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CASE REPORT

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α-Amylase Kinetic Test in Bodily Single and Mixed Stains

ABSTRACT: Recently, in Italy, a murder and a putative sexual violence was accomplished on a child. A bodily fluids mixture on the child's underwear between the victim (female) and the suspect (male) was ascertained by short tandem repeat (STR) DNA typing and, due to the absence of seminal fluid, saliva from the suspect and urine from the child was hypothesized. In order to investigate the possibility of specifically and rapidly detecting saliva stains both alone and mixed with other bodily fluids, we used a quantitative spectrophotometric technique, named Amylase test, for the detection of α-amylases. We determined α-amylase activity and reaction kinetic curves in several samples collected from the child's underwear. In order to confirm our intuition, we first tested saliva, perspiration, and urine, singularly and in mixtures; second, several forensic stains including saliva, perspiration, urine stains, saliva/perspiration, and saliva/urine mixture stains were tested. Evaluating α-amylase activity values and time-course curves' behavior of α-amylase reactions we were able to recognize successfully, in all cases, the presence of saliva and to distinguish it specifically from other bodily fluids containing α-amylase. A further confirmation of our result was provided by STR DNA typing on several areas of the underwear: a clear correlation between α-amylases activity and male DNA was detected on all the samples evaluated.

KEYWORDS: forensic science, saliva presumptive test, α-amylase activity, kinetic test, STR DNA typing

In criminal investigation, it is not unusual to find at the crime scene saliva stains both as single biological fluid and mixed with other bodily fluids. These biological fluid can be found in many forensic samples such as postage stamps (1), cigarette butts (2), on balaclava used in robbery cases, (3,4) and, frequently, in sexual assault cases when oral sodomy has been perpetrated.

Over the years, to identify saliva stains some different methods have been routinely performed, mostly based on the detection of human α-amylase (1,4-α-D-glucan glucanhydrolases), the main salivary marker, via both chemical and immunological methods (5).

The α-amylases enzyme (1,4 α-D-glucan glucanohydrolase, EC 3.2.1.1) are proteins found in both plants and animals (6–8). In the latter, these enzymes are monomeric, calcium-binding proteins that are produced and stored mainly in the salivary glands and in the pancreas and, for this reason, are abundantly present in their secretions. As α-amylases are secreted into the digestive system, small amounts diffuse into the bloodstream and are eliminated through urine and perspiration; so are commonly found in several biological fluids, especially urine, perspiration, and, in addition, in vaginal secretions, breast milk, tear fluid, serum, male and female reproductive tissues, and feces (9–13); however, with the exception of feces, no other body fluid approaches the level of amylases activity in dried stains from the aforementioned biological fluids. In some pathological conditions, especially in pancreatic disorders such as pancreatitis (14) and in several kind of tumors affecting the digestive apparatus (15), the α-amylase levels may be drastically altered and tissue normally not expressing these enzymes may become relevant sources of α-amylases (16). α-amylases are also found in the liver, fallopian tubes, and small intestine; inflammation of these tissues also increases levels. During a mumps infection, amylases from the inflamed salivary glands increase. Moreover, even bacterial populations, such as those populating vaginal secretions, may produce appreciable levels of α-amylases (17).

Over the years, a wide heterogeneity of isoforms or isoenzymes has been described in human bodily fluids. Traditionally, two distinct isoenzymes, commonly marked S (salivary) and P (pancreatic), encoded by two separate gene loci on chromosome 1 (*Amy1* and *Amy2*, respectively), have been found, sometimes contemporarily, in several different body fluids. As a general rule, tissues that produce α-amylases often have distinct isoenzyme patterns while many bodily fluids, such as serum, urine, and perspiration, appear to be mixtures of isoenzymes from more than one tissue source (6,18). For example Merritt et al. (9) and Townes et al. (19) demonstrated that human serum and urine α-amylases are composed of contributions from both salivary and pancreatic isoenzymes. Additionally, these two main forms can undergo posttranslational modification, thus leading to the different α-amylases patterns found in bodily fluids. Although it is now well ascertained that the biochemical differences among α-amylases are also due to chemical changes including glycosilation and/or deamidation on the same genic product to give different isoforms, the existence of different types of α-amylases isoenzymes has been widely debated and the putative expression of different genic products continues to be investigated (6,20,21).

Regardless of the origin of α-amylase biological variability, based on these biochemical differences, several physical, chemical, or immunological methods to identify presumptively saliva stains (22–24), such as light sources, amylases activity measurements, and enzyme linked immunosorbent assay (ELISA) tests,

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have been used. Owing to α-amylase biochemical heterogeneity and to the wide distribution of similar isoforms on different body fluids, it is not easy for the forensic practitioner to characterize saliva stains unequivocally. In spite of the presence of high levels of α-amylase in fecal stains, which may resemble those of saliva stains, normally no interpretational issues arise as fecal stains can be visually differentiated.

At lower levels of α-amylase content in dried stains, however, the origin of the amylase is difficult to determine using traditional techniques. In oral sexual assault, the source of the amylase may be salivary or endogenous from vaginal secretion, or even bacterial.

Recently, two new methods have been developed to discriminate saliva from other bodily fluids. The first approach investigates the mRNA levels of genes uniquely and specifically expressed in saliva, such as statherin and histatin 3, and does not use α-amylases as a specific marker of saliva (25). This innovative technique, introduced for the presumptive identification of several different bodily fluids, is highly specific and affordable but its practical use in forensic science is strongly limited by both the relevant degree of mRNA instability (especially in forensic samples) and the complexity of the procedure.

The second technique is an immunological method based on an indirect ELISA technique utilizing monoclonal antisalivary α-amylases (26). This approach has been developed in order to distinguish salivary α-amylases specifically from other types of α-amylases. Nevertheless, while this technique is specific and highly sensitive, it is capable of detecting the salivary α-amylases type also in other fluids where it is present. Moreover the ELISA method is time-consuming (at least 2 workdays) and, compared with other techniques, is much more demanding for the analyst.

Presumptive identification of saliva may also be accomplished by evaluating α-amylases enzymatic activity levels by several methods using chromogen oligosaccharidic substrates and a spectrophotometric measurement (5,27). In 1991, Tsutsumi et al. (28) first applied the study of α-amylase-specific activity to the forensic identification of saliva in pure and mixed stains. They demonstrated the ability of this technique to identify saliva successfully in a wide range of concentrations and both alone or mixed with other bodily fluids at various ratios but their method was time consuming and required special equipment as α-amylase and total protein purification and quantification from each stain were performed in order to calculate specific activity (enzyme activity/total protein concentration).

In our laboratory, we routinely use a totally enzymatic method that uses 2-chloro-*p*-nitrophenol linked with maltotriose to give 2-chloro-*p*-nitrophenyl-α-D-maltotrioside ($CNPG_3$) as a chromogen substrate for the determination of α-amylase enzymatic activity on stains without α-amylases or total protein extraction procedures, thus effectively simplifying and quickening the procedure

$$10\,CNPG_3 \xrightarrow{\alpha-amylase} 9\,CNP + CNPG_2 + 9\,G_3 + G$$

α-Amylase hydrolyzes the $CNPG_3$ to release 2-chloro-4-nitrophenol and form 2-chloro-4-nitrophenyl-α-D-maltoside ($CNPG_2$), maltotriose (G_3), and glucose (G). The absorbance of 2-chloro-4-nitrophenol, stoichiometrically formed, can be measured spectrophotometrically at 405 nm. The rate of its release can be kinetically measured by the increase in color and is directly proportional to the α-amylases activity in the sample.

In several forensic cases in our laboratory, we observed a remarkably different kinetic behavior among samples containing saliva, urine and perspiration stains when they were tested with the colorimetric Amylase test. These differences were also maintained when biological mixtures of the above-mentioned fluids were tested. When saliva was present, both individually and mixed with the other two body fluids, a rapid chromatic change (in most cases within 2 min) of the solution test from uncolored to an intense bright yellow color was achieved. On the other hand, when either urine or perspiration was present, a much slower and less intense chromatic change was observed even waiting longer times after adding the test reagent. The observed kinetic differences were easily detectable even to the naked eye. These qualitative observations were further confirmed in a forensic casework consisting of a sexual oral assault between a male (offender) and a female (victim). Amylase test was performed on several samples collected from the victim's underwear, which clearly showed the abundant and diffuse presence of bodily fluid stains. Strongly positive results were obtained, which deposed for the abundant presence of saliva stains alone or, possibly, mixed with urine or vaginal secretions.

Based on these observations, the aim of our study was to determine whether the Amylase test, directly performed on a biological fluid or on a stain without any protein purification, could be used as a quantitatively reliable, rapid, and low-cost kinetic method to affordably distinguish the presence of saliva from that of perspiration, urine and to ascertain the presence of saliva in mixtures with perspiration or urine. For this purpose, on some samples from the above-mentioned forensic casework and, then, on several experimental specimens such as individual body fluids, mixed body fluids, stains composed by a single body fluid, and stains composed of mixtures of two bodily fluids, we carried out the Amylase test, followed by both enzymatic activity measurement and enzymatic time-course evaluation.

Materials and Methods

Samples Preparation

Casework samples—20 portions of 5 mm × 5 mm of the child's underwear involved in an oral rape case were chosen, cut, and tested twice with Amylase test. A further area of the underwear, lacking in biological stains, was also collected and assayed as a negative control for the Amylase test. The effects of substrate inhibition of α-amylase activity were evaluated by spotting 3 μL of saliva from a healthy donor on a 5 mm × 5 mm portion of the underwear fabric lacking in biological stains (positive control).

Experimental samples—10 healthy donors were chosen, and 1 mL of saliva, perspiration, and urine samples were collected from each.

In the first stage of the experiment 3, 6, and 9 μL of each biological fluid collected was first individually tested by the Amylase test and then five mixtures of saliva/perspiration and saliva/urine were prepared by mixing equal volumes of two biological fluids (saliva/perspiration and saliva/urine) and, then, 3, 6, and 9 μL of each sample were tested twice by the Amylase test.

In the second stage, 10 of the most common experimental forensic samples including cigarettes butts, underwear, and stamps, containing 9 μL of individual biological fluids (pure stains) or 9 μL of equal volumes of mixed biological fluids (mixed stains saliva/perspiration and saliva/urine 1:1 volume ratios) from the same donors, were prepared and allowed to dry. The substrates were completely soaked to saturation with the biological fluids and then a 5 mm × 5 mm portion of each substrate was cut and tested twice with the Amylase test. Again, several negative

controls of the test, constituted by portions of the substrates without biological stains, were assayed.

