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Ultraviolet-Visible Spectrophotometry (UV-VIS) and SALIgAE® Qualitative and Semi-quantitative Tools for the Analysis of Salivary Amylase

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Abstract

The goal of this research was to quantitatively measure the amount of salivary amylase in a sample by using UV-VIS and the SALIgAE® reagent. The samples included serial dilutions of standard human salivary amylase, human saliva, as well as other body fluids from human and animals. These were added to the SALIgAE® reagent and the absorbances of the solutions were monitored by the UV-VIS at 403 nm every 30 seconds over a 10 minute period. The lowest concentration of salivary amylase detected by UV-VIS was 1 ppm (parts per million), or 0.0010 units of amylase, while the color of the reagent was clear upon visual observation at this dilution. The absorbance change and kinetics of the reaction between the amylase and the SALIgAE® reagent were determined to be proportional to the concentration of the salivary amylase in a dilution. This relationship can be used to quantify the amount of amylase from items of evidence.

Keywords: Forensic science; Forensic serology; Amylase; Saliva; Ultraviolet-visible spectrometry

Introduction

Human saliva is often encountered in forensic investigations of violent crimes. Detecting the presence of saliva in a biological sample is essential for investigative purposes and for eventual DNA analysis. Usually saliva stains are identified by the detection of salivary amylase, an enzyme found in large quantities in saliva. If the test for salivary amylase is positive, then the conclusion is that saliva may be present [1]. There are two genetic loci on chromosome 1, Amy 1 and Amy 2, which code for two different types of this enzyme. Amy 1 codes for the "salivary" type of amylase [2-6] and Amy 2 codes for the "pancreatic" variety [3,7]. Other body fluids, such as serum and tissues of male and female reproductive organs may contain both varieties [8].

The SALIgAE® saliva test from *Abacus Diagnostics Inc.* is a simple, sensitive, and accurate colorimetric test for the detection of salivary amylase [9,10]. The color of the SALIgAE® reagent changes from clear to yellow when amylase is present in a sample. However, determining the color change solely by visual means can be subjective, especially if the saliva in an evidence sample is extremely diluted or highly degraded, due to challenging environmental conditions. Also, sometimes the sample is contaminated or deposited on colored substrate and the extract of the sample appears yellow. In such cases it is difficult to determine if the color changes of the solution are due to the presence of salivary amylase. Since both forms of amylase have a similar structure, pancreatic amylase may also react weakly with the SALIgAE® reagent giving it a faint yellow color. Therefore, various body fluids may react with the SALIgAE® reagent and generate a yellow color.

UV-VIS is an instrument often used to measure the absorbance of a sample at a certain wavelength. A typical UV-VIS includes four major components: a light source, a scanning monochromator, a sample/reference cuvette, and a detector. A beam is emitted from the light source and then separated into narrow bands of different wavelengths by the monochromator. When the beam passes through the sample, some radiation is usually absorbed by the target compound in the sample. As the beam continues to travel, the detector will measure the irradiance, P, of the beam, measured in units of watts/m^2. However, when the reference sample or solvent blank is placed in the path of the beam, no radiation is absorbed and the irradiance is denoted as P_0. The ratio of P and P_0, which is between 0 and 1, is the transmittance, T. The absorbance of the sample is –logT, which is directly proportional to the concentration of the target compound of the solution. Beer's law states that the amount of absorbance of a solution equals the product of the molar absorbtivity of a compound (ϵ), the light path of the sample (b), and the concentration of the compound in the solution (c):

$$\text{Absorbance} = \epsilon bc \quad (1)$$

Thus, if the path length and the molar absorbtivity are constant, and the absorbance is measured, the concentration of the substance, i.e. the yellow product generated by the SALIgAE® reagent and amylase, can be calculated.

Since the exact mechanism of the SALIgAE® test is currently unknown, the reaction between the SALIgAE® reagent and amylase can be represented as:

$$\text{SALIgAE®} + \text{Amylase} \rightarrow \text{Yellow Product} \quad (2)$$

As long as the amount of the SALIgAE® reagent is significantly larger than the amount of amylase, the concentration of the product is equal to the concentration of the amylase. Therefore, the amount of amylase in a sample can be quantitatively obtained by measuring the absorbance of the sample. Higher concentrations of the yellow product in a sample results in a more concentrated yellow color. Thus, absorbance can also provide a more accurate result for the SALIgAE® test than simple visual examination.

