



Review

Analysis of body fluids for forensic purposes: From laboratory testing to non-destructive rapid confirmatory identification at a crime scene

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ABSTRACT

Body fluid traces recovered at crime scenes are among the most important types of evidence to forensic investigators. They contain valuable DNA evidence which can identify a suspect or victim as well as exonerate an innocent individual. The first step of identifying a particular body fluid is highly important since the nature of the fluid is itself very informative to the investigation, and the destructive nature of a screening test must be considered when only a small amount of material is available. The ability to characterize an unknown stain at the scene of the crime without having to wait for results from a laboratory is another very critical step in the development of forensic body fluid analysis. Driven by the importance for forensic applications, body fluid identification methods have been extensively developed in recent years. The systematic analysis of these new developments is vital for forensic investigators to be continuously educated on possible superior techniques. Significant advances in laser technology and the development of novel light detectors have dramatically improved spectroscopic methods for molecular characterization over the last decade. The application of this novel biospectroscopy for forensic purposes opens new and exciting opportunities for the development of *on-field, non-destructive, confirmatory methods for body fluid identification at a crime scene*. In addition, the biospectroscopy methods are universally applicable to all body fluids unlike the majority of current techniques which are valid for individual fluids only. This article analyzes the current methods being used to identify body fluid stains including blood, semen, saliva, vaginal fluid, urine, and sweat, and also focuses on new techniques that have been developed in the last 5–6 years. In addition, the potential of new biospectroscopic techniques based on Raman and fluorescence spectroscopy is evaluated for rapid, confirmatory, non-destructive identification of a body fluid at a crime scene.

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1. Introduction

The detection and identification of body fluids at a crime scene are very important aspects of forensic science. Determining whether or not there is a body fluid present and subsequently identifying it allows the sample to undergo further laboratory testing including DNA analysis which is a very crucial step in a wide range of investigations. Sometimes just knowing the identity of a fluid can be enough to influence the outcome of a case. This is not always an easy task, however, since many body fluid stains are either invisible to the naked eye or similar in appearance to other fluids or substances. Even when the identity of a stain may seem obvious to a forensic investigator, absolute confirmation is necessary in order for the evidence to be used in court to either prove or disprove a fact in a case. This is especially important with the possible occurrence of mixtures. A stain could contain multiple body fluids from more than one donor. Physical tests performed on these questioned stains allow crime scene investigators and laboratory technicians to identify a fluid or to confirm the absence of one which can be of equal value in a case. The most common body fluids found at crime scenes are blood, semen, and saliva, but others such as vaginal fluid, urine, and sweat can also play important roles including the contribution of valuable DNA evidence. Each of these fluids has one or more screening tests that are presumptive in nature, and some of them have confirmatory tests that will conclusively identify their presence. There are also some tests which can identify the species of a particular fluid, and these are also considered to be confirmatory.

The main problem with these tests is the destruction of the sample. Sometimes a case can be broken with just the smallest amount of biological evidence, so it is crucial that these small quantities are examined as efficiently as possible by non-destructive methods at the crime scene. The most important reason for these tests to be non-destructive is the preservation of DNA evidence. Body fluids such as blood, semen, saliva, vaginal fluid, urine, and sweat all contain DNA evidence so it is imperative to develop identification tests that will protect this valuable data [1]. Another disadvantage of most of these current methods is that they are designed to detect a specific body fluid, so the investigator needs to decide which test to perform based on the fluid that is most likely present. There is a need for a universal confirmatory test that can be applied to an unknown stain which will be able to identify any of the body fluids that might be present.

Many of the common techniques used to identify particular fluids have been around for decades. Some of these techniques have changed very little such as the luminol [2] and crystal tests [3] for blood and the microscopic identification of spermatozoa to confirm the presence of semen [4]. Others such as the presumptive tests to identify heme in blood, acid phosphatase in semen, and amylase in

saliva have evolved over the years due to advances in technology, better understanding of the nature of the fluids, or even to prevent exposure to hazardous chemicals. A few new methods have been discovered, and the majority of these involve the detection of specific messenger ribonucleic acid (mRNA) markers to identify different body fluids [5–7]. Over time they will possibly be expanded upon and become more accepted by the forensic community.

An extensive and thorough book that describes the known identification tests for body fluids up to the year 1983 is *Sourcebook in Forensic Serology, Immunology, and Biochemistry* by R.E. Gaensslen [8]. It is a very in-depth analysis of the various methods studied up to that point in time. In the years since Gaensslen's publication, several other book chapters have summarized the identification of body fluids. These include Spalding's [9] and Greenfield's [10] chapters in *Forensic Science: An Introduction to Scientific and Investigative Techniques* in 2003, Shaler's [11] and Jones' [12] work in *Forensic Science Handbook Vol. II* (2002) and *Vol. III* (2005), respectively, Watson's 2004 chapter [13] in *Crime Scene to Court; The Essentials of Forensic Science*, and most recently portions of Li's book *Forensic Biology* [14] which was released in 2008. These books summarize the well accepted techniques, and Li mentions some of the new mRNA methods in his chapters. The above mentioned books and chapters describe in detail the presumptive and confirmatory methods of identification that are currently being used in forensic laboratories, with the exception of Li's book, which also discusses the more recently developed mRNA techniques.

The following review briefly summarizes all current and former methods of body fluid identification and focuses on the new developments in forensic science during the last 5–6 years. It discusses both significant improvements in conventional bioanalytical methods and developments of novel approaches. The review evaluates the advantages and disadvantages each method presents, and it concludes with the discussion of new biospectroscopy methods under development that offer non-destructive confirmatory identification of body fluid traces immediately at the crime scene [15,16]. It is important to emphasize that these new biospectroscopy techniques are still being developed and are not yet available. We believe that with further testing, these novel methods will be able to deliver results in a simple and automatic fashion at a crime scene that will be acceptable for court testimony. For specific and in-depth details about a particular test, including former tests, tests currently in use, and newly developed tests, it is best to consult the individual articles and books referenced throughout the review.

The review is organized as follows:

- A description of the composition of each body fluid and how the different biological components found in each fluid influence current identification methods.

- Summary of current and previous techniques used either at the crime scene or in the forensic laboratory. The body fluids discussed will include blood, semen, saliva, vaginal fluid, urine, and sweat. Each fluid will be reviewed individually in that order, and the tests for that fluid will be broken down into presumptive and confirmatory tests when applicable.
- Discussion of new methods of identification that have been published in the last 6 years. These methods have not been substantially reviewed by other sources. Again, each fluid will be covered individually, and this section on new techniques will fall at the end of each individual fluid section.
- Introduction of new non-destructive, confirmatory techniques based on fluorescence and Raman spectroscopies, which are applicable to all body fluids and their dry traces. The potential of these spectroscopic methods for forensic applications, specifically for identification of body fluid traces *on-field* at a crime scene, will be discussed.

2. Composition of body fluids

Each body fluid has a unique composition, and the presence of specific components in one fluid versus another is the basis of its identification. Table 1 shows the major components of blood, semen, saliva, vaginal fluid, urine, and sweat [8,10,14,17,18]. There are several components that are common among more than one fluid, but it is the difference in relative contribution which makes tests for these components effective. One example is the large amount of amylase in saliva compared to the smaller amounts in semen and vaginal fluid. Another example is the ratio of citrate to lactate when comparing semen and vaginal fluid. Urea is a component in urine, semen, and sweat, but it is used as an indicator of urine based on the much higher concentration in that fluid.

3. Blood

3.1. Current techniques

Blood is the most common body fluid encountered at crime scenes. There are several presumptive tests to identify blood as well as confirmatory tests. The following two sections explain techniques that are well known in the forensic community as well as some variations to these methods which are not currently in use. Table 2 summarizes all of the techniques for blood and the other body fluids.

3.1.1. Presumptive tests

The simplest test that crime scene investigators use to detect bloodstains that are not clearly visible is an alternate light source (ALS) such as ultraviolet light. This method is especially helpful when the stain is on a dark background [11]. An ALS can direct

attention to a latent stain at a crime scene, and then further presumptive tests can be utilized to form more conclusions about any body fluids that are present. A versatile light source product known as Polilight[®] contains a range of wavelengths and can even reveal stains covered by paint [19]. These light sources must be used with caution, however, since certain ultraviolet wavelengths can damage the DNA evidence in a sample. One study found that exposure of 30 s or more to 255 nm light damaged the DNA enough that none was detected during polymerase chain reaction – short tandem repeat (PCR-STR) quantification and amplification [20]. Another experiment found that restriction fragment length polymorphism (RFLP) patterns only weakened and were not falsified with exposure to UV light up to 5 days, but the wavelength used was not specified [21].

The luminol test is one of the first presumptive blood tests that investigators often use at a crime scene, and it has been around for over 40 years [22]. It is based on the ability of hemoglobin and derivatives in blood to enhance the oxidation of luminol in the presence of an alkaline solution and involves spraying a suspected area with an aqueous solution of luminol and an oxidant [8,9]. It is known to be the most sensitive of the current presumptive tests being used [23], and there are also several formulations available that have advantages and disadvantages regarding sensitivity, intensity and duration of illumination, and effect on subsequent DNA analysis [24,25]. It can even be used on an area that has been cleaned by a suspect [11]. One study found that a certain popular form of the luminol test known as the Grodsky formulation can have detrimental effects on subsequent DNA analysis when compared to the Weber, Weber II, and Bluestar[®] alternatives [24]. The luminol test remains popular due to the lack of false positives and false negatives in comparison with other screening tests as well as the fact that luminol is not as hazardous as other reagents [26]. However, it is limited to use in dark environments [14]. A similar, less popular fluorescence technique involving fluorescein depends on heme accelerating the oxidation of fluorescein to fluorescein in hydrogen peroxide [8,9]. Studies have shown that it is just as effective as luminol as a presumptive test for blood and also will not damage potential DNA evidence [27]. However, unlike luminol which will emit light on its own, fluorescein-sprayed stains need to be exposed to an ALS with a wavelength range of 425–485 nm [14]. Another technique based on chemiluminescence that gives positive results without damaging the DNA in a sample is Bluestar[®] [28], and studies have even shown it to be more sensitive and stable when compared to luminol [25].

