an older “young” sample and a newer “old” sample. Knowing the time when a blood sample was deposited at a crime scene can give investigators a wealth of information. The TSD could eliminate a sample as being part of the crime if it was found to be left long before the crime occurred, or if a blood sample is found to have been deposited at a particular time it could give a suspect an alibi. Overall, this simple but accurate biocatalytic-assay can determine a wealth of information about a suspect that would be pertinent to an investigation.
Conclusion:

The results of the biocatalytic-assay found that hydrolysis of para-nitrophenol phosphate (pNPP) to the yellow para-nitrophenol (pNP) through the enzyme alkaline phosphatase is capable of generating characteristic absorbance values that could be used to distinguish between “young” and “old” males and females. Unlike many other biocatalytic-assays that determine a personal attribute about a blood donor, the hydrolysis of pNPP to pNP by AP is not altered in a statistically significant manner by the biological sex of the blood originator. This means that the assay can distinguish between “young” and “old” without interference of the biological sex of the blood sample originator. Using statistical analysis of the AP hydrolysis of pNPP to pNP, it was determined that the assay could correctly distinguish between the “young” sample and “old” sample with a 99% and 100% probability for male and female samples, respectively. This is proved to be useful for forensic investigators because it can differentiate between a juvenile and an adult offender, and it can drastically eliminate a large portion of a suspect pool. Additionally, the biocatalytic assay is useful for forensic investigations because it can still differentiate between “old” and “young” blood samples that were left at crime scenes in open conditions for up to two days. The aging of samples proved the real world application of the assay to actual forensic investigations. The results of aging the mimicked blood samples also showed that the AP assay could also be used in reverse to determine the time since deposition of a sample (TSD). The results of the numerous experiments performed proved that the hydrolysis of pNPP by AP is highly accurate, precise, and useful to real world applications. The success of this project has even allowed the results to be submitted to the journal *Analytical Chemistry* for possible publication.
**Future Implications:**

The results of this experiment prove that the age range of a blood sample originator can be accurately determined in as little as 15 minutes through the conversion of pNPP to pNP by AP. This time to completion does not take into account the time it would take to get a blood sample to a laboratory to be analyzed. If done within an hour or two of the crime scene being discovered the total turnaround time would likely be just under three hours. While this would still be much faster than the turnaround time for DNA analysis to identify the age of a person, in the future this biocatalytic-assay could even be done on site. Small, portable UV/Vis spectrophotometers that plug into a laptop or that can be used with smartphones have been developed that can analyze a single cuvette of liquid. Using these type of small and portable UV/Vis instruments, a portable kit could be developed to test blood samples found on scene. All that would be needed is a stock of carbonate buffer solution that would be run through the cuvette as a blank, then the buffer, the blood sample, and a stock pNPP solution would all be used to activate the biocatalytic assay to get the absorbance of the blood sample. This method would require little training, is as simple as having bottles with labels of how much of each liquid to put in the cuvette, and could easily be done by forensic investigators at the scene. By increasing the turnaround time police officers can narrow down their suspect pool at the start of an investigation. This would help avoid wasting time looking into a suspect who does not fit within the age range, and would substantially aid investigators in situations where the first 48 hours are the most important time, particularly in murder and missing person’s cases.

Before this test could be applied fully to on-site, real world forensic investigations future experimentation should be done into the effects of more extreme environments on a blood samples resulting absorbance from the assay. When the crime scene mimicked conditions were done the samples were placed at about room temperature and were left inside the laboratory, which is humidity controlled. While this part of the experiment was necessary to prove that the assay could still distinguish between “young” and “old” after a sample was aged in ideal crime scene mimicked conditions, it would need to be taken another step further. The same procedure should be done but with samples incubated at very high temperatures to mimic the conditions of crime scene in the summer or in a hotter climate. Additionally, humidity levels should be varied to test if the assay would still be applicable to more humid climates. Also samples should be placed incubated in the freezer to mimic the freezing cold temperatures of winter. Lastly, precipitation tests should be run
where the samples would be left in the rain and a blind test would be performed to determine if the distinction between an “old” and a “young” sample could still be made. Further testing of the samples in various real world environmental conditions would solidify the assay’s potential to be used in all crime scene applications.

Future tests could be, and are currently being completed in the Halámek research laboratory, into combining this simple one enzyme assay into a more complex multi-analyte assay, like that shown in figure 1, that could distinguish more than the age range of a blood sample originator. As previously mentioned, the so called “unreliable” diagnostic markers often vary for different personal attributes. These markers could be coupled in a reaction to determine more than just the age range of the blood sample originator. For example, the previously mentioned CK, PK, LDH assay that distinguishes between the two African Americans and Caucasians, could be combined with the single enzyme AP assay to determine both age and ethnicity simultaneously.\(^{13-14}\) In the CK, PK, LDH assay PK takes in phospho(enol)pyruvic acid (PEP) to make pyruvate, which is then reduced by LDH to simultaneously oxidize NADH to NAD\(^+\).\(^{13-15}\) AP can also take in PEP and convert it to pyruvate. So in theory, in a CK, AP, LDH assay if the person’s blood sample had enough AP to hydrolyze PEP to pyruvate, the ethnicity of the person would be determined by CK and LDH, but the fact that there was enough AP to get a distinction between ethnicities would indicate that the person is also young. If there was very little or almost no signal, it would likely mean that the blood sample is from an older Caucasian person. This is just one theory of how combining the simple AP biocatalytic assay with another assay could be used in forensic investigations to distinguish more than one personal attribute of a blood donor on site at a crime scene.

Similarly, the combination method could also be used in the future to distinguish a more accurate age profile of a blood sample originator. Over the age of 25 some other enzymes lose their specific activity and become less reactive, mainly phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase.\(^{19}\) This loss in specific activity could also be measured using a biocatalytic-assay and in combination of with the AP assay developed in this experiment, the resulting absorbance could potentially differentiate between the age ranges of 0-18, 19-25, and 25+.\(^{19}\) These are the three age ranges that would be most important to distinguish between since crime is most commonly committed by people around the age of 20.\(^{18}\) Therefore, a multi-analyte system could not only give forensic investigators more information about other personal attributes
but it could even increase the distinction in age range of a blood sample originator. As a whole, this experimental development of the biocatalytic AP assay has tremendous potential to give forensic investigators reliable and pertinent information about a blood sample originator. As a proof of the concept of the AP assay’s applicability to forensics this experiment was a success, and has many directions that it could go in the future to make it more precise and reliable for on-scene use. Perhaps in the future the AP assay for blood analysis could become as common at crime scenes as the use of Luminol.
References:


