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Forensic analysis of biological fluid stains on substrates by spectroscopic approaches and chemometrics: A review

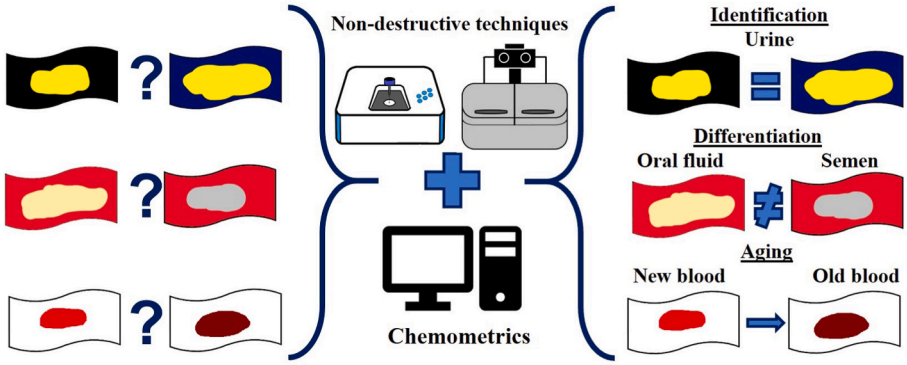
Cristina Cano-Trujillo [a,b], Carmen García-Ruiz [a,b], Fernando E. Ortega-Ojeda [a,b,c], Francesco Romolo [d], Gemma Montalvo [a,b,*]

[a] Universidad de Alcalá, Departamento de Química Analítica, Química Física e Ingeniería Química, Ctra. Madrid-Barcelona km 33,6, 28871, Alcalá de Henares, Madrid, Spain
[b] Universidad de Alcalá, Instituto Universitario de Investigación en Ciencias Policiales, Libreros 27, 28801, Alcalá de Henares, Madrid, Spain
[c] Universidad de Alcalá, Departamento de Ciencias de la Computación, Ctra. Madrid-Barcelona km 33,6, 28871, Alcalá de Henares, Madrid, Spain
[d] Università degli Studi di Bergamo, Dipartimento di Giurisprudenza, Via Moroni 255, 24127, Bergamo, Italy

HIGHLIGHTS

- In Forensics, most of the published studies analyse blood and semen stains.
- Bodily fluid stains are identified and differentiated using spectroscopy.
- Spectroscopy and chemometrics have potential to be applied in real criminal scenes.
- Body fluid stains analysis by vibrational spectroscopy is prevalent in forensics.
- Spectroscopic methods need validation before their use in real casework.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Bodily fluid stains are one of the most relevant evidence that can be found at the crime scene as it provides a wealth of information to the investigators. They help to report on the individuals involved in the crime, to check alibis, or to determine the type of crime that has been committed. They appear as stains in

Abbreviations: 2D CoS, two-dimensional correlation spectroscopy; ANN, artificial neuron network; AFM, atomic force microscopy; ATR FTIR, attenuated total reflectance Fourier transform infrared spectroscopy; EPR, electron paramagnetic resonance; GA, genetic algorithm; HSI-NIR, near infrared hyperspectral imaging; LASSO, least absolute shrinkage and selection operator; LDA, linear discriminant analysis; LIBS, laser-induced breakdown spectroscopy; LSR, least square regression; LVs, latent variables; MCR-ALS, multivariate curve resolution-alternating least squares; MLR, multiple linear regression; NDI, normalized difference image; NIR, near-infrared; OPLS-DA, orthogonal partial least squares-discriminant analysis; PCA, principal component analysis; PCR, principal component regression; PLS-DA, partial least squares-discriminant analysis; PLSR, partial least square regression; PSA, prostate-specific antigen; RF, random forest; rMANOVA, regularized multivariate analysis of variance; RMSE, root mean squared error; SERS, surface enhanced Raman spectroscopy; SVM, support vector machine; SVM-DA, support vector machine-discriminant analysis; SVMR, support vector machine regression; TSD, time since deposition; UV–Vis, ultraviolet–visible; Vis-NIR, visible-near-infrared.

* Corresponding author. Universidad de Alcalá, Departamento de Química Analítica, Química Física e Ingeniería Química, Ctra. Madrid-Barcelona km 33,6, 28871, Alcalá de Henares, Madrid, Spain.
E-mail address: gemma.montalvo@uah.es (G. Montalvo).

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Spectroscopy assisted by multivariate analysis
Forensic direct analysis
Aging

different types of substrates, some of them porous, which can interfere in the analysis. The spectroscopy techniques combined with chemometrics are showing increasing potential for their use in the analysis of such samples due to them being fast, sensitive, and non-destructive.

Findings: This is a comprehensive review of the studies that used different spectroscopic techniques followed by chemometrics for analysing biological fluid stains on several surfaces, and under various conditions. It focuses on the bodily fluid stains and the most suitable spectroscopic techniques to study forensic scientific problems such as the substrate's characteristics, the influence of ambient conditions, the aging process of the bodily fluids, the presence of animal bodily fluids and non-biological fluids (interfering substances), and the bodily fluid mixtures. The most widely used techniques were Raman spectroscopy and attenuated total reflection Fourier transform infrared spectroscopy (ATR FTIR). Nonetheless, other non-destructive techniques have been also used, like near infrared hyperspectral imaging (HSI-NIR) or surface enhanced Raman spectroscopy (SERS), among others. This work provides the criteria for the selection of the most promising non-destructive techniques for the effective *in situ* detection of biological fluid stains at crime scene investigations.

Significance and novelty: The use of the proper spectroscopic and chemometric approaches on the crime scene is expected to improve the support of forensic sciences to criminal investigations. Evidence may be analysed in a non-destructive manner and kept intact for further analysis. They will also speed up forensic investigations by allowing the selection of relevant samples from occupational ones.

1. Introduction

A special area in forensic science is the contribution to investigate crime scenes in an effective and reliable manner and to support criminal investigations. Bodily fluids are one of the most important traces in a crime scene. They allow to connect a person to the scene, the victim/s, or the suspect/s. To do so, it is essential to correctly identify the found bodily fluids, which can lead to the correct DNA extraction and analysis [1–3]. There are some environmental interferences, *i.e.*, substances that could give a false positive on a bodily fluid detection test or substances that resemble bodily fluid stains that must be differentiated from the real bodily fluid stains [4,5]. Moreover, determining the age of a stain let the investigators know when the crime was committed or what fluids were relevant to the case [1–3].