The UV-VIS used in this research can be programmed to measure

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the absorbance multiple times in a period. Thus, the kinetics of the reaction between the salivary amylase and SALIgAE® reagent can be revealed. Kinetics, also known as the reaction rate, describes the speed of a chemical reaction and provides information about the mechanism of the reaction and other characteristics. According to the manufacturer, the color of the SALIgAE® reagent changes from clear to yellow in a 10 minute interval upon the addition of amylase; the concentration of the yellow product, [Yellow], can then be estimated as a function of time, t:

$$[\text{Yellow}]_t = kt + [\text{Yellow}]_{t=0} \quad (3)$$

Equation (3) is the form of the integrated zero-order reaction law, which assumes that the rate is independent of the concentration of the species. The linear plot used to determine the reaction rate is a straight line, which is the slope k in equation (3). When visually observed, concentrated saliva samples generate yellower colors than diluted samples in a 10 minute interval. According to equation (3), if final [Yellow] is larger and time is constant, the term k has to increase to balance the equation. This relationship can also help determine the concentration of the amylase in a sample. Plus, if the pattern of the color change in a sample corresponds to the kinetics of reaction, the color change is most likely due to the presence of amylase rather than other contaminations. The goal of this research was to quantify the color change of the SALIgAE® reagent from a variety of samples by using UV-VIS.

Materials and Methods

Standard Sample Preparation

Standard human salivary amylase (Catalog Number: 8F16508) and pancreatic amylase (Catalog Number: 4G19406) were obtained from *Meridian Life Science, Inc* (Saco, ME). The salivary amylase sample was in powder form, which contained 0.4 g of protein, and the concentration of specific activity was 518 Units/mg Protein. 1 mL of distilled deionized water (ddH_2O) was added to the powder to reconstitute a 400 ppm (mg protein/L) solution. The standard 0.165 mL of pancreatic amylase solution contained a total 100 Units of protein, with a concentration of 605 Units/mL or 3.4 mg protein/mL. Thus the total amount of the protein in the sample was 0.561 mg. The sample was reconstituted to 400 ppm (mg protein/L) by adding 1.24 mL of ddH_2O. The stock solutions of salivary and pancreatic amylase were then diluted with ddH_2O to 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 ppm, and 30, 50, 80,100, 120 and 160 ppm, respectively.

Sample collection

Samples were collected following Institutional Review Board and Institutional Biosafety Committee guidelines of Pennsylvania State University. Human saliva samples were collected from six male and female volunteers whose ages ranged from twenty-four to sixty-eight. Each individual was assigned a random ID number: PSU-01 to PSU-06. Three samples were collected from each donor, at three different times during one single day: in the morning, in the afternoon and at night. Thus a total of eighteen samples were collected using sterile cotton swabs. The three samples from the donor with sample ID PSU-01 were then denoted as PSU-01a, PSU01b and PSU-01c. This numbering system was followed for the other five donors as well. The samples were extracted and diluted using ddH_2O, with dilution ratios ranging from 1:20 to 1:2. Other body fluids such as blood, semen and urine from humans, saliva from cats and dogs, and urine from pigs, cats, dogs, stallions and bulls were also used. The blood, semen and saliva samples were collected by sterile swabs and then extracted with ddH_2O. The urine samples were already in liquid form and used without dilution.

Approximately 2 µL of each extract were added to 600 µL of water and the absorbance was measured by the UV-VIS (Varian Cary 4000 Spectrophotometer, Palo Alto, CA) over a broad wavelength range (200 nm to 800 nm) to determine the peak wavelength. To create a calibration curve, 2 µL of each standard salivary amylase dilution were added to 600 µL SALIgAE® reagent (Catalog Number: 903295) from *Abacus Diagnostics, Inc.* (West Hills, CA). The changes in absorbance of each sample were monitored by UV-VIS at 403 nm in 30 second intervals over a 10 minute period. The starting time of the measurement was 60 seconds after the addition of the samples. The final absorbance of each dilution was used to create the calibration curve of standard salivary amylase. The absorbance values of the standard pancreatic amylase dilutions and the extracts of human and animal body fluids were also measured by UV-VIS in conjunction with SALIgAE® reagent with sample size of 2 µL. The final absorbance of the dilution of human saliva extract that were in the absorbance range of the standard calibration curve were recorded along with the dilution ratios. Also, 1 µL of each standard salivary and pancreatic amylase was added to 300 µL of SALIgAE® reagent to observe its color change visually.