There are several different catalytic tests commonly used to identify presumptively blood based on the peroxidase-like activity of the heme group [9]. The most utilized of these tests used to be the benzidine test. A positive result yields a blue color when blood reacts with the ethanol/acetic acid solution [8,9]. There are several

Table 1
Composition of body fluids.

Blood [17]	Semen [17]	Saliva [17]	Vaginal fluid [14,18]	Urine [10,17]	Sweat [8,17]
-Hemoglobin	-Acid phosphatase	-Amylase	-Acid phosphatase	-Urea	-Urea
-Fibrinogen	-Prostate-specific antigen	-Lysozyme	-Lactic acid	-Creatinine	-Lactic acid
-Erythrocytes	-Spermatozoa	-Mucin	-Citric acid	-Uric acid	-Chloride
-Albumin	-Choline	-Buccal epithelial cells	-Urea	-Chlorine	-Sodium
-Glucose	-Spermine	-Thiocyanate	-Vaginal peptidase	-Tamm-Horsfall glycoprotein	-Potassium
-Immunoglobulins	-Semenogelin	-Potassium	-Glycogenated epithelial cells		-Immunoglobulins
	-Zinc	-Bicarbonate	-Acetic acid		
	-Citric acid	-Phosphorus	-Pyridine		
	-Lactic acid	-Glucose	-Squalene		
	-Fructose	-Immunoglobulins	-Immunoglobulins		
	-Urea				
	-Ascorbic acid				
	-Immunoglobulins				

Table 2
Summary of all current or older tests^a.

Body fluid	Component	Classification	Method(s)
Blood	Whole fluid	ALS	<i>Polilight</i> [®]
		Chemiluminescent	Luminol, Fluorescein, Bluestar [®]
	Hemoglobin	Chemical	Benzidine, Kastle-Meyer, <i>O</i> -toluidine, TMB/Hemastix [®] , LMG
		Crystal test	Teichman, Takayama
	Elements	Spectroscopic	UV-vis, Fluorescence (hematoporphyrin)
		Chromatographic	PC, TLC
Isozymes	Immunological	SEM-EDX	
Antibodies	Immunological	LDH	
Semen	Whole fluid	ALS	<i>Wood's Lamp, Bluemaxx BM500, Polilight</i> [®]
		Chemical	SAP, LAP, GDA, CAP, γ -GTP
	Enzymes	Immunological	GGT ELISA
		Crystal test	Florence test
	Choline	Chemical	Choline oxidase
		Chemiluminescent	Choline oxidase/Luminol solution
	Spermine	Electrophoresis	Isotachopheresis
		Chromatographic	HPLC, PC, TLC
	Elements	Electrophoresis	Capillary tube electrophoresis
		Crystal test	Barberio test, Puanen's test
	Spermatozoa	Chemical	Zinc paperstrip test
		Spectroscopic	SEM-EDX
Microscopic		Christmas tree stain	
Immunological		PSA, SVSA	
Saliva	Whole fluid	ALS	<i>UV light, High intensity quartz are tubes</i>
		Chemical	Starch-iodine, Phadebas [®] , Amylose Azure, Rapignost [®] -Amylase
	Antibodies	Immunological	ELISA, Immunodiffusion
		Spectroscopic	SEM-EDX
	Elements	Chemical	PAS reagent
		Electrophoresis	Starch gel/L-valyl-L-leucine
Vaginal fluid	Gly. epithelial cells	Immunological	Oestrogen receptors
	Vaginal peptidase	Electrophoresis	Capillary isotachopheresis
	Antibodies	Electrophoresis	
Urine	Whole fluid	ALS	<i>UV light</i>
		Microscopic	<i>Visualize cells (no stains)</i>
	Epithelial cells	Chemical	Nessler's, DMAC, Urease/bromothymol blue
		Chemical	Jaffe test, Salkowski test
	Urea	Immunological	Sandwich ELISA, SP radioimmunoassay
	Creatinine	Chemical	TZ-UA One/Urea NB-test
	THP	Spectroscopic	SEM-EDX
	UA/UN	Chromatographic	HPLC, ESI-LC-MS, PC, TLC
Elements			
17-Ketosteroid conj.			
Sweat	Elements	Microscopic	SEM-EDX

Codes: grey: presumptive; dark grey: confirmatory; *italic*: non-destructive; and **bold**: applicable to multiple fluids.

^a It is most desirable for a test to be confirmatory, non-destructive, and applicable to multiple fluids, but this table contains no methods which fit this description.

false positives for this test such as chemical oxidants and fruit/vegetable peroxidases [8,29], but its main disadvantage is that benzidine is a known carcinogen, and this has led to its replacement as a presumptive reagent in the forensic laboratory [9,11,29].

A very popular presumptive catalytic method is the phenolphthalein test which is also known as the Kastle-Meyer test. Phenolphthalein will cause an alkaline solution to turn pink after its oxidation by peroxide when blood is present [9]. It can also have false positives similar to those of benzidine, but tests on other body fluids do not yield a positive result [8], and it is not carcinogenic. Although it is not as sensitive as luminol [27], it will still detect blood as dilute as 1 part in 10,000 [30].

Two derivatives of benzidine, ortho-toluidine and tetramethylbenzidine (TMB), were developed as catalytic presumptive tests to replace the hazardous benzidine reagent. Both of these tests are conducted under acidic conditions that involve color changes that are blue or blue-green, respectively [9]. After more consideration, however, it was found that ortho-toluidine was carcinogenic in rats, and the more sensitive TMB slowly replaced it in the laboratory [8].

The TMB reagent remains one of the more popular used in presumptive tests for blood and is the main constituent of the Hemastix[®] field test [9,23]. Although Hemastix can be used in the field, it tends to show even more false positives than the other popular presumptive tests [11].

One of the other most popular presumptive tests for blood used in forensics today is leucomalachite green (LMG) [11]. It is also performed under acidic conditions and involves a heme-catalyzed reaction with a resulting green color [9]. Similar to phenolphthalein, it has a sensitivity of 1 part in 10,000 [30]. Like all other presumptive tests for blood, it is not species specific and will not indicate whether suspected blood is human or not [13]. Leucocrystal violet is another form that can be used but is not as common in forensic investigations [8,11].

Additional non-catalytic presumptive tests have also become available to be used in conjunction with the previously mentioned screening tests. Some of these are Heme Select[™], ABACard[®] HemaTrace[®], and Hexagon OBTI which all use immunological methods to identify primate blood. The latter has been recently compared to other catalytic tests and was found to be inferior as a

presumptive test for blood but rather a good method to supplement the results of the more sensitive catalytic tests due to its specificity. Hexagon OBTI works well on aged or degraded material [9], however it does produce false negatives [30].

3.1.2. Confirmatory tests

Once a positive result is obtained from a presumptive blood test, several confirmatory tests are available to identify absolutely an unknown stain to be blood. These tests can be categorized as microscope tests, crystal tests, spectroscopic methods, immunological tests, and chromatographic methods.

The simplest of microscope tests involves the identification of blood cells by directly visualizing them in liquid blood [11]. It is believed that visually identifying the red and white blood cells along with fibrin is definite proof that blood is present. Numerous stains have been developed to aid this process [8], but other more popular confirmatory tests have replaced cell identification as a technique [11]. An expansion on this method involves the use of a scanning electron microscope (SEM) which allows scientists to study the morphology of an unknown stain and to analyze its chemical composition using an energy dispersive X-ray (EDX) analyzer. Very small or dilute stains can be detected with this method [31].

Crystal tests are the most common confirmatory tests for blood that are used, and the Teichman and Takayama crystal tests are the two most popular [9,11]. The Teichman test is based on the formation of hematin by heating a dried stain in the presence of a halide and glacial acetic acid [8,11]. This test forms brown, rhombic crystals and is very sensitive to under- and over-heating [9]. The Takayama test is based on the formation of hemochromogen by heating a dried stain in the presence of pyridine and glucose under alkaline conditions [9], although acidic conditions have also been used [8]. A positive result yields needle-shaped crystals, and this test is preferred due to advantages such as less heat sensitivity [8], ease of use, and a wider variety of stain compatibility [11]. Both of these crystal tests were improved upon in another experiment so that they became easier to use and more sensitive [32].

Spectroscopic methods, such as UV–vis absorption, are considered highly reliable in confirming the presence of blood in a stain [8]. Many different derivatives of hemoglobin have a characteristic strong absorbance band around 400 nm that is called the Soret Band [11]. Many of these tests can distinguish between different hemoglobin derivatives and also show a positive result for older stains that have a negative result with other presumptive tests and crystal tests [11]. However, there are many conditions which can interfere with the spectral results such as water submersion, sunlight exposure, heating, and rust [8]. A microspectrophotometer can be used to measure an absorption spectrum after a sample has been treated with the Takayama reagent [33]. Another spectroscopic test is based on the fluorescence of hematoporphyrin from excitation with ultraviolet light. This method has been found to be especially successful when dealing with bloodstains on oxidized metals, bloodstains up to 10 years old, and bloodstains exposed to conditions not suitable for UV–vis absorption such as heat, sunlight, and humidity. Best results can be obtained by analyzing a saline extract of the bloodstain, and it is important to keep in mind that there are possible false positives since porphyrin compounds occur frequently in biological systems [8]. These spectroscopic tests have not been used in many years for the investigation of crimes, but it is important to include them in this discussion in order to help to understand the evolution of body fluid testing.