Over the years, many techniques and tests have been developed to correctly identify the bodily fluids. Two kinds of tests are being used for this regard: presumptive and confirmatory tests. For instance, to identify urine samples, urea and creatinine can be targeted using both a presumptive test, like the ELISA immunoassay, or a confirmatory test, such as reverse phase high performance liquid chromatography [6]. The main problem with immunoassays and chromatography is that they are destructive, not allowing the sample to be analysed afterwards with different techniques or to extract DNA. Another problem is that some presumptive tests could give false positives; for example, prostate specific antigen (PSA) test is a test for seminal fluid that gives positive results with other fluids such as urine [7]. Mass spectrometry is also employed to identify bodily fluids in laboratories. It has high sensitivity, good limit of detection (LOD), and specificity. However, it is also a destructive (the stain is not analysed as such but requires processing and extraction of the fluid before the analysis can be carried out) and time-consuming technique [8] that cannot be used *in situ* at the crime scene. The extraction and analysis of mRNA and miRNA can be used to identify stains, being bodily fluid specific, but the technique is also destructive. Although miRNA and DNA can be extracted and analysed simultaneously [2], the crime scene investigation should be conducted as quickly as possible. Instead of that, in recent times, more and more scientists in the scientific community are searching for direct (no need for sample processing), non-destructive, and fast techniques. Consequently, vibrational spectroscopy is being widely studied in forensic sciences as it meets these two requirements.

To the best of our knowledge, there is no review focusing on the study of various bodily fluid stains affecting the analysis on certain surfaces such as glass, fabrics, construction materials, etc. Many factors change in the bodily fluids as they leave the body. Ambient conditions, such as temperature, humidity, or oxygen concentration, are way different from the bodily conditions and they affect the samples' aging, characteristics, or behaviour.

In a crime scene, the fluid evidence is not going to be in an ideal condition. If the fluid is deposited on a porous substrate, it will be absorbed into the substrate while, in non-porous materials, the fluids will stay on the surface. For example, it was proven that depending on the material on which the blood was deposited on, it will be affected differently by the environmental agents [3]. McLaughlin et al. [9] established that the detection of a fluid was different according to the fluid being on the substrate surface or into it. When the fluid is absorbed into the substrate, some light might penetrate the substrate too and cause substrate interference. Another important factor that affects bodily fluids when they exit the body is contamination, either from microorganisms or agents like sand or dust [3,10]. Sometimes, bodily fluids do not appear alone, but in mixtures [3]. These mixtures can be from the same person or not, from the same fluid or two or more different fluids, and they can be from humans or from animals. All these can complicate their detection, characterization, classification/differentiation, and individualization.

The analysis with spectroscopic techniques results in a large amount of data to be processed. Therefore, chemometrics is becoming increasingly relevant in the field of forensic sciences. Chemometrics allows for the pre-processing and analysis of chemical data, such as those obtained from spectroscopic analysis [11]. The objective of pre-processing the data is to eliminate its variability or the physical phenomena that may affect the data, improving the models and the analyses to be carried out later [12]. After pre-processing, the data can be used to generate different types of models depending on the objective of the study. These models can be used to visualize the data, to classify them into different classes, or to compare the results of the analysis with previously obtained information [11].

Up to date, some reviews focused on the spectroscopy study of biological fluids from the point of view of the fluids aging. For instance, Bremmer et al. [1] in 2012, reviewed the techniques employed to study the blood stains aging. More recently, Weber and Lednev [2] wrote an extensive review about blood stain aging and the techniques developed throughout history for their dating purposes, similar to the one published by Das et al. [3] one month later. Other reviews gathered the analytical techniques employed for the analysis of forensic traces, not only for biological samples, but for paint, fibres, or gunshot residues too; they also include the latest technological advances. Considering these previous studies, this work aimed at assembling a comprehensive review of the studies that used different spectroscopic techniques for analysing biological fluid stains on different surfaces, and under different conditions having as first aim a suitable classification of traces for forensic purposes. This document focuses on the bodily fluid stains and the most suitable spectroscopic techniques to study forensic scientific problems such as the substrate's characteristics, the influence of ambient conditions, the bodily fluid mixtures, etc. This work is expected to allow

selecting the most promising non-destructive techniques for the effective *in situ* detection of biological fluid stains at crime scene investigations and to facilitate their technological developments.

2. Methodology

For this review, a total of 63 articles were analysed. These articles were selected after numerous searches in various search engines using different keywords and filters. As we wanted to make a comprehensive review while not missing important articles, we used six search engines: SciFinder^{n}, Scopus, Pubmed, Web of science, Google Scholar, and the *Universidad de Alcalá* Library database. We started by using general terms, like "bloodstains", "forensics" and "spectroscopy". Then, we combined them using Boolean operators to make more complex combinations: (crime AND forensic*) AND (tsd); "bodily fluid" AND stain* AND forensic* AND spectroscopy; forensic* AND semen AND Raman, etc.

Apart from the active searching, some articles were cited in papers we were studying, or they appeared as similar to other ones we had found in the search engines. Finally, to make sure all the articles were analysed, we searched by author of interest.

3. Biological stains on different substrates from a forensic perspective

Bodily fluids are one of the most valuable evidence that can be found at the crime scene. Those traces must be detected on-site as soon as possible before they degrade and loose forensic information important for criminal investigation. In the last years, the forensic laboratories demand the development of spectroscopic techniques for the optimization of the trace, detection, visualization, identification, and interpretation on-site, with a consequent reduction of the time and resources in the laboratory. Although the first spectroscopy articles related to body fluids were published in 1972 [13,14], the interest of the scientific community has risen exponentially (Fig. 1) since 2009 [15], which is undergoing a great development at present and, foreseeably, in the future.

In the reviewed papers, bodily fluid stains were studied employing different analytical technologies. Most of the stain studies were carried out using Raman spectroscopy and attenuated total reflection Fourier transform infrared spectroscopy (ATR FTIR). Nonetheless, other techniques such as atomic force microscopy (AFM) [16], electron paramagnetic resonance (EPR) spectroscopy [17], or digital imaging using a smartphone camera [18] were also used. Fig. 2 summarizes the main information of these papers regarding the bodily fluid studies (Fig. 2A), the substrates where the bodily fluid stains were studied on (Fig. 2B), and their pursued objective (Fig. 2C). Briefly, the main biological fluids studied as stains were blood, semen, vaginal fluid, and oral fluid (Fig. 2A). Moreover, the main substrates comprised aluminium foil, glass, fabrics, and construction materials (Fig. 2B). All the studies focused on the fluid's identification, differentiation, and aging (Fig. 2C).

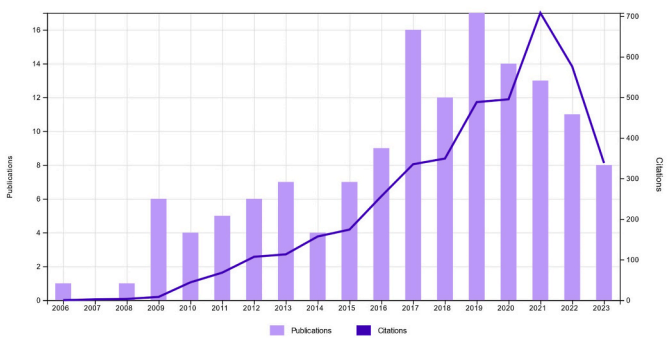

Fig. 1. Citation report created with the Web of science's tool using "body fluid", "spectroscopy", and "forensic" as search terms. This information was obtained on 24 July 2023.