All experimental samples were prepared in order to simulate realistically, both as sample volumes and as bodily fluid ratios in mixtures, forensic stains commonly found at the crime scene. Particularly, in the second stage of the experiment when experimental mixed stains were set up, 1:1 volume ratios were chosen as, especially in oral rapes, equal volumes of bodily fluids are often detectable on the victim.

Amylase Test

To detect α-amylases activity, we performed the Amylase test (Sclavo Diagnostics International S.p.A, Siena, Italy), a colorimetric assay based on the absorbance of 2-chloro-nitrophenol that can be measured at 405 nm. The rate of its release can be kinetically measured by the increase in color and, if the measurements are conducted when the reaction is of first order (linear dynamic range between enzymatic activity and absorbance), it is directly proportional to the α-amylases activity in the sample.

In a 1.5 mL eppendorf tube, 1 mL of the ready-to-use reagent provided by the manufacturer and containing $CNPG_3$ 2.25 mM, sodium chloride 350 mM, calcium acetate 6 mM, potassium thiocyanate 900 mM, sodium azide 0.1%, and MES buffer at pH 6 (Sclavo Diagnostics International S.p.A.) was added to the sample quantities as described above. Biological samples or forensic stains were directly added to the test reagent without α-amylase or total protein extraction. Both reagent and samples were maintained at a stable temperature of 37°C. In all cases, the first absorbance lecture was conducted 5 s after mixing the reagent with the sample (time 0). The tubes were immediately placed into the TU-1901 UV/Vis Double Beam Spectrophotometer (GenTech Scientific Inc., Arcade, NY) and absorbance measurements were conducted, by using UVWin Software (supplied with the spectrophotometer), for both casework samples and experimental samples at 37°C at a wavelength of 405 nm at different times, depending on the samples tested in order to be in the linear range between absorbance and enzymatic activity (first-order reaction). The following absorbance measurement was conducted at 20 sec after mixing the reagent with the sample in the case of pure biological fluids or biological fluid mixtures. When samples from underwear or experimental forensic stains were tested, the measurement was conducted at 100 s after mixing the reagent and the sample. Reaction solution absorbance was always measured twice, and a blank constituted by the regent alone was tested before the samples.

For each measurement, enzymatic activity was calculated according to the formula reported on user's sheet of the test and was expressed as International Units per liter (U/L).

The enzymatic activity data were statistically evaluated by calculating mean and standard deviation (SD).

Moreover, the kinetic behavior of α-amylases in the samples was comparatively evaluated up to 1000 s, when the reaction was at its maximal rate.

Combur[10] Test® (Urobilinogen Test)

To exclude that a positive result from the Amylase test on the child's underwear could be due to the presence of diluted fecal material containing relevant amounts of α-amylases, an urobilinogen detection test was performed on 10 portions of 5 mm × 5 mm of the child's underwear previously tested with the Amylase test. Urobilinogen is a colorless product of bilirubin reduction that is formed in the intestines by bacterial action. Even if it may be poorly detectable in urine, it is mostly excreted with other bilinogen/bilin compounds such as urobilin and stercobilin in the feces. Thus, a measure of these bilinogen/bilin compounds in the stain may be considered an orientative test for the presence of fecal material even when it is extremely diluted (unpublished data). We used the Combur[10] Test® (Roche Diagnostics S.p.A., Milano, Italy), a firm plastic strips to which are affixed several separate reagent areas for the determination of several catabolites and, among these, urobilinogen. The urobilinogen test area was soaked with about 20 μL of distilled water and placed contact with a small piece of the underwear stain to be tested with the Amylase test: if urobilinogen is present, a rapid chromatic change to a pink color is observed almost immediately. This urobilinogen test is based on a modified Ehrlich reaction (29) in which 2.9% w/w *p*-diethylaminobenzaldehyde, balanced with buffer and nonreactive ingredients, reacts with urobilinogen in a strong acid medium to produce a pink color. This test area gives qualitative results and will detect urobilinogen in a concentration as low as 0.4 mg/dL (7 μmoL/L).

Short Tandem Repeat (STR) DNA Typing

DNA from 20 samples collected from the child's underwear (casework sample) was extracted by using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sixteen STR *loci* were simultaneously amplified by using the AmpF*l*STR® Identifiler™ PCR Amplification kit (Applied Biosystems, Foster City, CA). The separation, detection and analysis of amplified products were accomplished with the ABI Prism® 3100 Genetic Analyzer 16-capillary array system (Applied Biosystems), following manufacturer's protocols. Data collection was performed with Data Collection v 2.0 software (Applied Biosystems) and samples were analyzed by GeneMapper® v. 3.2 software (Applied Biosystems).

For each *locus*, male to female peak ratios were calculated from peak area data (30,31) and mean values for all *loci* were plotted and compared with the respective α-amylases activity data.