A modern and currently used confirmatory test for blood that can also distinguish species involves the cross reaction of the hemoglobin in a bloodstain with anti-human hemoglobin. Precipitin lines observed after immunodiffusion confirmed the presence of not only hemoglobin, but specifically human hemoglobin. This method has been expanded upon to detect numerous different species of

blood [8]. Isozyme analysis is another technique that can confirm the presence of blood by comparing the differences in lactate dehydrogenase (LDH) isozyme patterns of different fluids [29,34]. Enzyme-linked immunosorbent assay (ELISA) is another method that has been used to identify blood [35] as well as identify blood groups using different antibodies [36].

Finally, chromatographic methods can be used to confirm the identity of blood. Paper chromatography was the first technique and involved the separation of hemoglobin and its derivatives. An alcoholic benzidine spray reagent in conjunction with a hydrogen peroxide spray was used to detect spots, and the hematoporphyrin component was visualized due to fluorescence without spraying. The main disadvantage of this technique was a lengthy development time and the necessity of pre-saturation. Thin-layer chromatography has also been utilized with a similar developing technique [8]. These tests are also not currently used in forensic laboratories.

3.2. Emerging techniques

The following section will describe new and emerging techniques in the area of forensic blood detection. These techniques are almost all confirmatory in nature, and most of them involve immunological markers and can simultaneously identify the species of the unknown blood sample. Table 3 summarizes the new techniques that have emerged in the last 5–6 years for blood and the other body fluids.

The majority of new techniques being investigated for the identification of blood found at a crime scene involve RNA with a focus on mRNA. This form of RNA carries information about proteins from the DNA to the ribosomes in cells where proteins are synthesized [37]. Some of these new techniques have been mentioned in a review on the applications of RNA in forensic science [38]. In 2004, Alvarez et al. revealed a method to isolate simultaneously RNA and DNA from the same stain extract. Performing an mRNA expression analysis on the RNA extracts will yield information regarding the identity of the stain, and the DNA analysis will reveal the donor's identity. The marker being used to identify blood in this case was β -spectrin (SPTB). The ability to analyze both of these characteristics at the same time will help to identify better the number of different fluids or donors in a mixture [39].

In 2005, a multiplex reverse transcription-polymerase chain reaction (RT-PCR) method was proposed by Juusola and Ballantyne to identify different body fluids including blood [42]. This was an expansion on their previous work that concluded that RNA could be retrieved from a blood stain and be analyzed for identification purposes [40]. The RT-PCR process involved the detection of specific mRNA markers for gene expression analysis, namely the SPTB and porphobilinogen deaminase (PBGD) genes for blood. A patent was developed for the same technique [41]. Sensitivity was in the range of 6 ng of blood for a positive result for both genes. Specificity was proven when these genes were only detected in the blood samples, and menstrual blood was even exclusively detected by the presence of the matrix metalloproteinase-7 (MMP-7) enzyme [42]. This MMP-7 enzyme had previously been studied and determined to be a conclusive marker for menstrual blood since it was not detected in vascular blood or other fluids [43]. Bauer and Patzelt have also explored this menstrual blood enzyme and the importance of a negative result that shows a stain is not menstrual blood [44]. A similar study was conducted in 2008 [6] using the same SPTB and PBGD blood genes that Juusola and Ballantyne worked with, and it was found that positive results could be obtained on stains that were up to 15 months old.

Another RT-PCR approach was developed by Nussbaumer et al. in 2006 and focused on the detection of the alpha locus 1 (HBA)

Table 3
Summary of techniques recently developed^a.

Body fluid	Component	Classification	Method(s)
Blood	Whole fluid Hemoglobin Antibodies mRNA	ALS	<i>IR light</i>
		Spectroscopic	<i>XRF (iron)</i>
Semen	Whole fluid Elements mRNA Semenogelin	Immunological	Lateral flow test strip – Antiglycophorin A SPTB/PBGD, HBA, ALAS2/SPTB (RT-PCR)
		ALS	<i>Lumatec Superlight 400</i>
		Spectroscopic	<i>XRF (zinc)</i>
Saliva	Whole fluid Amylase	Immunological	PRM1, PRM1/PRM2, KLK (RT-PCR)
		Immunological	RSID-Semen Test, Nanotrap Sg
	Antibodies	Chemical	<i>Lumatec Superlight 400</i>
		Spectroscopic	SALigAE [®]
mRNA	Immunological	Fluorescence spectroscopy (swabbed from skin)	
	Immunological	RSID-Saliva Test	
Vaginal fluid	Antibodies mRNA	Immunological	Amylase ELISA, Lateral flow test strips
		Immunological	HTN3, STATH/HTN3 (RT-PCR)
Sweat	Antibodies	Immunological	E2-17 β ELISA
		Immunological	MUC4, HBD-1/MUC4 (RT-PCR)
		Immunological	G-81 ELISA

Codes: grey: presumptive; dark grey: confirmatory; *italic*: non-destructive; and **bold**: applicable to multiple fluids.

^a It is most desirable for a test to be confirmatory, non-destructive, and applicable to multiple fluids, but this table contains no methods which fit this description.

RNA marker in blood [7]. In 2007 Juusola and Ballantyne continued to make alterations and improvements to their technique with the introduction of a triplex system that detects the blood-specific genes erythroid δ -aminolevulinic synthase (ALAS2) and SPTB along with the housekeeper gene GAPDH. This housekeeping gene normalizes the expression of the blood-specific genes to maintain high specificity [5].

In 2008, Zubakov et al. comprehensively analyzed the whole-genome gene expression on aged bloodstains in order to generate a set of stable RNA markers. After an initial selection of about 1000 possible markers for blood, the options were narrowed by comparing them to the GNF SymAtlas tissue database. Gene markers were chosen based on high expression in blood and low expression in other fluids. In the end, nine were chosen based on these parameters and the production of positive results on stains as old as 180 days [45].

One more new technique for confirmatory identification of human blood has recently been developed which uses a lateral flow test strip containing an antiglycophorin A antibody that will react with an aqueous blood sample when contact is made. This is the first test which detects this specific sialoglycoprotein, and the test claims to overcome the problem of the high dose Hook effect encountered by other similar methods. The basic theory uses a detection antibody which binds to human blood, then migrates up the membrane and forms a visible complex with an immobilized capture antibody [46].

A completely different form of presumptive identification of blood that is non-destructive has been suggested by Trombka et al. The method involves a unique NASA technology involving portable X-ray fluorescence (XRF) that was originally designed for elemental analysis during planetary exploration. It can detect the abundance of iron present in the hemoglobin of blood, and it

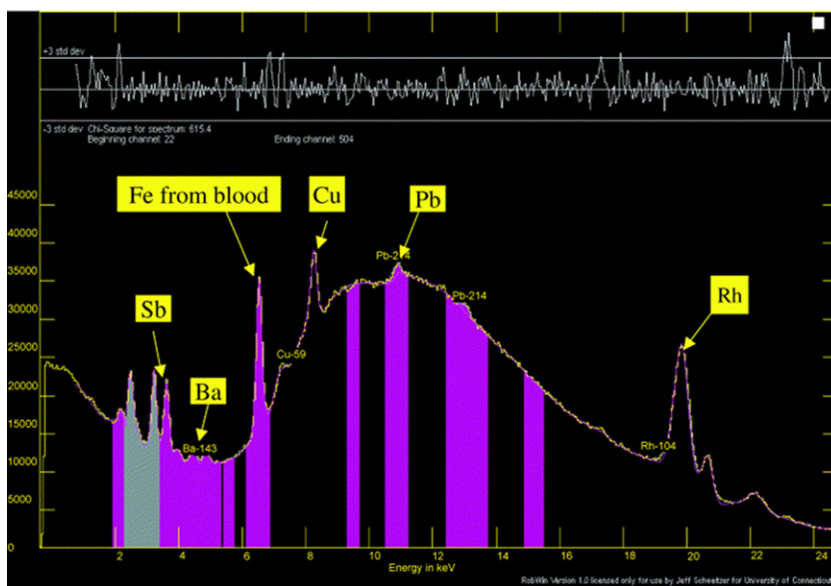


Fig. 1. A non-destructive XRF spectrum of the elemental composition of gun shot residue spattered with blood. A portable device was used which was developed for NASA for planetary exploration. Preliminary results have been obtained for blood and semen based on the presence of iron and zinc, respectively. This is a good screening technique that does not damage the sample but is not confirmatory. (Reprinted from [47] with permission from Elsevier.)

has potential to be a valuable aid in the identification of blood at a crime scene since the device is portable [47]. Fig. 1 shows an XRF spectrum displaying a peak for iron. The fact that it is non-destructive is unique compared to the other known techniques and is very helpful in preserving DNA evidence.

Another non-destructive screening test for blood was suggested in 2007 by Chun-Yen Lin et al. and involves the use of infrared (IR) light as a means to identify latent blood traces. This method is similar to the more familiar techniques involving ultraviolet light. Bloodstains as dilute as 1:8 were detected on black fabrics when a digital camera with an IR sensitive CCD captured pictures of the stains illuminated under IR light. Although this technique is not as sensitive as other presumptive tests and does not work well on some fabrics, it can be helpful due to its non-destructive nature which allows preservation of DNA evidence while quickly searching a crime scene for bloodstains [48].

4. Semen

4.1. Current techniques

Semen is one of the other most commonly encountered body fluids at crime scenes. There are also several presumptive tests to identify semen as well as confirmatory tests. The following sections explain techniques that are either well known in the forensic community or have been summarized in previous literature.