Regarding the purpose of the papers (Fig. 2C), it is important to understand that the "identification" they refer to, was an "analytical identification". However, such identification is different in the forensic science field, which corresponds to the first two steps in the process of interpreting the scientific evidence or trace:

I. Classification. The attribution/assignation of an object or trace to a definite class/group. This is based on a comparison between the unknown specimen to be analysed and one reference sample of known origin that serves to infer if they belong to the same class.
II. Individualization. The possibility to distinguish one object or trace from all considered objects or traces. This is based on a comparison between the specimen of unknown origin and one reference sample of known origin that serves to determine if they come from the same unique source [19,20].

Identification of the fluids is paramount for a crime scene investigation due to many reasons: to determine the kind of crime it has been committed, to correctly extract DNA, to establish a relationship between the people involved as being suspect/s or victim/s [21], etc. Aging studies are scarcer owing to their complexity, but they must be further studied due to the forensic importance of estimating the time when a crime was committed and of helping to establish the veracity of the alibis.

Considering the different biological stains studied, this section discusses, from a forensic perspective, the different biological stains on various substrates, organized by the highest bodily fluid frequency occurrence (Fig. 2A and B).

3.1. Blood

Blood is the most important fluid found at the crime scene. It is composed of red and white blood cells, platelets, and a liquid called plasma. It occurs whenever a wound bleeds, such as in cases of homicides, sexual assault, robbery, hit-and-run, or battery. It is important to identify a sample as blood; if the fluid is from a human or from an animal; if it is peripheral or menstrual; and how long it has been since the fluid was placed on the substrate. Knowing all of this information, the investigator can determine if the blood found at the scene is relevant to the case or not (for example, blood from a cut while preparing food), if it is related to it (for example, in hit-and-run cases, if the blood is human or from an animal), or the time at which the event took place. This would make it possible to verify alibis or the testimonies of those involved and witnesses [2,22].

3.1.1. Blood stains on aluminium foil

As shown in Table 1, in 19 out of the 47 papers where blood was studied, the fluid was deposited on aluminium foil [4,5,9,10,23–37]. Raman and fluorescence spectroscopy were used for the blood stains analysis along with chemometrics models. Many studies used aluminium as a support because it does not generate noise in the measurement of the fluids spectra and does not influence the Raman scattering [35]. The Raman technique is rapid, non-destructive, selective, and sensitive, as it can analyse very small sample amounts [2]. However, it also has limitations, like a high laser power that can damage the sample [3] and background fluorescence that can mask certain Raman signals [38]. Nevertheless, other techniques such as Infrared spectroscopy (IR) or laser-induced breakdown spectroscopy (LIBS) can overcome these problems.

Assessing the analysis of blood stains contaminated with environmental factors, Sikirzhytskaya et al. [10] analysed blood contaminated with sand, dust, and soil and were able to classify and differentiate pure and contaminated blood from the contaminants. Boyd et al. [25] used surface enhanced Raman spectroscopy (SERS) to analyse blood stains

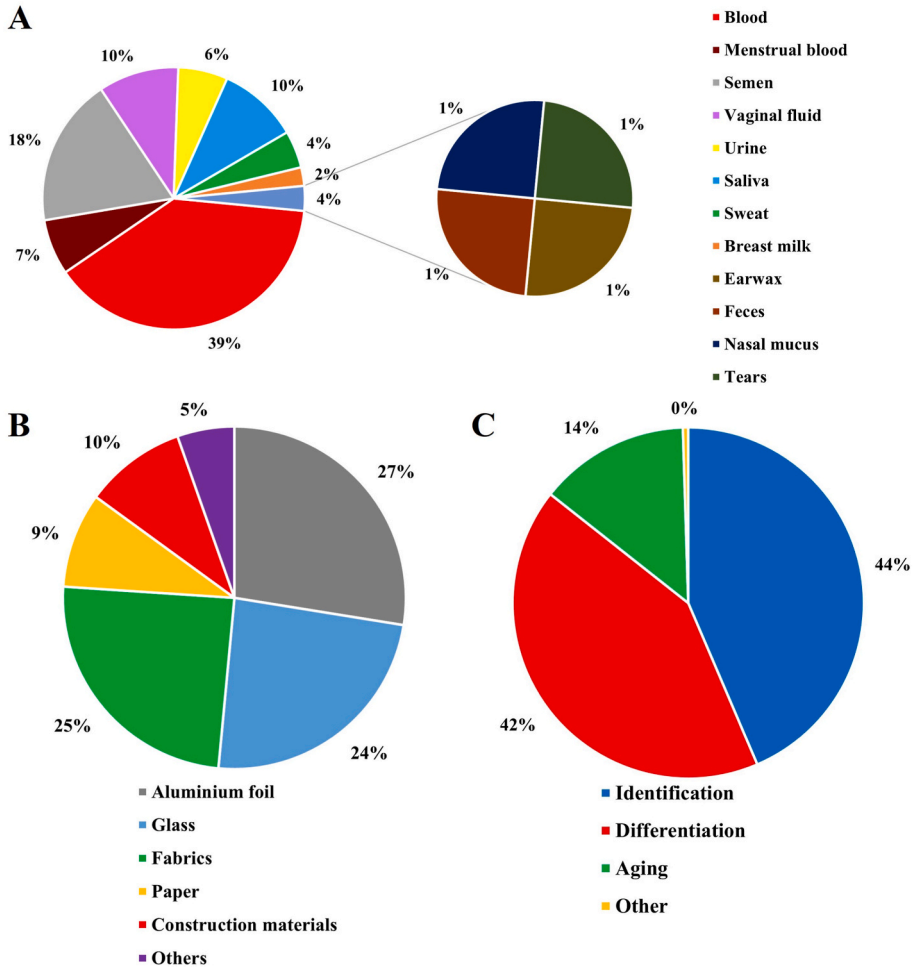

Fig. 2. A) Percentage of the types of bodily fluid stains that are studied in the articles reviewed for this work. B) Percentage of the studied substrates with bodily fluid stains in the reviewed literature. C) Objectives of the reviewed literature in relation to the analysis of bodily fluid stains.

with higher sensitivity and saw that the blood could be detected up to a dilution of 1:100 000 and that the substrate luminescence did not influence the samples measurements (Table 1). SERS is more sensitive than conventional Raman, and the fluorescence can be avoided by swabbing or mixing the blood stain on/with a SERS substrate. However, this Raman spectroscopy mode also implies modifying or destroying the stain sample [25].