Results and Discussions

Case Report

Recently, in the center of Italy a child was killed after a presumptive sexual oral assault. STR DNA typing analyses and the most common presumptive immunological and chemical assays were conducted on a relevant number of samples from different areas of the victim's underwear in correspondence of the vaginal region. A DNA mixture composed of the profiles from both the suspect and the child was ascertained in all samples tested, while no seminal fluid was detected by using both an immunochromatographic PSA-based test and by optical microscope observation (data not shown). Thus, we performed an Amylase colorimetric test, and we observed a rapidly increasing and intense change of the reagent from colorless to bright yellow, while if the test was performed on an area of the underwear lacking in biological stains (negative control) no detectable chromatic changes were achieved. These kinetic differences were easily detectable even to the naked eye. Based on these results, achieved only by a simple qualitative statement, we stated that most likely this result was due to the presence of saliva from the suspect, whereas the defense attorney forensic specialist declared that the high amylases activity depended on the presence of the child urine and/or vaginal

secretions, and that the DNA mixture derived from the child urine and vaginal secretions and from the man's perspiration.

To demonstrate that these test results were principally due to salivary α-amylases activity we used an easy-to-use, rapid, and affordable quantitative measurement of α-amylases activity differences among different bodily fluids, both pure and mixed, which are more commonly found in forensic caseworks, especially when an oral sexual assault is suspected and DNA STR typing procedures confirm the presence of DNA from both the victim and the suspect.

α-Amylases Kinetic Data

Casework samples (samples collected from a child's underwear)—Once the presence of seminal fluid was excluded, to understand the body fluid composition of each of the chosen portions of the child's underwear (20 samples), the Amylase test was performed and enzymatic activity data were measured at 100 sec after mixing the reagent and samples. In 40% of the samples tested (eight samples, group 1 samples) we ascertained enzymatic activity values ranging from 322 to 488 U/L, with a mean value of about 410 ± 20 U/L, whereas in the remaining 60% of the samples (12 samples, group 2 samples), α-amylases activity was notably lower, the mean value being about 57 ± 5 U/L (data not shown). Relevant kinetic differences were also detectable between the first and the second group of samples: a typical hyperbolic time-course curve achieving the plateau level at about 1000 sec was observed for group 1 samples, while group 2 samples showed an apparently linear curve easily distinguishable from the previous. When the test was performed on an area of the underwear lacking in biological stains (negative control), no detectable chromatic changes and negligible activity values were achieved. Finally, no substrate inhibition effects were detected by testing a fabric sample from the underwear soaked with saliva (positive control).

Some kinetic curves referred to samples from both the groups are shown in Fig. 1.

To exclude that a positive result in the Amylase test could be due to α-amylases contained in extremely diluted and not easily visible fecal material we tested the same 20 stains for the presence of urobilinogen (and, more generally, of bilinogen compounds), which could be considered a presumptive marker for feces. The rapid test used, namely the Combur[10] Test®, allowed us to exclude a detectable presence (down to 0.4 mg/dL) of fecal matter (data not shown).

According to biochemistry and forensic literature these data led us to suspect effectively the presence of saliva stains (alone or mixed with urine or vaginal fluids) in most of the areas of the underwear and the presence of urine or vaginal fluids in the remaining samples. To further investigate our hypothesis, we carried out an α-amylases activity analysis on several experimental body fluids and stains, both alone and in mixture conditions, with special attention on α-amylases kinetics in mixed bodily fluids stains as the forensic literature considerably lacks in the evaluation of these situations.

Experimental samples—Individual body fluids from 10 donors and five body fluids mixtures between saliva and urine or perspiration from the same donors were tested, enzymatic activity was measured 20 s after mixing the sample to the reagent in order to allow the reaction to be in the linear range between absorbance and enzymatic activity (first-order reaction) and the time course of the enzymatic reaction was evaluated up to 1000 s.

α-amylases-activity data measured in all saliva samples showed, for each quantity of saliva tested, relevant differences from that observed in both perspiration and urine, the average ratio between α-amylases activity in saliva and that in perspiration and urine being, in most cases, greater than 20. Considerable differences were approximately maintained even comparing the minimum activity value of saliva (490 U/L for 3 μL at 25 s) with the maximal activity values from perspiration (88 U/L for 9 μL at 25 s), or urine (81 U/L for 9 μL at 25 s): in all samples tested, an activity ratio of at least 6 was always found.

Moreover, the kinetic behavior of the reaction catalyzed by α-amylases was clearly different when saliva, perspiration, and urine were tested (Fig. 2). At each time (up to 1000 s) and with all volumes of biological fluid tested, saliva showed a clear hyperbolic time-course curve of α-amylases activity (first-order kinetics), achieving its maximum rate at 150 s (zero-order kinetics), whereas for both perspiration and urine an apparently linear time-course curve of α-amylases activity was detected up to the last measurement performed (1000 s).