4.1.1. Presumptive tests

Like with blood, semen can also be detected using an ALS such as ultraviolet light. It is routine procedure to search a crime scene for semen and other fluids using this simple and non-destructive method [29]. The Wood's Lamp (WL) is a specific device that emits wavelengths from about 320–400 nm, and it is small, inexpensive, safe, and easy to use. However when the WL was put to the test against other fluids, it was not very specific and sometimes did not even detect semen stains, and it gave false positive results for ointments and creams [49]. Another commercial ALS, the Blue-maxx™ BM500, was tested in a similar way and was 100% sensitive to semen stains. Also, physicians using the ALS were able to distinguish semen from other products 83% of the time after receiving training on how to use the device [50]. Another ALS that has been used on several fluids including semen is Polilight® which has a wavelength range of 415–650 nm as well as white and ultraviolet light [19].

The most popular and accepted presumptive test for the presence of semen is the test for seminal acid phosphatase (SAP). This enzyme has the ability to catalyze the hydrolysis of organic phosphates which forms a product that will react with a diazonium salt chromogen to cause a color change [10]. This is the basic principle behind all the variations of the SAP test. One popular substrate/color developer combination is alpha-naphthyl phosphate and Brentamine Fast Blue. Other combinations that have been successful are beta-naphthol with Fast Garnet B, and alpha-naphthol with Fast Red AL [8]. Additional reagents used have been sodium thymolphthalein monophosphate due to its high selectivity, stability, and low hazardous risk [51], and a combination of *p*-nitroaniline, NaNO₃, α-naphthyl phosphoric acid, and aqueous magnesium chloride [52]. Of course there are false positives such as some plant materials and even vaginal acid phosphatase (VAP), so this technique cannot be considered confirmatory [8]. One way to avoid a potential false positive for VAP is to observe a color change that only occurs between 5 and 30 s since VAP has never given a positive result that quickly [12]. Other methods that have been developed to distinguish between SAP and VAP involve the separation of the two acid phosphatases using isoelectric focusing

[53], and by using acrylamide gel electrophoresis [54]. Further disadvantages of SAP tests are that the enzyme can degrade when exposed to heat, mold, putrefaction, or chemicals [12].

There are similar presumptive tests for semen based on the presence of other enzymes, but these tests are not as popular. In a comparison study with the SAP test, the leucine aminopeptidase (LAP) test had fewer false positives with other human body fluids, fruits, and vegetables, and only showed negative results with semen from other species. However it was less sensitive to high dilutions [55]. Another enzyme that has been tested is glycylproline dipeptidyl aminopeptidase (GDA). Results have shown that stains as old as 24 years can give a positive reaction; false positives include vaginal fluid, feces, strawberries, broad beans, and onions. The sensitivity was only tested as low as a 1:4 dilution in which all were positive [56]. Another enzyme-based test relies on the detection of cystine aminopeptidase (CAP). It is about 100 times more prevalent in semen than other fluids. When tested on other body fluids including vaginal fluid and feces, there were no false positive results as with some of the previously mentioned enzyme tests. There were also only negative results for several fruits and vegetables, including strawberries [57]. A test for the enzyme γ-glutamyltransferase (γ-GTP) using Fast Garnet GBC salt and α-naphthylamine as indicators showed positive results on stains as old as 23 years, but there were several false positives including breast milk, vaginal fluid, green peas, broad beans, onions, strawberries, apples, and plums [58]. Finally, the test for seminal zinc has been studied and proposed to be a better and less degradable marker than SAP. This study reported no false positive results on other body fluids, fruits, or vegetables, and it detected semen on stains that were 25 years old [59]. A zinc test paperstrip method was compared to an SAP paperstrip test, and in the end both tests appeared to be similar in both sensitivity and specificity [60].

Another presumptive test for semen that has been around for a long time but no longer regularly used is the test for the presence of choline. One test for choline is the Florence test which involves placing an extract of a questioned stain on a microscope slide, treating it with a solution of iodine and potassium iodide, and observing the brown needle-like crystals that form [61]. The possibility of false negatives is great due to low sensitivity [61], and this test is negative for other body fluids including vaginal fluid as well as semen from other species [8]. Additional methods developed for the detection of choline are based on a reaction with choline oxidase [62] including a chemiluminescent test involving a choline oxidase/luminol solution [63]. A more complicated method to detect choline known as isotachopheresis was found to have no false positives with other body fluids, fruits, or vegetables. Stains up to 10 years old still showed some positive results, and the test showed positive results for semen in vaginal fluid swabs taken from deceased females [64].

One more presumptive test that has been studied in the past but is not currently in use is the detection of the seminal polyamine known as spermine (SPM) which has the highest concentration in semen among all the different forms of polyamines. High-performance liquid chromatography (HPLC) combined with a simple extraction method has been found to be a simple and sensitive way to detect SPM in comparison to paper chromatography, TLC [65], capillary tube isotachopheresis [66], and an enzyme method [67]. The most prevalent false positive came from soy sauce, and no spermine was measured in body fluids including urine, blood, sweat, breast milk, and saliva. Fruit and vegetable juices also showed no positive results [68]. Another test for spermine that has been used in the past is called the Barberio test and involves the microscopic confirmation of yellow crystals that form when semen is exposed to an aqueous solution of picric acid. This test is considered more reliable than the Florence test for

choline, and it still gave positive results on stains as old as 3 years and ones that had been heated to 150 °C [8]. A similar method known as Puanen's test is another crystal test that uses Naphthol Yellow S as a reagent and forms orange crystals in the presence of semen [8].

Finally, a completely unrelated method to the ones previously mentioned has also been used to identify semen stains but is not as popular when compared to SAP tests. It applies scanning electron microscopy (SEM) and energy dispersive X-ray microanalysis to the detection of sodium, phosphorus, sulfur, chlorine, potassium, calcium, and other metal trace elements. These elements occur in varying proportions among different body fluids, and identifying an unknown stain's element ratio will distinguish it from other fluids. Chlorine was the largest peak detected in the semen samples, but calcium can be used as an identification marker. The method was flawed, however, since the substrate spectrum will dominate the fluid spectrum, and subtraction of the substrate will also remove peaks from the fluid that are present in both. In the end, this method will work best to identify one fluid stain as being identical to another [69]. This technique can only be classified as presumptive due to the interference of the substrate.

4.1.2. Confirmatory tests

The most reliable and widely accepted confirmatory technique for the detection of semen is the microscopic identification of sperm cells. Semen is the only body fluid which possesses sperm cells, and the large amount of DNA in the heads can be treated with a stain to make the sperm visible [13]. The most popular stain used is the Christmas tree stain, and it is known for its characteristic colors which stain the heads red and the tails green. There is an additional technique using a solution of proteinase K that will denature epithelial cells and make the unaffected sperm heads more visible [10]. Other stains that are less effective than the Christmas tree stain that have been tested are Gram modified Christmas tree, hematoxylin and eosin, Baecchi's, Papanicolaou's, and Wright's [12]. Of course the largest disadvantage of this microscope technique is if the semen donor is azoospermic due to natural causes or by vasectomy; for this reason, other chemical tests have been developed.

The most popular confirmatory test for semen beyond looking for sperm cells is the test for prostate-specific antigen (PSA). The semen from azoospermic males will still contain this antigen which is present in the seminal plasma; other body fluids contain a very low level of PSA and do not interfere [10]. These low levels require that PSA tests are not too sensitive so that there are no false positives. An important aspect of detecting PSA involves the ability to detect it on contaminated or scarce samples including laundered fabrics and decomposed cadavers [70]. The original methods involved immunoelectrophoresis or ELISA [71], and some specific techniques involved a dot blot immunoassay with a radiolabeled Protein A [72] as well as a dot-immunobinding method called the membrane aspiration test (MAT) [73]. Another used thin-layer immunoassay (TIA) and showed no false positive results with blood, saliva, urine, sweat, and tears [74]. Now several test kits have emerged that depend on antibody-antigen reactions and are much quicker and easier to use [12]. One commercially available kit is the Biosign[®] PSA test, and it was also found to be cheaper to operate than the traditional ELISA method [75]. The OneStep ABACard[®] is another commercial test kit. It also relies on the technique of a mobile monoclonal anti-human PSA antibody which binds to human PSA and migrates along the strip to the immobilized polyclonal anti-human PSA antibody and forms a visible line [76]. PSA in semen diluted 10⁶ times is able to be detected with this test, and only male urine samples gave a false positive result [77]. Other commercial tests that have been developed include Chembio, Medpro, Onco-screen [70], PSA-

check-1, and Seratec[®] PSA Semiquant [76]. The Seratec[®] test was found to show false positives with semen-free vaginal samples as well as some readily available contraceptive foam [78]. A comparison study among the PSA Rapid Test Kit, Rapid PSA, and SMITEST determined that all three had equal specificity, but SMITEST [79] had the greatest sensitivity [80]. An automated test kit that allows simultaneous analyses is the Hybritech Tandem-E PSA Immunoenzymetric Assay and uses less labor and funds than traditional methods [81].

MHS-5, also known as immunoglobulin G₁ and seminal vesicle-specific antigen (SVSA), is an antigen that will only react with the epithelium in human seminal vesicles [82]. The major gel-forming proteins in human semen, semenogelin I and semenogelin II, both contain SVSA and are recognized by a monoclonal MHS-5 antibody. Sema[®] is an ELISA kit designed to detect the presence of semen based on the reaction between the MHS-5 antibody and SVSA. This method is sensitive for semen samples diluted as many as 10⁶ times, but not nearly as specific as typical PSA tests and is no longer used [12]. Other immunological methods that have been developed to detect semen involve the presence of semenogelin [83], semenogelin II [84], 19-OH F_{1α}/F_{2α} prostaglandin [85], and a monoclonal antibody called 1E5 [86]. Finally, an ELISA technique for the detection of a monoclonal antibody to SAP has been developed which showed no false positives with other body fluids and is sensitive with semen dilutions up to 1:100,000 [87].