Results and Discussion

The peak wavelength of all the biological samples and the corresponding absorbance are listed in Table 1. Salivary amylase had a single peak wavelength at 282 nm; no other body fluid had similar peak wavelength. Some of the samples had no peak wavelength and some of them had multiple peak wavelengths. The results indicate that it is possible to exclude samples that are suspected to have originated from non-salivary source by using a simple UV-VIS absorbance screen. For example, if a sample generated multiple peak wavelengths, such as observed in this study using human urine, it is not very likely to be a sample containing only salivary amylase. In these cases a mixture of saliva and urine should be suspected.

The peak wavelength of the SALIgAE® reagent after the addition of the salivary amylase was 403 nm. The best quantification range of the absorbance measured by UV-VIS is 0.2 to 2, which is well accepted by the forensic science community. The concentration of salivary amylase dilution just above the lower end of the range was 1 mg/L, or 1 ppm, which corresponded to 0.0010 Units of salivary amylase. At this concentration, SALIgAE® reagent had no color change when examined visually. Figure 1 contains the results of five SALIgAE® Test tubes with the addition of five different concentrations of the standard human salivary amylase, from 1 ppm to 5 ppm, separately. The results

Sample ID	Final color	Peak wavelength (nm)	Absorbance
Salivary amylase (400 ppm)	colorless	282	0.0988
Pancreas amylase (400 ppm)	colorless	N.A[a]	N.A
Feline (cat) saliva	colorless	N.A	N.A
Canine (dog) saliva	colorless	N.A	N.A
Human semen	colorless	N.A	N.A
Human blood	colorless	N.A	N.A
Human urine	colorless	203	-0.1567
Porcine (pig) urine	colorless	223	1.2935
Cattle (bull) urine	colorless	237	0.3429
Canine (dog) urine	colorless	237	1.2374
Stallion (horse) urine	colorless	236	0.9316
Feline (cat) urine	colorless	236	0.9359

[a] N.A – no peak λ was observed or the peak λ was beyond the detection limit of UV-VIS

Table 1: Results of the absorbance of biological samples mixed with water.

Figure 1: Results of SALIgAE® test of five known dilutions of standard human salivary amylase with concentrations of 1 ppm, 2 ppm, 3 ppm, 4 ppm and 5 ppm from left to right.

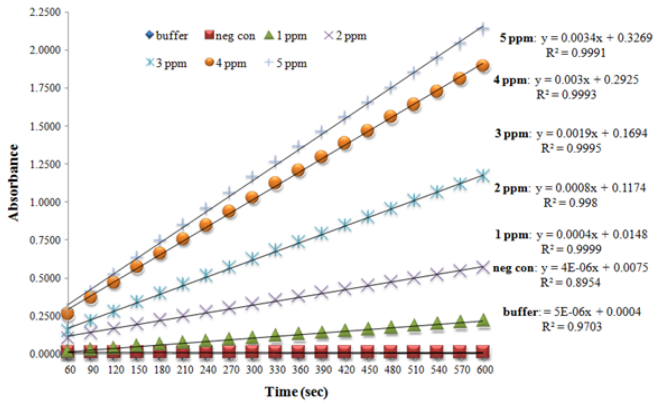

Figure 2: The curve of absorbance versus time of five known dilutions of standard human salivary amylase with concentrations: 1 ppm, 2 ppm, 3 ppm, 4 ppm and 5 ppm. The function of each curve is listed on the right side.

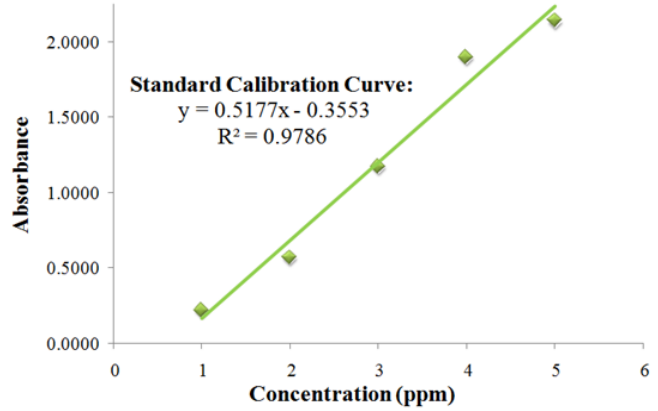

Figure 3: Standard calibration curve of standard human salivary amylase with absorbance range of 0.2161 to 2.1392. The equation of the calibration function is listed with $R^2 = 0.9786$.

were read at exactly 10 minutes after adding the samples. The colors of the solution gradually became more yellow as the concentration of the amylase was increased. The tube with 5 ppm, show strong yellow color, while the tubes with 2 ppm to 4 ppm amylase indicate much lighter yellow colors.