Analogously, when mixed samples of equal volumes of saliva and perspiration or saliva and urine were tested, both the enzymatic activity values and kinetic curve (Fig. 3) were found to resemble strictly the previous data of α-amylases activity in saliva. The main effect of mixing saliva with perspiration or urine on kinetic curve was to lower its hyperbolicity and to increase the time for the curve to reach the plateau level. Correspondingly, only a small decrease in activity values was observed in mixed

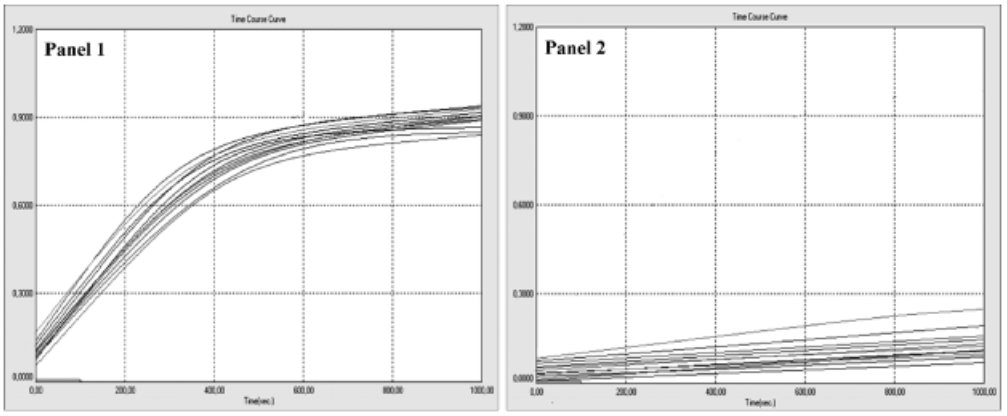

FIG. 1—*Amylase test time-courses up to 1000 s conducted on several samples collected from the child' underwear. Panel 1 shows kinetic behavior of some samples showing higher α-amylases activity (group 1), whereas Panel 2 shows some samples showing lower α-amylases activity (group 2). Both the abscissa (absorbance) and ordinate (time) are reported with the same scale in order to allow a direct comparison of kinetics.*

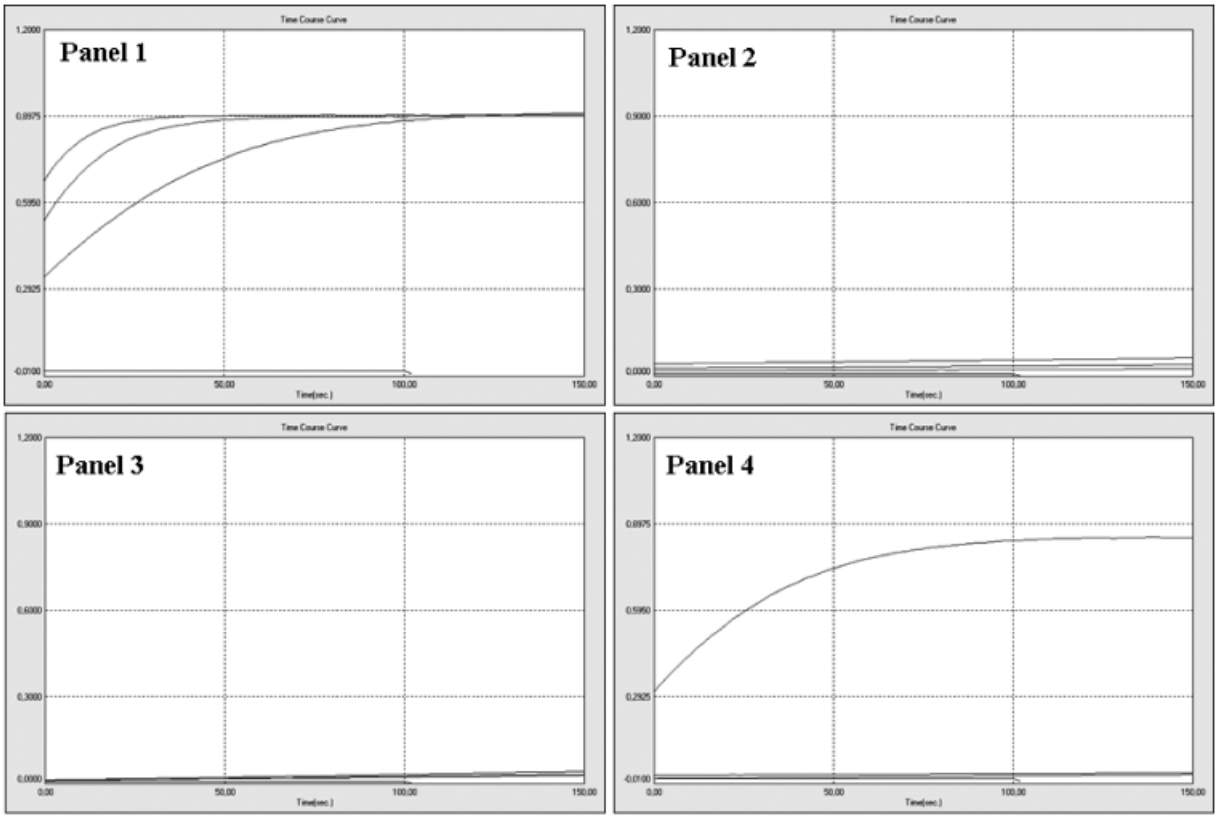

FIG. 2—*Amylase test time-courses up to 150 s conducted on 3, 6, 9 μL of saliva (panel 1), 3, 6, 9 μL of perspiration (panel 2), and 3, 6, 9 μL of urine (panel 3). Upper curve: 9 μL; lower curve 3 μL; and middle curve 6 μL. Panel 4 shows the overlapping of time courses of Amylase test conducted on 3 μL of saliva, perspiration and urine from the previous panels. Both the abscissa (absorbance) and ordinate (time) are reported with the same scale in order to allow a direct comparison of kinetics.*

samples when compared with pure saliva samples most likely due to a dilution of salivary α-amylases with a less concentrated body fluid such as perspiration or urine. Consequently, relevant kinetic differences were maintained among biological fluid mixtures depending on the presence of saliva.