As mentioned earlier in the discussion about confirming the presence of blood, an LDH isozyme can also play a role in confirming the presence of semen. It was discovered to have properties in between LDH-3 and LDH-4 and was soon named LDH-X. It is unique to human sperm, however, so like the Christmas tree staining technique, the LDH method will only work if the donor's semen contains sperm [8,88]. The isozyme is detected by electrophoresis, and this technique will give positive results on stains at least 30 weeks old [88] and on post-coital vaginal swabs [29]. Sperm can even be detected in a mixture with blood and vaginal fluid using this method [89]. This method is not commonly used in modern forensic laboratories.

Another isozyme that can be used to detect semen is γ -glutamyl transpeptidase (GGT). It is much more active in seminal plasma than other body fluids and is believed to be spermic or testicular in nature [29]. A few other isozymes have been studied such as "sperm" diaphorase, creatine phosphokinase (CPK), and various esterases, but these methods have not proved to be as effective as the more popular PSA tests [29].

4.2. Emerging techniques

The following section will describe new and emerging techniques in the area of forensic semen detection. Like with blood, these techniques are almost all confirmatory in nature, and most of them involve immunological markers and can simultaneously identify the species of the unknown semen sample.

Many of the new methods being developed to identify semen stains involve mRNA markers and are the same methods already mentioned for the detection of bloodstains. Bauer's review of the uses of RNA in forensic science also covers methods to detect semen [38]. The same RNA and DNA co-isolation method described by Alvarez et al. can also be applied to semen samples. Protamine 1 (PRM1) was the semen-specific marker under investigation in this study [39].

The RT-PCR method proposed by Juusola and Ballantyne in 2005 also applies to semen. The process involves the detection of the semen-specific genes PRM1 and protamine 2 (PRM2) [42]. A patent was developed for the same technique [41]. Sensitivity for the semen samples was the highest among body fluids being detected with less than 200 pg of input RNA needed for a positive

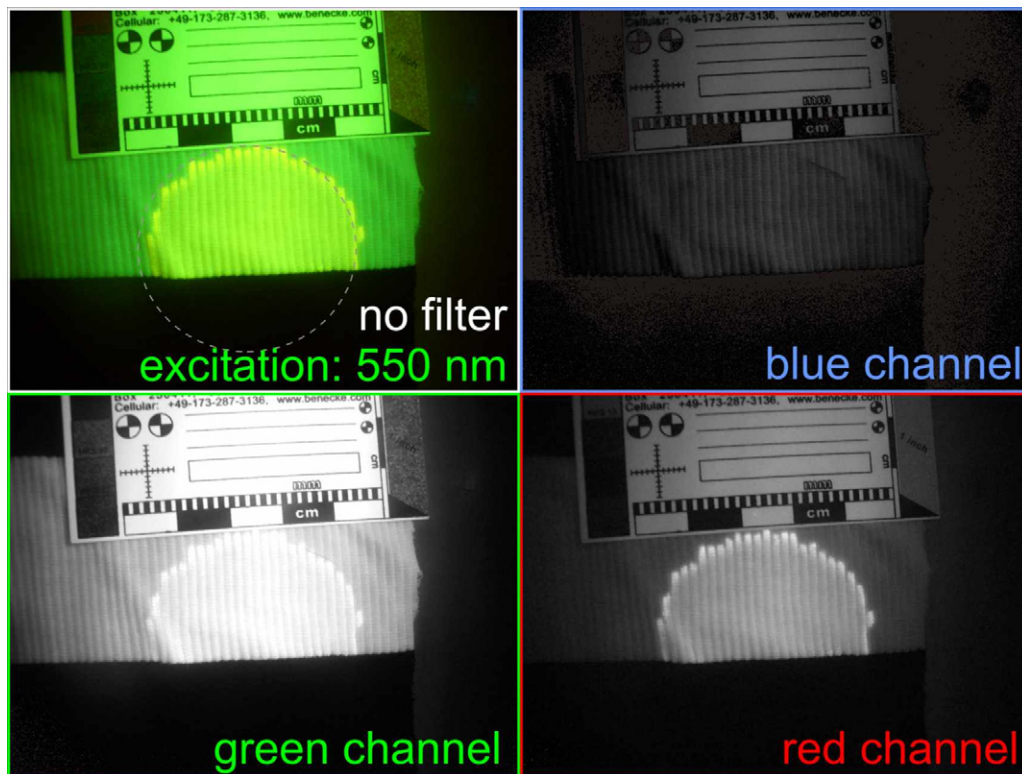


Fig. 2. A white and black cloth containing semen is illuminated with the Lumatec Superlight 400 at 550 nm. The device has a range from 320 to 700 nm and will work in darkness and daylight. Semen was detectable 100% of the time, and saliva was only found 60% of the time. Dark fabrics absorbed fluorescence and made the stain visibility decrease. (Adapted from [92] with permission from the author.)

result with both genes. Specificity was proven when these genes were only detected in the semen samples. This octaplex RT-PCR method produced no false positives, no false negatives, and no single gene drop outs [42]. The updated version of this experiment that was presented in 2007 involves a triplex system as previously mentioned during the bloodstain discussion, and the genes involved are PRM1 and PRM2 for semen along with the GAPDH housekeeping gene [5]. A similar study was conducted in 2008 using the same PRM1 and PRM2 semen genes that Juusola and Ballantyne worked with, and it was found that positive results could be obtained on stains that were up to 15 months old [6].

The RT-PCR approach developed by Nussbaumer et al. in 2006 focuses on the kallikrein 3 (KLK) marker for semen (also known as PSA). There was no cross reactivity with samples from other fluids such as blood, vaginal secretions, and saliva, and none of these samples showed positive results for the KLK assay. Also, this specific semen mRNA marker showed great stability and was equally detected after 10 days of room temperature storage with no stabilizing buffer. This technique proved to be more sensitive than popular protein-based assays for PSA [7].

In 2007 Pang and Cheung [90] compared a new commercial rapid stain identification (RSID)-Semen Test to the previously mentioned ABACard[®] PSA test for the detection of semen stains. The RSID-Semen Test is based on the detection of semenogelin (Sg) using monoclonal anti-human Sg antibodies. Both tests involve the same immunochromatographic membrane assay technology. The RSID-Semen Test was found to be more sensitive by detecting Sg at a semen dilution of 1:100,000. The several different species of semen that tested positive for spermatozoa all showed negative results for Sg. This test also showed no false positives with other body fluids, and the analysis takes only 10 min [90]. Another commercial test kit that detects Sg is called Nanotrap Sg. It has a similar sensitivity as the RSID-Semen Test, but more than three repeats of freezing and thawing semen samples caused the

sensitivity of the results to drop. This test was able to detect semen in 67% of samples that contained no spermatozoa. The ability for this method to find male DNA in samples showing no spermatozoa makes it valuable for subsequent DNA analysis [91].

As with blood, Trombka et al. have introduced a non-destructive method of presumptive semen identification. The method which involves a unique NASA technology involving portable XRF can detect the abundance of zinc present in the semen stains, and it also has potential to be a valuable aid in the identification of semen at a crime scene since the device is portable. The process only takes about a minute, and it has a precision of better than 10% in measuring 1 ml of semen distributed over a 40 cm² area [47]. The non-destructive nature of the method will be very helpful in sexual assault cases that rely on DNA evidence not being destroyed by early screening tests.

Finally, a newly developed presumptive test for semen stains is a UV-vis light source called Lumatec Superlight 400. It emits light from 320 to 700 nm and was tested on human and boar semen samples on different types and colors of fabrics. Fig. 2 shows the results of illuminating a stain on light and dark fabric with different filters. The Superlight was able to detect stains both in darkness and in the presence of daylight; storage times of 3 and 5 weeks showed no difference in results. Semen was best detected using a range of 415–490 nm with orange or red goggles. Poor results were obtained on dark fabrics and on fabrics that had been washed, but different types of fabrics showed similar results [92].

5. Saliva

5.1. Current techniques

In addition to blood and semen, saliva is another commonly encountered body fluid at crime scenes. There are a few well known and accepted presumptive tests for saliva, but there are no

currently used confirmatory tests that are specific to saliva. The following section explains presumptive techniques that are either well known in the forensic community or have been summarized in previous literature.

Like blood and semen, saliva can also be located using an ALS. Saliva stains will appear bluish-white when being viewed under an ultraviolet light, though this will not distinguish it from another body fluid stain [8]. Saliva is also harder to detect than semen due to the lack of solid particles in the saliva sample [12]. One study that compared two different argon laser light sources to a high intensity quartz arc tube found the quartz arc tube to be superior based on portability, cost, sensitivity, and power output. The lifetime of the quartz tubes was found to be the largest disadvantage, though they were cheaper to replace than the laser light sources [93].

The most popular and widely accepted technique to test presumptively for saliva is based on the activity of amylase. Two different forms are found in the human body. Amylase found in saliva, breast milk, and perspiration is coded by the AMY1 locus on chromosome 1, while amylase found in the pancreas, semen, and vaginal secretions is coded by the AMY2 locus [10,29]. Although AMY1 is found more in saliva than any other fluid, it can still only give presumptive information since it is not exclusive to saliva [10]. A radial diffusion assay has been used to distinguish sources of AMY1 and AMY2 [94]. The starch-iodine test is based on the fact that starch appears blue when in the presence of iodine, and salivary amylase will break down the starch to cause a color change. However, competing proteins such as albumin and gamma-globulin in blood and semen will also break down iodine to form a false positive result. The Phadebas[®] test reagent, which includes procion red amylopectin, has been applied in tube tests and press tests which both can detect saliva diluted up to 1:128 [10]. A study testing the Phadebas[®] reagent for false positive results found that hand cream, face lotion, washing powders, urine, and feces tested positive using Red-Starch paper [95]. Another method utilizing an insoluble amylase/dye complex called Amylose Azure as a substrate will yield a blue color upon hydrolysis in amylase. This technique is more sensitive since it can detect saliva dilutions as high as 1:1000, it requires a shorter incubation time, and it will detect saliva present in body fluid mixtures. A commercial test strip known as Rapignost[®]-Amylase used to detect amylase in urine has also been applied to saliva samples. The method is simple and requires only 30 min of reaction time [96].