The curves of absorbance versus time of salivary amylase at five concentrations are shown in Figure 2. From the graphs, the final absorbance of a sample with higher concentrations is larger than the absorbance of a sample with smaller amount of amylase. Since more concentrated amylase sample generated yellower color, absorbance is directly proportional to the color of the solution and the concentration of amylase in the sample.

A calibration curve of absorbance versus the concentrations was created in order to use the standard amylase samples for the estimation of the concentration of an unknown sample (Figure 3). The function of the calibration curve was listed, which can be used to calculate the concentration of an unknown sample, with $R^2 = 0.9786$

$$y = 0.5177x - 0.3553 \quad (4)$$

The range of the absorbances covered by this calibration curve was 0.2161 to 2.1392.

The absorbances of four other dilutions of the standard salivary amylase, 1.5 ppm, 2.5 ppm, 3.5 ppm and 4.5 ppm, were measured and their concentrations were calculated by using equation (4) and shown in Table 2. The percentage errors of the calculated concentrations of the four dilutions range from 0.63% to 11.17%. This implies that the absorbance of an amylase sample can be used to estimate its concentration and provide a relatively accurate result.

Another observation from Figure 2 was that the color of the solution became more intense as the reaction between the amylase and the SALIgAE® reagent was prolonged. The curves are in the shape of a straight line, which indicates that, the rate of reaction between amylase and SALIgAE® reagent were indeed zero-order reactions. The function of each curve shown in the figure is in a form similar to that of equation (3), while x represents time, and y represents absorbance; the slope of the function represents the kinetics k. Table 3 has listed the kinetics of each dilution, which increase as the concentration of the amylase increases. The final absorbance of each sample and the results of visual examination of SALIgAE® test in Figure 1 are also shown.

Similar conclusions were drawn from the results of serial dilutions of human saliva samples obtained from six donors. For example, Figure 4 has the curves of absorbance versus concentration of three human saliva samples provided by donor PSU-01 at different times of the day: in the morning, in the afternoon and at night. Each sample had its absorbance measured at 4 dilutions; more concentrated dilutions generated higher absorbance.

The estimated concentrations of amylase in all 18 human saliva

Conc. (ppm)	Absorbance	Calculated Conc. (ppm)	Percentage Error
1.5	0.3717	1.4042	6.38
2.5	0.9308	2.4842	0.63
3.5	1.6591	3.8910	11.17
4.5	2.1390	4.8180	7.07

Table 2: Approximated concentrations of four unknown dilutions of standard human salivary amylase.

Conc. (ppm)	Final Absorbance	Kinetics	Color of SALIgAE® Reagent
1	0.2161	0.0004	Colorless
2	0.5664	0.0008	A trace of yellow
3	1.1705	0.0019	Light pale yellow
4	1.8975	0.0030	Light bright yellow
5	2.1392	0.0034	Bright yellow

Table 3: Results of standard human salivary amylase.

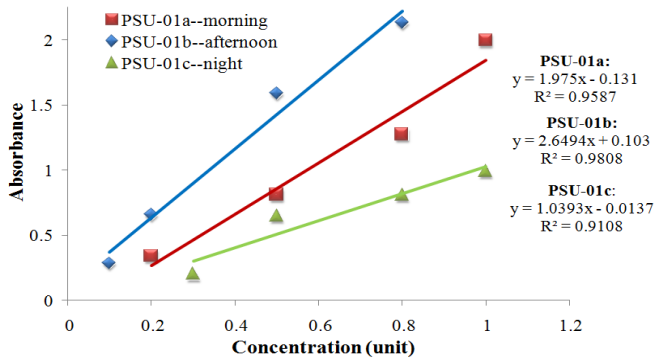

Figure 4: Curves of absorbances versus concentrations of three human saliva samples (PSU-01a, PSU-01b, and PSU-01c) taken at different times during the same day: in the morning, in the afternoon and at night, respectively. The function of each curve is listed on the right side.