Based on these experimental data, we tried to investigate whether also the common forensic stains involving saliva alone or mixed with other body fluids could have the same kinetic behavior. Thus, we first performed an Amylase test on saliva, perspiration and urine stains adsorbed on several kind of substrates separately and, then, we tested mixture stains including saliva and perspiration or urine. Only α-amylases activity data and time-courses for cigarette butts (pure stains) and for underwear (mixed stains) (data referred to the other forensic samples have not been shown) at 100 s after mixing reagent and sample have been reported in Fig. 4.

At 100 s, when the reaction is still of first order, saliva stains yielded a mean activity of 448 ± 22 U/L, perspiration stains of 63 ± 5 U/L, and urine stains of 51 ± 3 U/L, showing relevant differences from activity values observed for pure body fluids. However, this enzymatic activity decrease appeared to affect, with the same relevance, α-amylases contained in saliva, perspiration, and

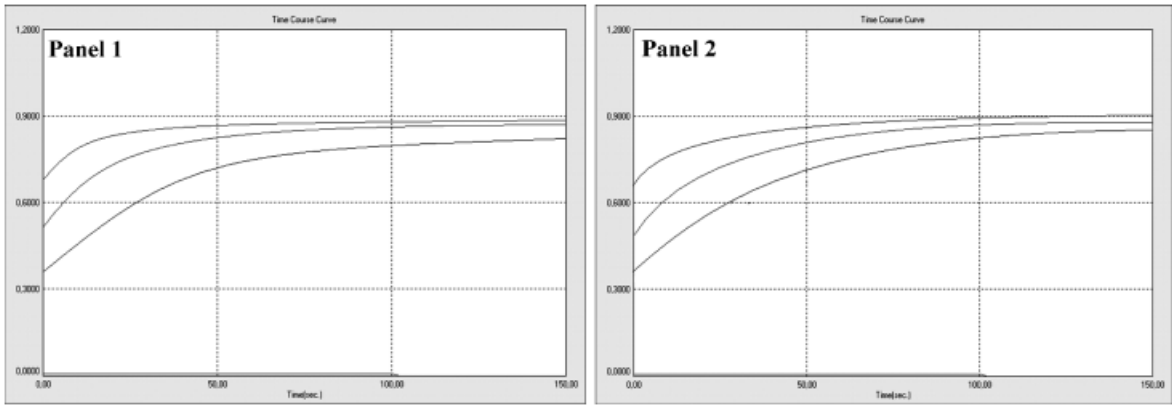

FIG. 3—*Amylase test time courses up to 150 s conducted on 3, 6, and 9 μL of mixed body fluids (1:1 volume ratios). Panel (1) Amylase test performed on a saliva/perspiration mixture, whereas panel 2 on a saliva/urine mixture (panel 2). Upper curve, 9 μL; lower curve, 3 μL; and middle curve, 6 μL. Both the abscissa (absorbance) and ordinate (time) are reported with the same scale in order to allow the direct comparison of kinetics.*