Muro et al. [29] created a model that allowed to classify and differentiate distinct bodily fluids. For this purpose, they analysed blood, semen, vaginal fluid, oral fluid, and sweat. Fig. 3 shows the spectra of the bodily fluids after pre-processing and averaging. They built a support vector machine-discriminant analysis (SVM-DA) system with a genetic algorithm (GA), achieving 100% of accuracy for the external predictions. Using a random forest (RF) model, Rosenblatt et al. [5] correctly identified and differentiated environmental interferences for blood stains not only from blood, but also from the other relevant bodily fluids (semen, vaginal fluid, oral fluid, and sweat). The environmental interferences were selected because they resembled a blood stain, or they gave false positives in blood tests. They established a threshold of 70% classification probability (if a sample had <70% the stain was marked as unassigned). This allowed achieving 100% accuracy with 19 bodily fluids and 132 environmental interferences samples [5].

One of the first studies about bodily fluid mixtures was published by Sikirzhytski et al. [24] in 2012, who used Raman spectroscopy for the analysis. The studied blood/semen mixtures are relevant in forensic investigations because they are frequently encountered at sexual assault crime scenes. The studies combined classification and regression support vector machine (SVM) models. They found that mixtures with more than 80% of blood were similar to pure blood, while mixtures with 5% of blood were distinguished from pure semen in the regression SVM (SVMR) model [24]. In 2017, Schlagetter et al. [31] published a paper where they analysed mixtures of bodily fluids by Raman. They identified eight of the nine bodily fluid mixtures without using chemometrics, failing with one blood-semen sample in which the blood was three times that of semen.

Regarding the differentiation of various types of blood stains, Mistek et al. [28] proved that a race differentiation can be achieved by analysing blood stains. 160 spectra were obtained from 20 donors by Raman spectroscopy. These spectra were used to build an SVM-DA model, which had an area under the curve (probability of the model to distinguish among races) of 71% based on a single spectrum or 83% based on a single donor. One year later, Sikirzhytskaya et al. [32] were able to differentiate the donor's gender from the blood samples using an artificial neuron network-genetic algorithm (ANN-GA) model. 59 out of 60 (98%) of the blood samples were correctly classified. The implementation of these two models would quickly provide highly relevant information about the people involved in the crime, both victims and suspects. For that, they had to improve or maintain, respectively, the model's predictive capacity and validation after testing more donors. Nichols and Lednev [36] tested a previous SVM-DA model created by Muro et al. in 2016 [29] that discriminated different body fluids to see if the model was able to correctly classify blood from donors with various diseases. The donors had blood-affecting diseases such as Celiac Disease, Sickle Cell Anaemia, and Type 2 Diabetes. The model achieved 100%

Table 1
Blood stains on aluminium. The references are arranged according to the article's objective (identification, differentiation, and aging). Abbreviations: 2D CoS: two dimensional correlation spectroscopy; ANN: artificial neuron network; GA: genetic algorithm; LASSO: least absolute shrinkage and selection operator; NIR: near-infrared; mW: milliwatts; PCR: principal component regression; PLS-DA: partial least squares-discriminant analysis; PLSR: partial least squares regression; RF: random forest; rMANOVA: regularized multivariate analysis of variance; SERS: surface enhanced Raman spectroscopy; SVM-DA: support vector machine-discriminant analysis; TSD: time since deposition.

Analytical methodology	Main information	Reference
Raman spectroscopy (785 nm, 10 s, 11.5 mW) + chemometrics	Spectroscopic signature of blood.	[23]
Raman spectroscopy (785 nm, 8.2 mW) + PLS-DA	Blood can be identified in highly contaminated samples.	[10]
SERS (532.1 nm, 50 mW)	Identification of blood stains with higher sensitivity.	[25]
Raman micro-spectroscopy (406.7, 457.9, 488, 514.5, 647.1 and 785 nm, 10 s, 0.5–100% power) + chemometrics models	Careful wavelength selection can circumvent the problem of substrate interference.	[9]
Raman spectroscopy (785 nm, 10 s, 110 mW) + SVM-DA	Blood was used to build the model. Identification of seminal fluid and azoospermatic semen as semen.	[33]
NIR Raman micro-spectroscopy (785 nm, 10 s, 10 mW) + PLS-DA + SVM-DA	Menstrual blood identification and differentiation from peripheral blood and vaginal fluid.	[26]
Raman spectroscopy (785 nm, 10–30 s, 4-65-130 mW) + SVM-DA + PLS-DA	Identification and differentiation of bodily fluids.	[29]
Raman spectroscopy (785 nm, 10 s, 40–80 mW) + SVM-DA + RF	Five bodily fluids were identified and differentiated from 24 environmental interferences.	[5]
Raman spectroscopy (785 nm, 10 s, 10 mW) + SVM-DA	Blood-semen mixtures were identified and differentiated.	[24]
Raman spectroscopy (780 nm, 20 s, 10 mW)	Bodily fluids were identified and differentiated from each other (not oral fluid). 8/9 mixture samples were identified.	[31]
Raman spectroscopy (785 nm, 10 s, 130 mW) + RF	Blood was used to build the model. Identification and differentiation of semen from other bodily fluids and environmental interference samples.	[4]
Raman spectroscopy (785 nm, 10 s, 4.0 mW) + SVM-DA	Two races were correctly classified.	[28]
Raman spectroscopy (785 nm, 10 s, 6.0 mW) + SVM + ANN/GA + ANN	Male and female blood stains were properly differentiated.	[32]
Raman spectroscopy (785 nm, 10 s) + SVM-DA + GA	Blood from donors with celiac disease, sickle cell anaemia, and type 2 diabetes is identified as blood.	[36]
Raman spectroscopy (785 nm, 15 s, 1.5 mW) + 2D CoS + PLSR	One month old blood was distinguished.	[27]
Raman spectroscopy (785 nm, 15 s, 1.5 mW) + PLSR (TSD) + PCR (TSD) + SVM-DA (differentiation)	Prediction of the TSD of two-year-old blood stains.	[30]
Raman spectroscopy (785 nm, 15 s, 2 mW) + LASSO	Age determination of blood stains on any surface.	[34]
Raman spectroscopy (785 nm, different power) + rMANOVA	Rotating mode reduced the risk of subsampling errors.	[35]
Fluorescence spectroscopy	Changes in fluorescence intensity of peripheral, menstrual blood, and vaginal fluid stains.	[37]

accuracy as the 208 spectra were correctly classified as blood, without false negatives.

Focusing on the blood stains aging, Doty et al. [27,30] published two relevant articles, one in 2016 [27] and another one in 2017 [30]. They studied how the characteristic blood bands and properties changed over time and showed that the time since the deposition (TSD) can be estimated by Raman spectroscopy. In the paper from 2016, they analysed blood stains up to one week (168 h) and created a partial least squares regression (PLSR) model to estimate the samples TSD. The model had a calibration root mean squared error (RMSE) = 0.05 and $R^2 = 0.99$, and a cross-validation RMSE = 0.13 and $R^2 = 0.97$ [27]. In the study from 2017, they were able to predict TSD up to two years (17 760 h for female samples and 19 008 h for male samples) [30]. Gautam et al. [34] studied the blood stains for two weeks (336 h) to estimate the samples age. They created a least absolute shrinkage and selection operator (LASSO) model and were able to predict the samples TSD on different substrates. Also in 2020, Menżyk et al. [35] analysed blood for three weeks, but their main objective was to avoid the sample degradation and subsampling errors by testing different experimental conditions. For improving the blood sampling, they rotated the blood sample during the acquisition (a kind of sample mapping), and compared it to the single-point procedure, which is the standard procedure. The rotating mode could be the solution for the damage sometimes caused by the laser high power [3], as was previously mentioned.