Sample ID	Dilution	Absorbance of Dilution	Estimated Conc. of Dilution (ppm)	Conc. of the extract	Average Concentration of each donor
PSU-01a	1:2	0.8090	2.2490	4.4980	5.2875
PSU-01b	1:2	1.5844	3.7467	7.4934	
PSU-01c	1:2	0.6468	1.9356	3.8712	
PSU-02a	1:10	0.2942	1.2693	12.6933	20.6532
PSU-02b	1:10	1.1577	2.9567	29.5673	
PSU-02c	1:10	0.6527	1.9699	19.6990	
PSU-03a	1:10	0.6867	2.0363	20.3632	12.0047
PSU-03b	1:5	0.3610	1.3998	6.9992	
PSU-03c	1:5	0.5301	1.7304	8.6518	
PSU-04a	1:2	0.3801	1.4372	2.8743	8.1856
PSU-04b	1:5	1.1546	2.9508	14.7540	
PSU-04c	1:2	1.4174	3.4643	6.9286	
PSU-05a	1:2	0.5677	1.8037	3.6074	3.0959
PSU-05b	1:2	0.7035	2.0691	4.1382	
PSU-05c	1[a]	0.4338	1.5420	1.5420	
PSU-06a	1:10	0.6948	2.0523	20.5226	12.7650
PSU-06b	1:5	0.5606	1.7899	8.9493	
PSU-06c	1:5	0.5476	1.7646	8.8230	

[a]The original extract of this sample was used for analysis without dilution since the amount of amylase in dilution was too low to be detected by UV-VIS

Table 4: Estimated concentrations of salivary amylase in the human saliva extracts from six individuals taken at three different times of the same day.

samples were calculated by using the equation (4), and the results are shown in Table 4. The concentrations of salivary amylase in the saliva from six individuals were very different. Donor PSU-02 had the highest average concentration of salivary amylase in one day and the saliva from donor PSU-05 contained the lowest average concentration of amylase

Also, the concentrations of the salivary amylase in the donors varied in amount during the same day. For instance, the sample taken in the afternoon from donor PSU-01 had the highest absorbance, or most concentrated salivary amylase, and the sample at night had the lowest absorbance. The kinetics of the three samples or the slopes of the functions have also indicated this pattern. However, the research indicated that the level of amylase varied during the day. In other words, the samples with highest amylase level were not always the one taken in the afternoon for the six donors. Samples taken in the morning from donor PSU-03 and PSU-06 contained the most concentrated salivary amylase.

Although the concentrations of the salivary amylase in human saliva samples were calculated by the calibration curve, it is difficult to calculate the exact concentration of the amylase in a forensic sample by using this method. The reason is that a typical forensic sample may contain contaminated, degraded saliva or a mixture of saliva and other body fluids. However, the UV-VIS method, in conjunction with the SALIgAE® test, can provide an estimate of the concentration of the amylase in a sample, or semi-quantitatively measure the concentration.

The results of pancreatic amylase indicated that it does react with the SALIgAE® reagent and could cause false positive results. Table 5 has the absorbance, kinetics and the visual examination of SALIgAE® test of pancreatic amylase samples. Similarly, the more concentrated pancreatic amylase samples have generated higher absorbance, larger kinetics and yellower color. The lowest concentration of pancreatic amylase dilution that fell within the quantification range of UV-VIS was 30 ppm, which corresponded to 0.010584 units of pancreatic amylase. By comparing the results with the salivary amylase in Table 3, the concentration of the salivary amylase sample that had similar absorbance and kinetics to the 30 ppm pancreatic amylase is 1 ppm. The salivary amylase with 5 ppm concentration had almost identical absorbance and kinetics to the 160 ppm pancreatic amylase sample. Therefore, the reaction between the SALIgAE® reagent and the pancreatic amylase was much less aggressive than the reaction with the salivary amylase.

Other human body fluids including semen, blood, urine, and body fluids from animals such as saliva and urine, did not yield positive results with the SALIgAE® UV-VIS method, i.e. the final absorbances were less than 0.2. No color change was detected in the SALIgAE® reagent when using these samples. Figure 5 shows the curve of the absorbance versus time of these samples and most of them generated a curve with negative

Conc. (ppm)	Absorbance	Kinetics	Color of SALIgAE® Reagent
30	0.3028	0.0005	Colorless
50	0.6131	0.0010	A trace of yellow
80	1.1327	0.0018	Light pale yellow
100	1.3138	0.0022	Light yellow
120	1.6333	0.0026	Light yellow
160	2.1493	0.0034	Light yellow

Table 5: Results of standard human pancreatic amylase.