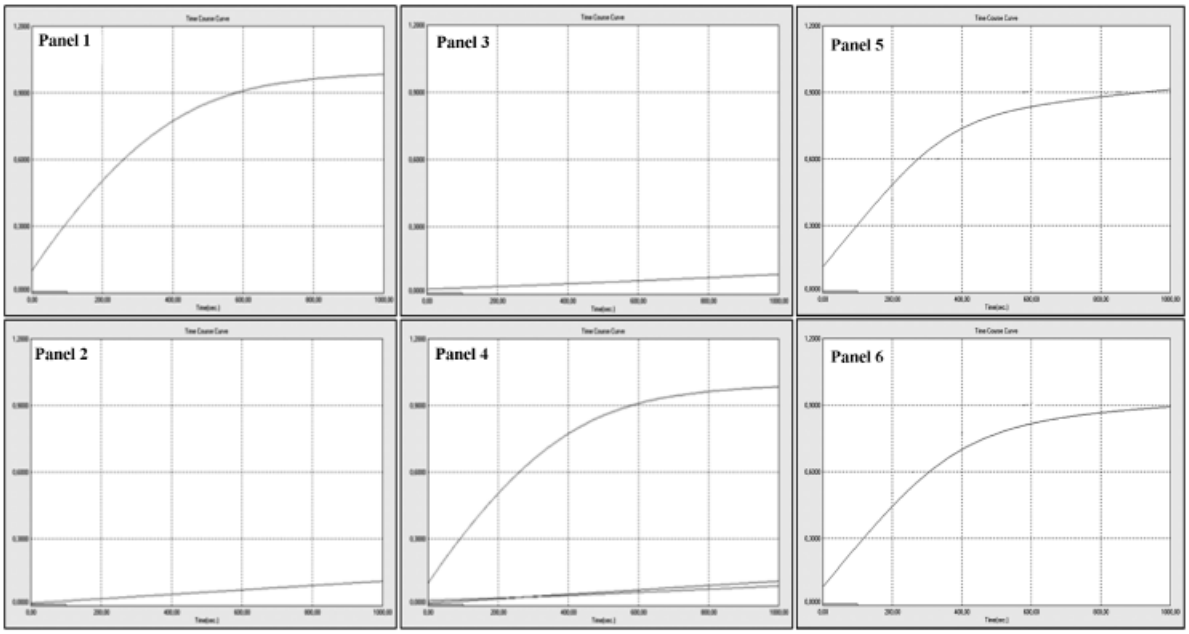

FIG. 4—*Amylase test time-courses up to 1000 s conducted on 9 μL of saliva (panel 1), urine (panel 2), and perspiration, (panel 3) forensic stains (cigarette butts). Panel 4 shows the overlapping of time courses of Amylase test conducted on 9 μL of saliva, perspiration and urine stains from the previous panels. Panels 4 and 5 shows time course of Amylase test on 9 μL of mixed stains between saliva and perspiration and saliva and urine (1:1 volume ratios), respectively. Both the abscissa (absorbance) and ordinate (time) are reported with the same scale in order to allow a direct comparison of kinetics.*

urine, the decreases in activity values being approximately linear among the three body fluids. Analogous data obtained for forensic samples containing mixtures saliva/perspiration and saliva/urine (mixed stains) compared with body fluid mixtures confirmed these observations as saliva/perspiration and saliva/urine mixture mean activities were, respectively, 413 ± 18 and 406 ± 17 U/L. As in our casework sample, when the test was performed on the same substrates lacking in biological stains (negative controls), no detectable chromatic changes and negligible activity values were revealed.

These data strictly resembled α-amylases activity mean value achieved for our forensic casework samples (underwear).

Correspondingly, the reaction rate of both forensic pure stains and mixed stains was clearly diminished in all cases compared with pure body fluids and mixed body fluids with saliva, and the kinetic curve showed a decreased hyperbolicity with respect to the corresponding curves of body fluids (Figs. 2 and 3). More notably than in the previous assays using body fluids when about 150 s were needed for the reaction to become of zero order, an increasing time for the curves to reach the plateau levels was again ascertained, being requested to the reaction almost 1000 s elapsed from the reaction start to become of zero order. Also, this kinetic behavior confirmed the time-course curves obtained for both groups 1 and 2 samples collected from the child's underwear demonstrating that the first group encompassed stains involving saliva, alone or mixed with other bodily fluids, while the second group included stains containing urine or perspiration.

The effective decrease in the activity of α-amylases contained in the tested body fluids was probably due to the presence, in forensic samples, of inhibiting substances affecting, in some instances, the enzymatic properties of α-amylases and, in the case of mixed stains, to the additional effect of dilution of saliva stains with perspiration and urine (containing a much lower α-amylases activity).

Even considering these effects, α-amylases activity values and time-course curve behavior of saliva in forensic pure samples showed relevant differences from those of the other body fluids investigated, and mixed stains containing saliva with either perspiration or urine still substantially resembled salivary α-amylases kinetic properties and showed α-amylases activity values much more similar to those of saliva than to those typical of perspiration or urine. Again, a reliable discrimination among samples containing saliva and samples containing the other bodily fluids tested was effectively achievable, simply based on different α-amylases kinetic behaviors.

STR DNA Profiling Data

In the case of the reported oral sexual assault, a further confirmation of our result was provided by STR DNA typing on several areas of the underwear, which revealed the presence of a DNA mixture from both the suspect and the victim (data not shown). In order to understand whether male DNA was derived from suspect saliva stains or from child body fluids, we compared male DNA levels with α-amylases activity values in each of the samples collected from the underwear.

As reported in Fig. 5, in all 20 samples investigated an evident direct correlation between α-amylases activity (ordinate) and male DNA signals (male to female peak area ratios average values from 15 *loci*, abscissa) was detected on all the samples evaluated with a mean correlation coefficient (R^2, Excel, Microsoft Corporation, Seattle, WA) exceeding 0.99. While a direct correlation between enzymatic activity and male DNA was evident for both groups 1 and 2 samples from the underwear, only for group 2 was it possible to argue that a male DNA source could be epithelial cell interspersed in saliva stains left from the suspect. α-amylases levels detected in group one samples were mostly due to urinary/vaginal fluids from the female victim.