There have been some immunological methods presented for the identification of saliva, though they are still not completely exclusive or widely used. An ELISA method using a horseradish peroxidase conjugate combined with monoclonal antibodies has been used to detect α -amylase activity in saliva stains [36]. Rabbit antisera against α -amylase have been used in conjunction with α -amylase purified from human submaxillary glands in a traditional immunodiffusion experiment. This experiment did not test other body fluids, so it can only be used as a species indicator once the sample has already been identified as saliva [97]. Many other experiments have been conducted involving immunoelectrophoresis and saliva antigens, but there were too many cross reactions with other fluids such as serum to lead to a reliable technique that was conclusive for saliva [8].

There are also some techniques that have dealt with microscopy. Like with semen, the use of SEM coupled with EDX can identify the relative concentrations of sodium, phosphorus, sulfur, chlorine, potassium, calcium, and other metal trace elements in the questioned sample. In the saliva samples tests, potassium was the largest peak and can be used as a basis of identification. As previously mentioned, this technique can only be used to screen a sample and determine if it is identical to another. The dominance

of the background spectrum and subsequent subtraction will lose valuable data about the fluid [69].

5.2. Emerging techniques

The following section will describe new and emerging techniques in the area of forensic saliva detection. Like with blood and semen, most of these methods involve mRNA markers and can potentially be considered confirmatory in nature. The application of some of these techniques to forensic casework could be substantially helpful.

Many of the same methods already discussed for blood and semen can also be applied to saliva. Bauer's review of the uses of RNA in forensic science also covers methods to detect saliva [38]. The same RNA and DNA co-isolation method described by Alvarez et al. can also be applied to saliva samples, and for this fluid histatin 3 (HTN3) is being detected [39].

The RT-PCR method proposed by Juusola and Ballantyne in 2005 also applies to saliva. The saliva-specific genes statherin (STATH) and HTN3 were the ones under investigation in this study [42]. A patent was developed for the same technique [41]. Sensitivity was similar to that of blood with only 9 ng of input RNA needed to detect both genes. Specificity was slightly less than that of blood and semen. Although the main components STATH and HTN3 were only detected in saliva, the larger and less specific histatin 1 (HTN1) was detected using the HTN3 primer and showed some slight false positive results in semen. Likewise, a smaller version of STATH was minutely detected in menstrual blood [42]. The updated version of this experiment that was presented in 2007 involves a triplex system as previously mentioned during the bloodstain and semen discussions, and the genes involved are again STATH and HTN3 for saliva along with the GAPDH housekeeping gene [5]. A similar study was conducted in 2008 using the same STATH and HTN3 semen genes that Juusola and Ballantyne worked with, and it was found that positive results could be obtained on stains that were up to 15 months old with much better sensitivity than tests for amylase [6].

In 2008, Zubakov et al. comprehensively analyzed the whole-genome gene expression on aged saliva stains in order to generate a set of stable RNA markers. After an initial selection of about 500 possible markers for saliva, the options were narrowed by comparing them to the GNF SymAtlas tissue database and targeting genes for tissues like the salivary gland, tongue, trachea, and tonsils. Gene markers were chosen based on high expression in blood and low expression in other fluids. In the end, five were chosen based on these parameters and the production of positive results on stains as old as 180 days [45].

An ELISA method developed by Quarino et al. uses a monoclonal anti-human salivary amylase antibody to detect saliva stains, and it shows no cross reactivity with other forms of amylase such as pancreatic or bacterial. The salivary amylase can be quantitatively detected by absorption at 405 nm directly from the sample well and there is a direct relationship between absorption and amylase activity. The results showed that 100% of the saliva samples and only 13% of other body fluids showed absorption. However, the false positive absorption results were ten times weaker than the lowest saliva result [98].

In 2007 Karl Reich presented a lateral flow test strip as a method to confirm the presence of saliva rapidly, accurately, and with high sensitivity. The technique uses nine antibodies against human salivary amylase which can be monoclonal, polyclonal, or recumbent antibodies. This test is species specific and can be applied to many different types of samples. The test strip is immunochromatographic in nature and uses a mobile and stationary antibody. Positive results were obtained from samples taken from buccal swabs, plastic bottles, plastic mugs, ceramic

mugs, cigarette butts, and soda cans. There was only some cross reactivity with feces and breast milk, and only human saliva yielded positive results [99].

Another recently released colorimetric assay test kit for saliva that is presumptive in nature is called SALIgAE[®] and is also available as a spray [14]. Myers and Adkins performed a study that compared this test to the Phadebas[®] and starch–iodine mini-centrifuge test. They found that SALIgAE[®] is much less sensitive than the other two, and it even had detection limits that were higher than the average α -amylase concentrations in human saliva. It could only detect dilutions of neat saliva as low as 1:10. Waiting longer than 10 min sometimes revealed positive results that were not formerly visible [100]. Another study compared SALIgAE[®] to an immunochromatographic test known as the RSID-saliva test and the Phadebas[®] test [101]. The RSID-saliva test uses a mobile and stationary monoclonal anti-human salivary α -amylase antibody that forms a visible pink line in the presence of antigen [14]. The results of the experiment were different than that of Myers and Adkins. The RSID-saliva test could detect saliva diluted up to 1:10,000, SALIgAE[®] up to 1:2000, and the Phadebas[®] test only up to 1:100. Species specificity was also highest with the RSID-saliva test with only rat saliva testing positive [101].

Finally, some new spectroscopic and ALS techniques have been recently presented. Soukos et al. developed a rapid and non-destructive method to detect dried saliva swabbed from skin using fluorescence spectroscopy [102]. Emission spectra were measured from solutions containing the dissolved swab contents in KCl in the range of 345–355 nm. Compared to a water control, the emission spectra showed greater intensity. The fluorescence spectra of saliva were similar to that of pure aqueous amylase and tryptophan [102]. The ALS technique introduced by Fielder et al. in 2008, which was previously mentioned in regards to semen identification, can also be applied to saliva. The Lumatec Superlight 400 emits light from 320 to 700 nm and was tested on human saliva samples on different types and colors of fabrics. It was able to detect stains both in darkness and in the presence of daylight; storage times of 3 and 5 weeks showed no difference in results. Saliva was also best detected using a range of 415–490 nm with orange or red goggles. Saliva was only detected in 60% of the cases, but the rate of positive results was much higher than with other reported ALS techniques [92].

6. Vaginal fluid

6.1. Current techniques

Although not as common at crime scenes as blood, semen, and saliva, vaginal fluid evidence can play an important role in sexual assault cases. However, there are not very many tests available to test for the presence of this fluid mainly because it is not very well defined. The constituents can change based on the menstrual cycle of the female, and this makes testing for specific components very difficult [8]. A few presumptive tests that have been established are mentioned in this section.

One test is based on the detection of glycogenated epithelial cells using a periodic acid-Schiff (PAS) reagent. This reagent will stain glycogen in the cytoplasm a magenta color, and the intensity of the color is rated to determine the concentration of cells. However, since glycogenation varies based on the menstrual cycle, this test is not very reliable. Also, some females will likely show no glycogenated cells if they are pre-pubescent or postmenopausal, so this technique can easily have false negative results. False positive results can emerge from the mouth or urethral tract in males. Finally, the test uses a large amount of sample and will destroy valuable DNA evidence [10].

Another older method involves an enzyme known as vaginal peptidase that has been found in vaginal fluid samples. The technique uses electrophoresis in a starch gel at a pH of 7.4 in which the vaginal peptidase hydrolyses the dipeptide substrate L-valyl-L-leucine. It was found that no other body fluid showed positive results using this method, and the vaginal fluid samples tested positive in 64% of the cases. Positive results were also obtained for mixtures of vaginal fluid and semen or blood [103]. Other studies have suggested additional possible components for vaginal fluid originating from the epithelial linings such as esterase, alkaline phosphatase, β -glucuronidase, and DPNH-diaphorase [8].

Another study investigated whether oestrogen receptors could be detected in vaginal fluid samples using monoclonal antibodies via immunohistochemical techniques. Samples were tested from living females and female corpses as well as male prepuces and urethral mucosa. Oestrogen receptors were detected in all vaginal biopsy samples taken from the living females regardless of age, though the superficial layer showed no positive results. The postmortem female samples also showed no positive results even with a short postmortem interval of only 8 h. False positive results were obtained from the male prepuce and urethral mucosa samples [104].

Finally, the ratio of lactic acid and citric acid present in vaginal fluid can be compared to the ratio found in semen to identify the presence of vaginal fluid either by itself or in a mixture. Lactic acid is present in large quantities in vaginal fluid when compared to semen, and semen has larger quantities of citric acid. Capillary isotachopheresis was used for the assay, and the results showed that all semen samples had a much higher concentration of citrate compared to lactate, and vaginal fluid samples showed the opposite. The levels of citrate present in post-coital vaginal fluid samples decreased with time which shows the diminishing presence of semen in the samples. Saliva and urine showed small amounts of both carboxylic acids, but there were not enough to cause confusion among the semen and vaginal fluid samples [105].

6.2. Emerging techniques

Almost all of the recently developed techniques for vaginal fluid are based on mRNA markers and can also be applied to the other body fluids already discussed. Juusola and Ballantyne's method of multiplex mRNA profiling using RT-PCR [41] can identify vaginal fluid based on the presence of the human beta-defensin 1 (HBD-1) and mucin 4 (MUC4) markers. These genes were also amplified in menstrual blood, but they were not detected in vascular blood, semen, or saliva. This fluid had the least sensitivity when compared to blood, semen, and saliva, requiring 12 ng of input RNA to identify both genes [42]. Haas et al. used the same two markers with success on 15 year old stains [6]. The MUC4 gene was also detected using the mRNA and DNA co-isolation technique developed by Alvarez et al. [39] as well as the RT-PCR technique presented by Nussbaumer et al. [7].