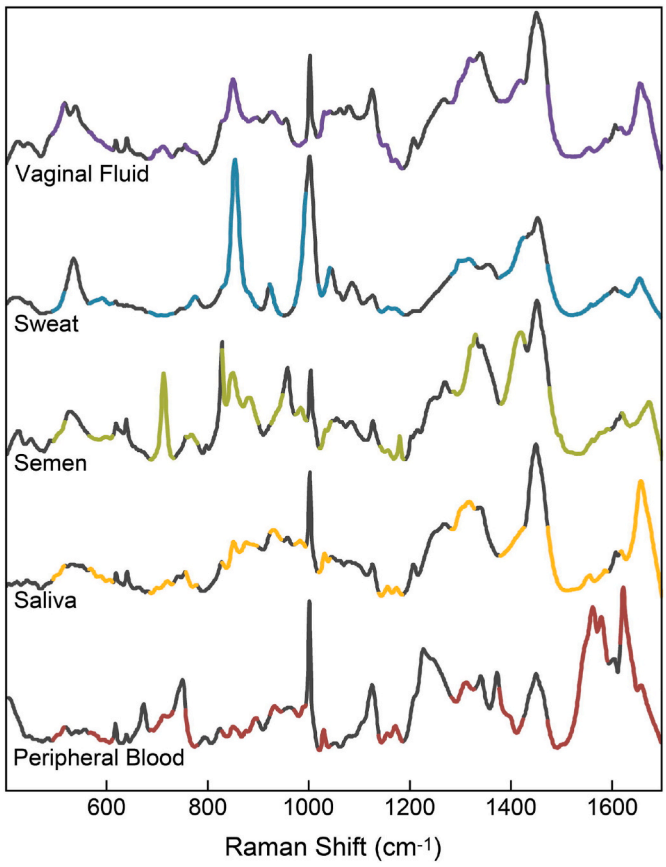

Fig. 3. Pre-processed mean Raman spectra of dry traces of peripheral blood, oral fluid, semen, sweat, and vaginal fluid. In colour appear the most informative variables for classification selected by GA. The black variables are the ones discarded by the model. Reprinted from Ref. [29] C.K. Muro, K.C. Doty, L. de Souza Fernandes, I.K. Lednev, Forensic body fluid identification and differentiation by Raman spectroscopy, Forensic Chem. 1 (2016) 31–38 10.1016/j.forc.2016.06.003, Copyright (2016), with permission from Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Weber et al. [37] wanted to study the blood stains aging with fluorescence spectroscopy. For that purpose, peripheral blood, along with menstrual blood and vaginal fluid, were deposited on aluminium foil and analysed for 24 h. This study is based on a previous one [39] were

they studied the menstrual blood aging (this article is mentioned at the *Menstrual blood* section). Peripheral and menstrual blood have similar fluorescence properties and kinetic changes: A decrease in the tryptophan bands, an increase in the NADH bands, and an intensity decrease in the first 3 h followed by an intensity increase in the flavins bands after those 3 h. Peripheral and menstrual blood differ in the relative intensity of tryptophan and NADH, which can be explained for the presence of vaginal fluid mixed with menstrual blood.

3.1.2. Blood stains on glass

Some authors used more than one substrate (or more than one fluid) in their research. Thus, in 22 articles, blood was analysed on glass using different techniques, not only Raman spectroscopy [9,16,18,21–23,40–54]. As seen in Table 2, FTIR plays a major role as analytical technique in the case of blood stains analysed on glass as substrate. Infrared spectroscopy is a non-destructive technique with limitations in quantitative analysis, especially because it gives a very large water signal, which masks other bands that may be relevant [8].

In 2009, Virkler and Lednev [41] used near-infrared (NIR) Raman spectroscopy and chemometrics to identify the species of blood stains (human, feline, or canine blood). They created a principal component analysis (PCA) model, which had six principal components (PCs). The first three explained 90% of the variation and separated (no overlapping) the three species with 99% confidence intervals each. This method, in which the samples seem to separate depending on the species, may be relevant to certain crimes, such as hit-and-run cases [41]. One year after, in 2010, they published the multidimensional spectroscopic signature of blood to classify it from other bodily fluids and red substances of artificial nature found at a crime scene [23]. However, ATR FTIR is the most widely used technique for analysing blood stain and their mixtures on glass. For these studies, each type of bodily fluid was deposited onto glass and the corresponding residue layers were scrapped and placed directly on the diamond crystal surface of the ATR FTIR for its analysis. Thus, Quinn and Elkins [43], in 2017, tried to differentiate (classify) venous from menstrual blood and other three bodily fluids: semen, oral fluid, and breast milk. They were able to differentiate them by their characteristic bands even on different substrates without using chemometrics. In 2020, Mistek-Morabito and Lednev [22] used ATR FTIR spectroscopy to differentiate (classify) human and animal blood from eight species by a partial least squares-discriminant analysis (PLS-DA) model with a genetic algorithm. A six-latent variable (LVs) model correctly classified 50/50 human samples and 239/240 animal samples from the external validation dataset. Kumar et al. [47] also studied how to differentiate human from animal blood (pig and goat) and the aging process for 175 days. They created curve estimation, multiple linear regression (MLR), and PLSR models to determine the samples TSD. The curve estimation model had an error of ~6 ± 5 days; the MLR model, ~3 ± 1 days, and the PLSR model, ~4 ± 1 days. Wang et al. [51] also used ATR FTIR to analyse human and animal (cattle and sheep) blood on glass, cotton, and napkin. They created PLS-DA models to discriminate the human from the animal blood on cotton and on napkin. Those models proved be also useful to discriminate between cattle and sheep blood on both substrates.

Takamura et al. [45] used ATR FTIR and chemometrics to differentiate between blood, semen, oral fluid, sweat, and urine and to study them for eight months. Fig. 4A-E shows the collected and averaged spectra of the five bodily fluids. They created a model combining PLS-DA (which was able to discriminate the five fresh bodily fluids) (Fig. 4F), linear discriminant analysis (LDA), and Q-statistics test, allowing all the bodily fluid spectra to be correctly classified whilst excluding the non-bodily fluids. To achieve the correct classification of the aged bodily fluids, they combined the PLS-LDA-Q model with hierarchical clustering and a dichotomous classification tree.