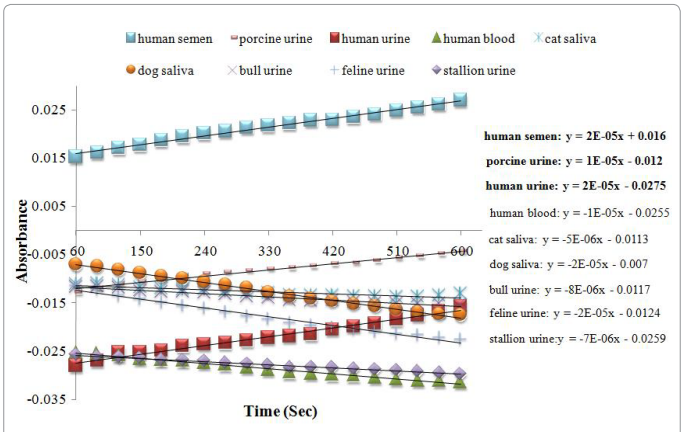

Figure 5: The curves of absorbance versus time of other body fluids and animal fluids samples with positive kinetics: human semen, porcine urine and human urine. The function of each curve is listed on the right side and the ones with positive slope are bolded.

slope, i.e. a negative kinetics. This implies that the color of the reagent became less yellow over time and no reaction occurred between these samples and the SALIgAE® reagent. Three body fluids gave very small positive slopes, namely human semen, porcine urine and human urine. Reactions seemed to have occurred between those samples and the reagent; however, they are highly unlikely to cause false positive results with forensic samples unless the concentrations of these body fluids are extremely high.

Conclusion

This research indicates that the SALIgAE® reagent, in conjunction with the UV-VIS method, can be used to qualitatively and semi-quantitatively measure the amount of salivary amylase in an evidence sample. Measuring the absorbance of a sample tested with SALIgAE reagent is a more accurate way to determine whether Amylase 1 (the salivary variety) is present in a sample than depending solely on visual observation. The UV-VIS method is also able to approximate the quantity of salivary amylase in a sample. In addition, the kinetics of the reaction between the sample and the SALIgAE® reagent can reveal whether the color changes of the reagent were due to the presence of salivary amylase, and thus eliminate false positive results. Research is underway to study the change in absorbance when saliva is mixed with other body fluids and added to the reagent. Future research will also explore the correlation between the detection limit of salivary amylase and the quality of the DNA profile obtained from that limiting amount. Also, the amount of salivary amylase in human saliva will be monitored by using this method at different situations, such as before and/or after eating and drinking different types of food and beverages. Different colorimetric presumptive tests of saliva will be used to substitute the SALIgAE® reagent and the results will be compared. Research is being conducted to compare the results of SALIgAE® reagent with Phadebas® test, and with radial diffusion assay. Both of these tests are used for the presumptive testing of saliva. The data of the comparative study will be published once the study is completed. Overall, estimating the amount of salivary amylase in a forensic sample can improve the technique of detecting saliva.

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References

1. Willott GM (1974) An improved test for the detection of salivary amylase in stains. J Forensic Sci Soc 14: 341-344.
2. Merritt AD, Rivas ML, Bixler D, and Newell R (1973) Salivary and Pancreatic Amylase: electrophoretic characterizations and genetic studies. American Journal of Human Genetics (25): 510-522.
3. Karn RC, Rosenblum BB, Ward, C and Merritt AD (1975) Genetic and Post-translational Mechanisms Determining Human Amylase Isozyme Heterogeneity, in Isozymes: IV Genetics and Evolution, edited by CL Market, New York: Academic Press 4: 745-761.
4. Fridhandler L, Berk JE, Montgomery KA, Wong D (1974) Column-chromatographic studies of isoamylases in human serum, urine, and milk. Clin Chem 20: 547-552.
5. Blake ET, Sensabaugh GF (1974) Genetic Markers in Human Semen-A Review. Journal of Forensic Sciences 21: 784-796.
6. Skude G, Mårdh PA, Weström L (1976) Amylases of the genital tract. I. Isoamylases of genital tract tissue homogenates and peritoneal fluid. Am J Obstet Gynecol 126: 652-656.
7. Morrison AI (1972) Persistence of spermatozoa in the vagina and cervix. Br J Vener Dis 48: 141-143.
8. Rosenmund H, Kaczmarek MJ (1976) Isolation and characterization of isoenzymes of human salivary and pancreatic alpha-amylase. Clin Chim Acta 71: 185-189.
9. Miller DW and Hodges JC (2005) Validation of Abacus SALIgAE® Test for the Forensic Identification of Saliva. WV State police Forensic laboratory, WV.
10. Myers JR, Adkins WK (2008) Comparison of modern techniques for saliva screening. J Forensic Sci 53: 862-867.