One additional new technique that has not yet been discussed is the detection of 17 β -estradiol (E2-17 β) which has the strongest oestrogenic activity of all the female hormones [106]. It was strongly detected in all 6 extracts from vaginal fluid stains using GC-MS and only in 1 of 10 semen stains and 1 of 9 female saliva stains. A commercial ELISA kit utilizing a monoclonal antibody to E2-17 β was also tested and had similar results to the GC-MS experiment. There were some false positive results from semen, female urine, and female saliva, and there were other problems with the test such as the sample adhering to the glass tube during preparation and a prozone phenomenon of the antigen-antibody reaction. This hormone has potential to identify vaginal fluid stains after further experimentation [106].

7. Urine

7.1. Current techniques

Like vaginal fluid, urine is a difficult body fluid to detect due to low sensitivity of the available tests and false positive results. It can be useful in sexual assault, harassment, and mischief cases. The nature of the fluid causes it to spread out and become diluted on fabric surfaces, and this makes it hard to find. Odor can be an indicator, but it will cover an entire item and not localize itself to the stained area [10]. This section will mention the many different techniques that have been established to identify presumptively a urine stain.

As with other fluids mentioned, urine will also fluoresce when exposed to ultraviolet light [8]. This is not specific, of course, and urine is especially hard to locate since the stains tend to be more dilute than other body fluids and this makes them fluoresce much less [10]. Other basic microscopic methods have been established which rely on solids in the sample which consist of various crystalline materials and epithelial cells characteristic of the urinary tract linings. This only gives useful results in liquid samples as opposed to stains, however, so there is limited usefulness to forensic cases. Inorganic ions such as chloride, phosphate, and sulfate have also been used to identify urine. These ions are not unique to urine, but phosphate and sulfate are present in much higher concentrations than in other body fluids [8].

Urea is an organic compound that is found in higher concentrations in urine than any other body fluid. Tests for urea depend on the activity of the enzyme urease which releases ammonia and carbon dioxide upon breaking down urea. The ammonia can then be detected by either using Nessler's reagent as an indicator or *p*-dimethylaminocinnamaldehyde (DMAC) [10]. Another method that uses a spray reagent containing urease and bromothymol blue has been performed to identify urine stains. A positive result will yield a blue spot at the crime scene [107]. Another technique using urease and bromothymol blue is radial gel diffusion. This method is both qualitative and quantitative with the diameter of the reaction circle being proportional to the square root of the urea concentration. Semen and sweat stains showed slight false positive results due to their urea content [108].

Creatinine is another organic compound that is present in high concentrations in urine. This can be detected using a solution of picric acid in toluene or benzene on an extract taken from a questioned stain. The result in the presence of urine is creatinine picrate which is a red product [10]. This is known as the Jaffe test [8,14]. The Salkowski test, which uses sodium nitroprusside, will yield a blue product when the reagent is heated with creatinine. Potassium ferricyanide acts as an oxidizing agent. *O*-nitrobenzaldehyde can also be used [8,12].

An immunological method that has been attempted to identify urine in stains involves the detection of Tamm-Horsfall glycoprotein (THP). It is specific to urine, but is also present in other animal species [10]. One method involves two monoclonal antibodies to THP called THP-27 and THP-22. A sandwich ELISA technique with THP-27 as a capture antibody and THP-22 as a detector antibody shows no false positive results from other body fluids [109]. A similar ELISA technique using goat anti-human uramucoid also showed promising results even on samples 3 months old, and other than cross reactivity with some primate species, it was specific to human urine [110]. THP has also been detected in urine stains using a solid phase radioimmunoassay. This technique uses affinity-purified rabbit anti-human THP, and any positive results from other body fluids were less than half that of a 1:100 dilution of urine. The crab-eater monkey urine was the only significant positive result from other species [111].

A different technique is based on the ratio of uric acid (UA) to urea nitrogen (UN) found in urine. Commercial tests kits utilizing uricase-peroxidase and urease-indophenol were used to quantify UA and UN, respectively. When compared to other body fluids and plant juices, most had neither UA nor UN, and none contained both. Other species of urine had ratios that were either much lower than the human urine average or they were much higher. The centers of the urine stains tested were found to have a much higher UA content than the edges, so it is important not to use the peripheral part of the stain when doing an analysis [112].

As with semen and saliva, the use of SEM coupled with EDX can identify the relative concentrations of sodium, phosphorus, sulfur, chlorine, potassium, calcium, and other metal trace elements in the urine sample. Urine stains were made on handkerchiefs for the purpose of the experiment. Urine showed a much stronger chlorine peak than the other fluids, and was lacking the calcium peak. As previously mentioned, this technique can only be used to screen a sample and determine if it is identical to another. The dominance of the background spectrum and subsequent subtraction will lose valuable data about the fluid [69].

Finally, some other instrumental techniques have been used to study urine stains. These methods revolve around identifying 17-ketosteroid conjugates found in urine, and one study used high-performance liquid chromatography (HPLC) [113]. A spectrophotometer at 380 nm measured results, and five conjugates were detected in the human urine samples while only traces of some were detected in animal samples. Electrospray ionization liquid chromatography–mass spectrometry (ESI-LC–MS) was used to confirm these results [114] with a lower detection limit for each conjugate being 10 ng in scan mode [115]. Some older paper chromatography and thin-layer chromatography methods have also been suggested to identify components of urine such as urea, creatinine, urochrome, indican, purine and pyrimidine bases, and steroid derivatives [8].

7.2. Emerging techniques

There have been no recent publications on new techniques to identify urine stains since Nakazono et al. presented their method of HPLC and ESI-LC–MS in 2002 [114], but they did expand upon that project in 2008. The new technique involved the same HPLC and ESI-LS–MS analysis, but it was carried out simultaneously as DNA extraction on the same stain sample. This allowed less evidence to be destroyed via testing which is a positive attribute of any forensic analysis. It is noteworthy that about twice as much sample was needed to identify simultaneously male urine along with DNA analysis as opposed to female urine due to the abundance of cells in female samples [116].

8. Sweat

8.1. Current techniques

Sweat is the least common body fluid found at crime scenes in comparison to the other five that have been mentioned, but it can contain DNA evidence and has been extracted from sweatbands in hats and waistbands in pants [12]. It is similar in composition to urine but contains less urea and creatinine [8].

The only current technique that has been developed to presumptively test for the presence of sweat is the previously mentioned SEM coupled with EDX that can identify the relative concentrations of sodium, phosphorus, sulfur, chlorine, potassium, calcium, and other metal traces. The sweat analysis showed that chlorine and sodium were the only consistently clear peaks among the different samples, and potassium was sometimes visible. The large chlorine peak is used as the basis of comparison and identification [69].

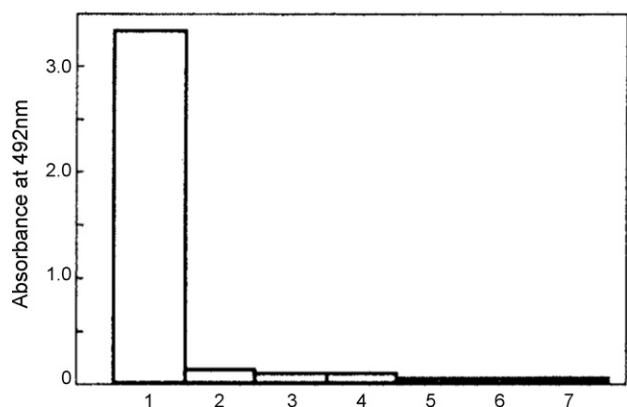


Fig. 3. A sweat-specific protein was discovered, and a monoclonal antibody (G-81) was developed for this protein. The G-81 antibody specifically forms a colored complex with sweat antigens. Absorbance measurements are shown for sweat (1), plasma (2), semen (3), saliva (4), milk (5), tears (6), and urine (7) at 492 nm after analysis by ELISA. The G-81-reactive protein was stable for up to 11 weeks in stains. (With kind permission from Springer Science + Business Media [117].)

8.2. Emerging techniques

In 2003 a study was released that involved the production of a monoclonal antibody for a sweat-specific protein [117]. The G-81 monoclonal antibody was chosen since it was specific to sweat but not to other body fluids such as serum, saliva, semen, milk, urine, and tears using an ELISA technique. Fig. 3 shows the specificity of this technique for the identification of sweat. G-81 only reacted with sweat glands in the skin when an immunohistochemical stain was used on different tissues. Using a Western blot, the G-81 antibody only reacted to the specific peptides in sweat and not the other body fluids that were examined. An antibiotic peptide known as dermcidin was found to have an identical 18-amino acid segment as the N-terminus of a peptide that reacted with G-81. Finally, ELISA analysis was able to detect the G-81 reactive peptide in sweat samples subjected to an 8192-fold dilution, and storage of samples at room temperature for 11 weeks still gave positive results [117].

9. Non-destructive confirmatory identification of body fluids

Table 2 reveals that many of the current methods of body fluid identification use presumptive and destructive chemical tests to identify certain components in each fluid. Looking at Table 3, it is evident that many of the emerging techniques use mRNA methods which are very specific and confirmatory in nature but still destructive to the sample. Unless a DNA profile is simultaneously analyzed as proposed by Alvarez et al. [39], additional sample material will be needed to perform a second DNA test. As previously mentioned, the ability to identify body fluids at a crime scene in a non-destructive manner is imperative in order to preserve the sample and DNA evidence. In addition to being non-destructive, a test that can be performed on site as opposed to being limited to the laboratory is very important because it provides instant feedback. This gives investigators valuable information about the unknown stain almost instantly so they can alter their investigation accordingly. There have been a few methods already mentioned that are non-destructive, but two more in particular show the best ability to confirm the presence of each fluid. These two new procedures involving fluorescence spectroscopy and Raman spectroscopy are still in the experimental stages of development and are not currently being used for forensic testing. The hope is that these techniques will ultimately be accepted by the forensic community and will be able to deliver confirmatory, non-destructive identification of body fluids at the

scene of a crime without needing lengthy laboratory testing. There may be a need for duplicate testing in the laboratory in the beginning stages of the application of these methods to show that the results are reliable and suitable for court testimony, but eventually the new methods should be able to produce acceptable results on site that are automatic and simple for a crime scene investigator or police officer to use.