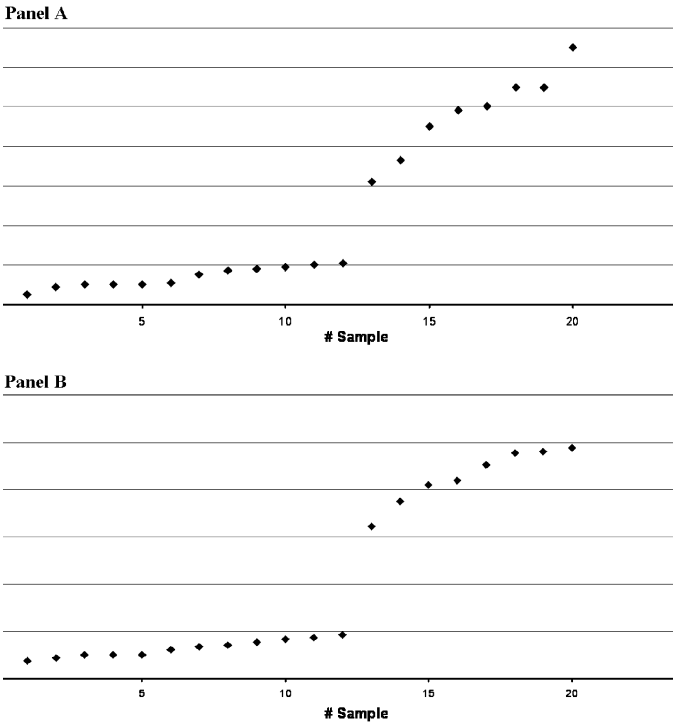

FIG. 5—*Male to female DNA peak area ratios (Panel A) and Amylase activity data (Panel B) for each of the 20 samples collected from the underwear. A direct correlation between enzymatic activity and male DNA is evident for both group 1 (cluster on the left area of the graph) and group 2 (cluster on the right area of the graph) samples.*

Conclusions

Saliva stains may be found at crime scenes on a wide variety of surfaces and both alone and mixed with other bodily fluids, especially in sexual assault cases. Among the most useful tests for saliva identification, the Amylase test is a rapid chromogenic enzymatic method used as a presumptive assay to screen substrates suspected to possess saliva stains by its capability to detect α-amylases. Nonetheless, due to the wide distribution in many bodily fluids of these enzymes, it is not easy, for the forensic investigator, to recognize unequivocally the presence of saliva, especially when it is mixed with other fluids, which is not an unusual situation. Recently, we dealt with an exemplary casework where a man had accomplished an oral rape on a female child and it was of the utmost importance to be able to understand the type of biological fluid from which we had detected male DNA, as saliva, perspiration, and urine contain α-amylases.

To evaluate the possibility to distinguish, by using this colorimetric test, the presence of saliva in a wide variety of stains and even in biological fluid mixtures, we first prepared these samples in order to simulate real forensic stains commonly found at the crime scene. Then, we focused on the measurement of α-amylases activity and on the investigation of the kinetic behavior of the hydrolysis reaction of the synthetic oligosaccharide included in the test catalyzed by α-amylases.

However, unlike other analytical methods of measuring α-amylases activity, we directly performed the test on bodily fluids or stains without previous α-amylases or total proteins' purification step, thus effectively simplifying and accelerating the procedure.

In both our forensic casework, where a sexual oral assault had been accomplished and saliva stains mixed with urine or vaginal secretions were present, and in all experimental samples tested, containing saliva alone or mixed with either perspiration or urine, we clearly demonstrate strongly different α-amylases activity values depending on the presence or on the absence of saliva. Correspondingly, time-course curves showed characteristic and clearly distinguishable behaviors between samples containing saliva and those containing perspiration or urine. Most notably, when saliva was mixed with either perspiration or urine, although a small decrease in reaction velocity probably due to α-amylases dilution effect was observable, both α-amylases activity data and kinetic curves in these mixtures strictly resembled those of saliva alone. In a previous paper (28), this α-amylases kinetic behavior had already been confirmed even when biological fluid mixtures with saliva at various ratios were prepared and tested. These data were also observed in experimental stains and in multiple stains from our casework, where the main effect was a more emphasized decrease in reaction velocity, most likely due to both the dilution effect on saliva by a less α-amylases concentrated body fluid and the presence on the substrates of some type of reaction inhibitors. As a matter of fact, it is well known that polyphenolic compounds such as tannins, widely distributed in plant-based food and materials (tea, fruits, wood, etc.), and possibly found together with saliva stains, have relevant inhibitory effects on the catalytic activity of α-amylases (32).

As an explanation attempt of these α-amylases kinetic differences among different biological fluids, we reported the considerably higher concentration of α-amylases in saliva than in all other bodily fluids commonly found at the crime scene and, additionally, the presence of different isoforms or even isoenzymes in the various bodily secretions that show different kinetic properties.

As the results obtained form these Amylase kinetic test on the stains detected on the child's underwear could also be attributed to the presence of fecal α-amylases we evaluate the presence of bilinogens/bilins as a putative marker for feces. Although fecal matter can be usually detected by the analyst with ease it is particularly difficult to recognize low-level, invisible fecal contamination. By using the Combur[10] Test® colorimetric assay, this possibility was excluded for all the stains from the underwear. Additional studies are ongoing in our laboratory to evaluate the effectiveness and reliability of the combination of separate tests for the contemporaneous detection of α-amylases, bilinogens/bilins, and uric acid to allow the discrimination of saliva, diluted feces, and urine, respectively.

A further confirmation of our results was provided by STR DNA typing on both several areas of the underwear from our forensic casework, which revealed the presence of a DNA mixture from both the suspect and the victim: in all cases investigated, an evident direct correlation between α-amylases activity and male DNA signals (male to female peak area ratios' average values from 15 STR *loci*) was detected on all the samples evaluated with a mean correlation coefficient exceeding 0.99 (Fig. 5).

In conclusion, regardless of the exact mechanism behind the α-amylases' kinetic differences among bodily fluids, we can note that the most common forensic stains containing either saliva or a biological fluids mixture including saliva are readily, quickly, and successfully distinguishable, at least under the typical conditions in which a biological stain may be found, by using our method, without any α-amylases or total protein extraction procedures.

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