Sharma et al. [52] also used ATR FTIR spectroscopy to discriminate blood from menstrual blood, semen, vaginal, and oral fluid, as well as to analyse blood in different conditions. First, blood was analysed after

Table 2
Blood stains on glass. The references are arranged according to the article's objective (identification, differentiation, and aging). Abbreviations: AFM: atomic force microscopy; ATR FTIR: attenuated total reflectance Fourier transform infrared spectroscopy; LDA: linear discriminant analysis; LIBS: laser-induced breakdown spectroscopy; LSR: least squares regression; MCR-ALS: multivariate curve resolution-alternating least squares; MLR: multiple linear regression; OPLS-DA: orthogonal partial least squares-discriminant analysis; PCA: principal component analysis; SVM: support vector machine; SVMR: support vector machine regression. Other abbreviations as in Table 1.

Analytical methodology	Main information	Reference
NIR Raman spectroscopy (785 nm, 10 s, 11.5 mW) + significant factor analysis + PCA	Identification of human, canine, and feline blood.	[41]
Raman spectroscopy (785 nm, 10 s, 11.5 mW) + chemometrics	Spectroscopic signature of blood.	[23]
Raman micro-spectroscopy (406.7, 457.9, 488, 514.5, 647.1 and 785 nm, 10 s, 0.5–100% power) + chemometrics models	Careful wavelength selection can circumvent the problem of substrate interference.	[9]
ATR FTIR spectroscopy + PLS-DA	Identification and differentiation of *ante-mortem* and *post-mortem* blood on different substrates.	[44]
ATR FTIR spectroscopy + PCA + LDA + PLSR	Menstrual blood identification and differentiation from bodily and non-bodily fluids.	[48]
ATR FTIR spectroscopy + PCA + LDA + PLSR	Blood was used to build a model. Semen identification and differentiation from bodily and non-bodily fluids.	[49]
ATR FTIR spectroscopy + PCA + LDA	Blood was mixed with vaginal fluid to make stains. Vaginal fluid identification on different substrates and differentiation from mixtures and non-bodily fluids.	[50]
ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
ATR FTIR spectroscopy + PLS-DA + GA	Differentiation of human blood from animal blood.	[22]
ATR FTIR spectroscopy + PCA + PLS-DA	Human blood stains were differentiated from animal (cattle and sheep) blood stains on three different substrates.	[51]
ATR FTIR spectroscopy + PLS-DA	Differentiation of peripheral and menstrual blood.	[21]
ATR FTIR spectroscopy + OPLS-DA	Differentiation of blood stains from three interferents.	[55]
ATR FTIR spectroscopy + chemometric methods (curve estimation, MLR, PLSR)	Differentiation of human and animal blood, and prediction of the TSD of blood stains.	[47]
ATR FTIR spectroscopy + PLS-DA + LDA + Q-test	Discrimination of bodily fluids and exclusion of non-bodily fluids. Discrimination of aged bodily fluids.	[45]
ATR FTIR spectroscopy + PCA + LDA	Blood stains can be identified on a variety of substrates and after 15 days post-deposition and differentiated from other bodily and non-bodily fluids.	[52]
HSI-NIR + PCA	Blood, urine, and semen stains were detected even in real-life criminal samples.	[53]
NIR spectroscopy + LSR	Loss of water during the first hour of aging. Bands due to proteins appear after 1 h.	[40]
Digital image analysis using a smartphone's camera + RF	Blood stain colour changes with time were quantified. Smartphone camera, temperature, humidity, light exposure, the addition of	[18]

(*continued on next page*)

Table 2 (*continued*)

Analytical methodology	Main information	Reference
	anticoagulant, and substrate colour affected the colour values.	
UV–Vis spectrometry (405 nm) + SVMR + SVM-DA	Prediction of the TSD of the dried bloodspot.	[42]
Atomic force microscopy (AFM)	Increase of the red blood cells' cellular parameters. There are differences among the supports. Force x distance curves to calculate the TSD of blood stains.	[16]
NIR Raman spectroscopy (785 nm, 20 s, 2.0 mW) + MCR-ALS	Prediction of the TSD of blood stains.	[46]
HSI-NIR + PCA + PLS	The TSD of blood, urine, and semen stains could be predicted.	[54]

deposition on 14 substrates to see the effects of substrates. In nine of them (*e.g.*, glass, iron, or wood) the blood was scrapped out of the substrate. In porous substrates, they identified the relevant blood bands, but the blood signal was weaker than in non-porous substrates. They made an extraction of the blood from those porous substrates with double-distilled water. They also tested if after washing the substrates with bleach, blood could still be detected, but they were not able to detect blood. To test the sensitivity limits of the technique, a series of dilutions were prepared, stablishing the limit of dilution at 1:512. Blood was left 15 days as stains on glass and on cotton to make an aging study. They were able to detect the characteristic bands of blood after 15 days on both substrates. Finally, they created a PCA-LDA model to discriminate blood from other biological fluids, which achieved 100% accurate classification of the 130 samples. These results are consistent with the potential of using ATR FTIR as a non-destructive technique for the analysis of biological fluids on different substrates, but the scraping of blood stains from non-porous substrates is a way of sample modification and destruction, which must be avoided when possible in forensic investigations [52].

Malegori et al. [53] used near infrared hyperspectral imaging (HSI-NIR) for the analysis of blood, urine, and semen on different substrates. This technique was assisted by chemometrics. They used PCA to explore the data, which allowed them to select characteristic spectral bands from the bodily fluids and from the substrates they were on. From this information, they were able to calculate a normalized difference image (NDI) by subtracting the reflectance value at the wavelength of the bodily fluid ($R_{\lambda f}$) from the wavelength of the support ($R_{\lambda s}$) and dividing by the reflectance value at the wavelength of the bodily fluid [($R_{\lambda s}$ - $R_{\lambda f}$)/$R_{\lambda f}$]. This allowed them to obtain an image with better contrast between the fluid stain and the substrate as it maximises the difference between the reflectance values. They first analysed the fluids on glass, and they saw that the spectral features of the three fluids allowed for their discrimination. When the samples were analysed on the different fabrics and paper, the fluids were all detected. They analysed real-life stains on fabrics and were able to detect the three fluids even though they were in small quantities (Fig. 5). This research is very relevant because 1) it is the first one that analysed real-life evidence from a crime and 2) they showed that HSI-NIR, which is a non-destructive, fast technique that combines imaging and spectroscopy can detect and analyse bodily fluid stains on different substrates from a real scenario.

In 2009, Botonjic-Sehic et al. [40] studied the blood stains aging on glass and gauze with NIR spectroscopy over one month. A least squares regression (LSR) model was created to predict the TSD, and this model had a standard prediction error of 2.3 h over 590 h.

Agudelo et al. [42] were able to determine, at the same time, the TSD of a sample and the age of its owner. To achieve this, they analysed the alkaline phosphatase by a biocatalytic assay during 48 h. This enzyme was added to the human serum at specific concentrations, deposited on glass, and then analysed at 405 nm. An SVMR model was used to determine the TSD of every sample from each group. In the young people's (0–17 years) group, the model had an $R^2 = 89\%$ and a RMSE of 6.2 h, while in the 18–60 years old group, the R^2 reached 91% and the RMSE of 6.4 h [42]. Considering that, this technique is already destructive, adding enzymes to the human serum does not really mimic the effects of time on the native enzymes present in these samples. Instead, it would be necessary to extract them from already aged whole blood [2].