9.1. Fluorescence spectroscopy

Fluorescence spectroscopy is a sensitive technique that relies on the presence of a fluorophore, which is a chemical group in an analyte that absorbs radiation (normally in the ultraviolet region) and emits light with a specific, longer wavelength. This technique uses no damaging reagents and does not cause contact with the sample, but its non-destructive nature is questionable due to the possibility of photodegradation upon exposure to ultraviolet light. A method to detect saliva using fluorescence spectroscopy has already been discussed [102]. In addition, Powers and Lloyd [16] have demonstrated that ultraviolet fluorescence spectroscopy can be applied to identify multiple body fluids. The ability of any single test to be applied to multiple fluids is a valuable characteristic since it eliminates the guesswork regarding what test to use based on what the unknown fluid might be. Many components found in body fluids can exhibit fluorescence such as nucleic acids, proteins and lipids, metabolite breakdown products in urine, heme in blood, and dried semen as a whole. The individual composition of each of the fluids (see Table 1) is unique, and their chemical fingerprint will correspond to characteristic emission spectra. Fig. 4 shows the emission spectra of semen, blood, saliva, and urine measured with an excitation wavelength of 260 nm [16]. The lowest excitation wavelength used was 260 nm, and it is unclear whether this wavelength will damage DNA evidence similar to the damage observed in another study using a 255 nm excitation [20]. This technique is ideal since it can quickly scan a large region of stained material and is very sensitive. The use of multiple excitation wavelengths and the detection of fluorescence over a wide range of wavelengths allows for the identification of a particular body fluid without the overlap of characteristics [16].

Another independent study introduces a handheld fluorescence instrument that could potentially be used at a crime scene to give instant feedback about the identity of body fluids [118]. The instrument was designed in 2003 to detect microbial contamination.

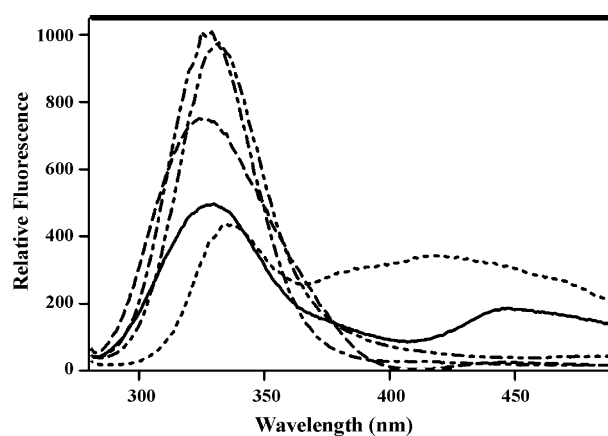


Fig. 4. The fluorescence emission spectra of semen (—), skin oil (.....), blood (- - -), saliva (- · - ·), and urine (— — —) excited at 260 nm. This technique uses no destructive reagents and makes no contact with the sample. The emission lines are broad and the sample must be analyzed at several wavelengths to be distinguishable from other body fluids. No data is available about the possible photodegradation from prolonged exposure to 260-nm UV light. (Adapted from [16] with permission from the author.)

tion, but its high sensitivity would make it ideal for the application of body fluid detection when merged with the right software.

9.2. Raman spectroscopy

Raman spectroscopy is another non-destructive bioanalytical technique which has a great potential for confirmatory identification of body fluids at a crime scene [15]. Raman spectroscopy, when compared to fluorescence spectroscopy, exhibits much higher selectivity and specificity to chemical and biochemical species despite having a lower sensitivity, and it could potentially be useful in resolving mixtures of multiple body fluids. The theory behind Raman spectroscopy is based on the inelastic scattering of low-intensity, non-destructive laser light by a solid, liquid or gas sample. Very little or no sample preparation is needed, and the required amount of material tested with a Raman microscope can be as low as several picograms or femtoliters. A typical Raman spectrum consists of several narrow bands and provides a unique vibrational signature of the material [119]. Unlike IR absorption spectroscopy, another type of vibrational spectroscopy, Raman spectroscopy shows very little interference from water [119], which makes it a great technique for analyzing body fluids and their traces. Typically, nonresonance Raman spectroscopic measurements do not damage the sample. The stain or swab could be tested in the field and still be available for further use in the laboratory for DNA analysis, and that is very important for forensic applications. The design of a portable Raman spectrometer is a reality now [120,121] which would lead to the ability to make identifications in the field. Fig. 5 shows a photo of a portable Raman



Fig. 5. A portable Raman spectrometer. This device has shown great promise in identifying explosives and polymers using an internal library, and it has potential to be applied to body fluid identification as well. Mixture analysis software can identify components of solid/liquid combinations and aqueous solutions, and it weighs less than 4 lbs. (With kind permission from C&EN and Mitch Jacoby.)

instrument designed to measure narcotics, explosives, white powders, chemical weapons, and industrial chemicals found at a crime scene [122]. Again, with the right software and database implementation, this portable device could potentially be used for the detection of body fluids on-field.

Raman microspectroscopy utilizing non-actinic (non-destructive) near-infrared light for excitation has been recently applied for analysis of dry traces of body fluids including blood, semen, saliva, vaginal fluid, and sweat [15]. The reported results are very promising and show that the Raman spectra obtained for each different body fluid are unique (Fig. 6). The distinct composition of each fluid listed in Table 1 makes each fluid unique, and this variation yields a characteristic Raman spectrum. The nature of Raman spectra to contain numerous sharp peaks as opposed to the few and broad peaks found in a fluorescence emission spectrum makes Raman spectroscopy a much more selective and specific method, and it is consequently a potentially more useful technique for forensic purposes. Future work will be necessary to evaluate the potential of Raman spectroscopy for analyzing body fluid mixtures as well as stains on various substrates. Potentially, mixtures of several body fluids can be characterized with Raman spectroscopy using advanced statistical analysis including significant factor analysis (SFA) and alternating least squares (ALS) using software like MATLAB 7.0. Each individual body fluid Raman spectrum contains peaks corresponding to specific biological components, and a mixture of fluids will yield a spectrum which is a combination of all these peaks which can be resolved mathematically into individual basis spectra. In addition, Raman spectroscopy is capable of distinguishing between human and canine semen [15]. The ability to distinguish species of body fluids at a crime scene is also very important, and some of the previously mentioned techniques can perform this task only in a destructive manner. Another study was unable to distinguish cat, dog, and human blood samples using Raman spectroscopy [123]. More studies are needed to determine the capability of this method to identify different species.

10. Future developments

There are numerous techniques, both new and old, which can be applied to the forensic identification of body fluids found

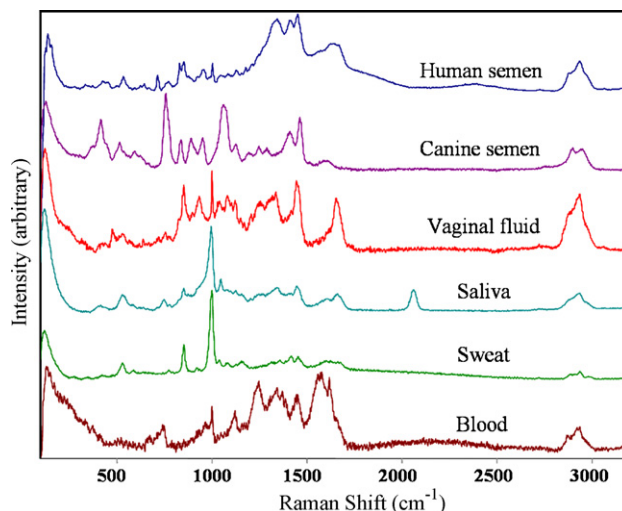


Fig. 6. Raman spectra of human semen, canine semen, vaginal fluid, saliva, sweat, and blood with 785-nm excitation. This is a non-destructive and highly specific technique that yields a unique spectrum for each fluid. The peaks on each spectrum correspond to the characteristic composition of that body fluid, and there is great potential for the analysis for mixtures. (Reprinted from [15] with permission from Elsevier.)

at a crime scene. Some are confirmatory and can conclusively identify the presence of a particular fluid, and others can only be used as screening tests for further testing. Most of these techniques are destructive and will prohibit subsequent DNA analysis on the same sample, and they are also not universal and will only work for one specific body fluid. In addition, some methods can only be performed in a laboratory which can lead to delayed results due to a back-log of cases. The application of non-destructive biospectroscopy to the universal rapid identification of body fluids can greatly assist investigators at the scene of a crime to identify an unknown stain without damaging DNA evidence. We are currently working with experts from the New York State Police Forensic Investigation Center and Northeast Regional Forensic Institute (NERFI) to further develop the new method of Raman spectroscopy to identify body fluids at crime scenes, and these experts are expecting the same outcome that the results need to be simple to obtain and automatic. Raman spectroscopy also shows great potential to determine the identities of multiple body fluids in a mixture, but more research is needed in this area. Additionally, our laboratory is currently working on an advanced statistical technique to distinguish different species of blood including humans, cats, and dogs. This type of analysis can potentially be applied to mathematically separating and identifying different body fluids from the same species as well. With the development of portable fluorescence and Raman devices as well as advanced statistics software, the technique of biospectroscopy has the potential to be very valuable.

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