Cavalcanti and Silva [16] used atomic force microscopy (AFM) to estimate the age of the red blood cells for 28 days. They found that the height, perimeter, area, and volume increased over the 28 days of study. They also learned that there are differences between the samples on different substrates. Furthermore, they observed that the force x distance curves can be used to estimate the TSD. Despite this, the technique needs to be deeply studied to be used for blood dating at crime scenes.

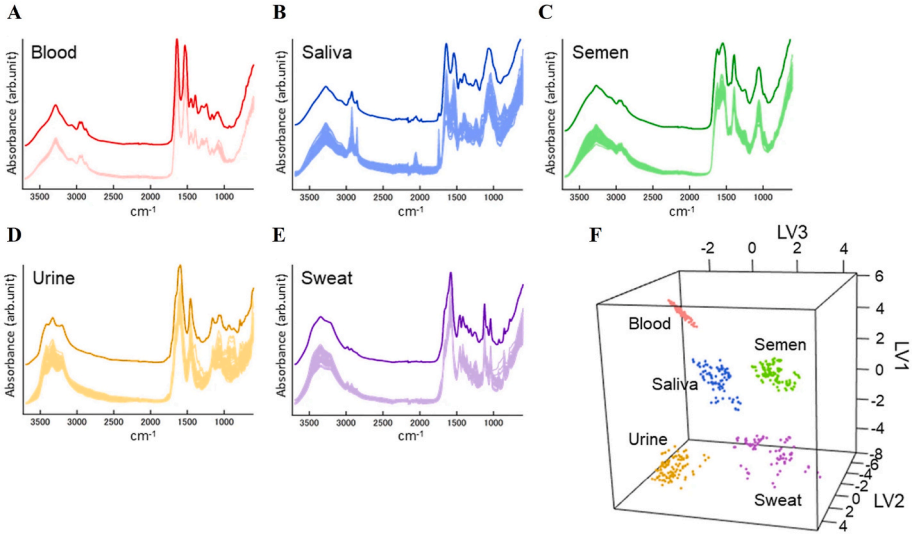

Fig. 4. ATR FTIR spectra of A) peripheral blood, B) oral fluid, C) semen, D) urine, and E) sweat. F) PLS-DA 3D scores dot plot for the ATR FTIR spectra of the five bodily fluids: peripheral blood (pink), oral fluid (blue), semen (light green), urine (yellow), and sweat (light purple). Extracted and modified from Ref. [45] A. Takamura, K. Watanabe, T. Akutsu, T. Ozawa, Soft and Robust Identification of Body Fluid Using Fourier Transform Infrared Spectroscopy and Chemometric Strategies for Forensic Analysis, Sci. Rep. 8 (2018) 8459-10 10.1038/s41598-018-26873-9. Under a Creative Common licence: http://creativecommons.org/licenses/by/4.0/.(For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

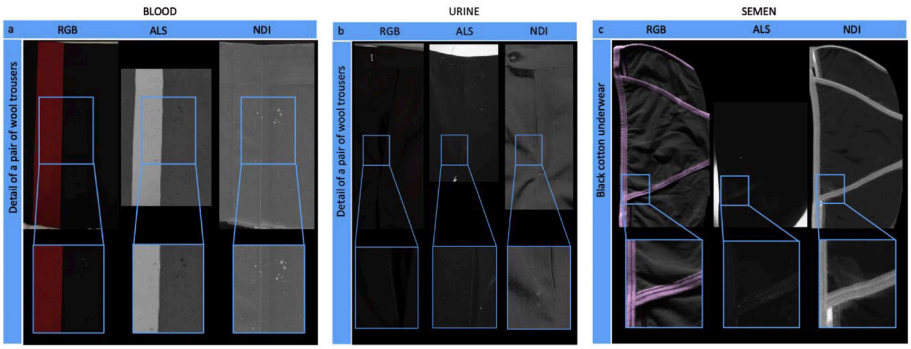

Fig. 5. Unknown samples. Blood and urine stains are found in the wool trousers (leg or belt-buckle position) and the semen stain is in a black underwear. Reprinted from Ref. [53] C. Malegori, E. Alladio, P. Oliveri, C. Manis, M. Vincenti, P. Garofano, F. Barni, A. Berti, Identification of invisible biological traces in forensic evidences by hyperspectral NIR imaging combined with chemometrics, Talanta. 215 (2020) 120911 https://doi.org/10.1016/j.talanta.2020.120911, Copyright (2020), with permission from Elsevier.

This is because the substrate characteristics or the morphology of the cells themselves may render different results. Moreover, the technique must be performed in the laboratory [56]. In the same year, Takamura et al. [46] studied the blood stains aging with Raman spectroscopy and created a kinetic model of the process by spectral deconvolution.

Manis et al. [54] used HSI-NIR to study the aging of blood, semen, and urine on different substrates for 19 days. After the analysis of the fluids on glass, they analysed the fluids on a hydrophilic substrate (cotton) and on a hydrophobic substrate (polyester). The PLS models showed that the age of the stains on the hydrophilic substrate were more predictable than the age of the stains in hydrophobic substrates. Comparing the three fluids, better results were obtained for the prediction of the blood aging than the semen or urine aging. The PLS models for the first 48 h predicted the TSD of the samples worse than the PLS models for the 19 days.

3.1.3. *Blood stains on other substrates*

Table 3 shows the studies regarding blood stains on several substrates including fabrics (cotton, denim, leather, facial tissue, gauze, or polyester), paper, construction materials, such as bricks, tile, ceramic, metal, wood, or other materials. All these substrates can be found on the crime scene.

For classification and individualization purposes, McLaughlin et al. [9] studied how to avoid the influence of the substrate in the analysis of blood stains. For this task, they used Raman micro-spectroscopy with six different excitation conditions whilst the laser power was adjusted between 0.5 and 100%. They also used the following substrates: un-dyed cotton swatches, denim, a pale-yellow glazed bathroom tile, and glass microscope slides. Blood on aluminium was used as reference. They stated that the wavelength selection required to consider the fluid and substrate nature since it can help to avoid the substrate interference. In 2017, Fujihara et al. [57] used portable Raman spectroscopy to classify human and animal blood (cow, horse, sheep, pig, rabbit, chicken, cat, dog, rat, and mouse) deposited on gauze and studied them for three months. The same year, Takamura et al. [44] used ATR FTIR along with chemometrics to differentiate *ante-mortem* and *post-mortem* blood on different substrates. They could differentiate these blood samples by developing PLS-DA models that considered the difference in the lactic acid concentration, which is higher in *post-mortem* blood than in *ante-mortem* blood. With the stains on cotton, polyester, and denim, the model could not identify the blood spectra due to the intensity of the substrate signals, so they subtracted the substrate signals to be able to identify the blood samples [44].

ATR FTIR was also used by Cano-Trujillo et al. [55] to differentiate blood stains from three interferents (chocolate, tomato sauce, and ketchup) on five common substrates (plywood, metal, gauze, denim, and glass). For that purpose, orthogonal partial least squares-discriminant analysis (OPLS-DA) models were created, which were able to discriminate the blood stains from its interferents in every substrate with prediction capacities above 80%. The stains were not scraped or removed from the substrates but measured directly on them. This method and models show high potential for their future application in forensic investigations, as the stains are directly analysed without previous treatment or modification.

To study the aging process, electron paramagnetic resonance (EPR) spectroscopy was used by Fujita et al. [17] to analyse blood samples for 775 days. Light, temperature, and the effect of the substrates was accounted for. The double logarithmic plots of the H (low-spin ferric heme species)/g4 (ferric non-heme iron species) ratio *versus* the days since deposition can be used to estimate the age of a blood stain. Environmental factors, such as light and temperature, affected the results, so it was necessary to create different models under different conditions to correctly estimate the samples age. In 2010 [58] and 2011 [59], Hanson et al. [58,59] published two related articles in which they estimated the TSD of blood stains by correlating it to the shifts in the haemoglobin Soret band. First, they studied blood placed on cotton for one year, investigating the effects of temperature and humidity up to one week, made mock casework samples, extracted proteins, and isolated DNA. The Soret band shifts could be used to estimate the TSD of blood stains because there was a correlation between this shift and the age of the sample [58]. Then, they analysed blood stains on different substrates (denim, paper, polyester, and cotton) at three different temperatures for three months by UV–Vis spectroscopy. They found that the substrate did not influence the Soret band shift, but the temperature did, in the same way they previously reported [59]. Therefore, the main disadvantage of this method is that the Soret band shift was temperature-dependent [2].

Edelman et al. [56] used NIR reflectance spectroscopy to identify and estimate the TSD of blood samples placed on multiple substrates. The blood was deposited on white cotton and studied for 77 days; on blue, red, green, and black cotton, and studied upon 28 days. Also, non-blood samples were deposited on white cotton and analysed after one week. The blood was identified by comparison against three blood stains spectra from a library, and the TSD estimated by PLSR. The presence of water and substrates absorbing light at the NIR wavelengths could complicate the analyses when using reflectance spectroscopy. In addition, NIR bands have characteristics (weak, broad, and overlapping bands) that do not allow determining the composition of the analysed substance.

Thanakiatkrai et al. [18] were the first group to study the blood stains aging using smartphones. They are user-friendly, rapid, and cheap, but the environmental and substrate conditions must be considered as they may hinder the stain analysis [2]. Blood was placed on multiple substrates: filter paper, denim, gypsum board, glass, leather, and white cotton to study the effect of the substrate. Blood stains were

Table 3
Blood stains on different substrates. The references are arranged according to the substrate employed to make the stains. Abbreviations: EPR: electron paramagnetic resonance; UV–Vis: ultraviolet–visible; Vis-NIR: visible-near-infrared. Other abbreviations as in Tables 1 and 2

Substrate		Analytical methodology	Main information	Reference
Fabrics	Cotton Denim	Raman micro-spectroscopy (406.7, 457.9, 488, 514.5, 647.1 and 785 nm, 10 s, 0.5–100% power) + chemometrics models	Careful wavelength selection can circumvent the problem of substrate interference.	[9]
	Cotton Denim Flannel	SERS (532.1 nm, 50 mW)	Identification of blood stains with higher sensitivity.	[25]
	Cotton	ATR FTIR spectroscopy	Identification and characterization of bodily fluids and differentiation from non-bodily fluids.	[62]
	Gauze	Potable Raman spectroscopy (532 nm, 20 s, 18.7 mW) + PCA	Human blood traces were separated from non-human blood traces. Blood identification for up to three months.	[57]
	Cotton	ATR FTIR spectroscopy + PCA + PLS-DA	Human blood stains were differentiated from animal (cattle and sheep) blood stains on three different substrates.	[51]
	Cotton Denim Polyester	Raman spectroscopy (780 nm, 20 s, 10 mW)	Bodily fluids were identified and differentiated from each other (not oral fluid). 8/9 mixture samples were identified.	[31]
	Cotton Denim Polyester	ATR FTIR spectroscopy + PLS-DA	Identification and differentiation of *ante-mortem* and *post-mortem* blood on different substrates.	[44]
	Cotton Gauze	ATR FTIR spectroscopy + OPLS-DA	Differentiation of blood stains from three interferents.	[55]
	Cotton Nylon	ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
	Cotton Denim Polyester	ATR FTIR spectroscopy + PCA + LDA	Blood stains can be identified on a variety of substrates and after 15 days post-deposition and differentiated from other bodily and non-bodily fluids.	[52]
	Cotton Denim	HSI-NIR + PCA	Blood, urine, and semen stains were detected even in real-life criminal samples.	[53]
	Cotton	UV–Vis spectroscopy Immunochromatography	Shifts of the hemoglobin Soret band correlated with the age of the blood stain.	[58]
	Cotton Denim Polyester	UV–Vis spectroscopy Immunochromatography	No significant effects from substrate in the shift. Temperature affected it.	[59]
	Coloured and un-coloured cotton	NIR reflectance spectroscopy for pure blood. Vis-NIR reflectance spectroscopy for blood on cotton. PLSR.	Differentiation of blood and non-blood substances. The TSD of one-month blood stains was estimated.	[56]
	Gauze	EPR spectroscopy	TSD estimation of blood stains for up to one year.	[17]
	Gauze	NIR spectroscopy + LSR	Loss of water during the first hour of aging. Bands due to proteins appeared after 1 h.	[40]
	Cotton Denim Leather Sneakers T-shirt	Digital image analysis using a smartphone's camera + RF	Blood stain colour changes with time were quantified. Smartphone camera, temperature, humidity, light exposure, the addition of anticoagulant, and substrate colour affected the colour values.	[18]
	Facial tissue	Raman spectroscopy (785 nm diode laser, 15 s, 2 mW) + LASSO	Age determination of blood stains on any surface.	[34]
	Cotton Polyester	HSI-NIR + PCA + PLS	The TSD of blood, urine, and semen stains could be predicted.	[54]
Paper		ATR FTIR spectroscopy	Identification and characterization of bodily fluids and differentiation from non-bodily fluids.	[62]
		ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
		ATR FTIR spectroscopy + PCA + PLS-DA	Human blood stains were differentiated from animal (cattle and sheep) blood stains on three different substrates.	[51]
		HSI-NIR + PCA	Blood, urine, and semen stains were detected even in real-life criminal samples.	[53]
		UV–Vis spectroscopy Immunochromatography	No significant effects from substrate in the shift. Temperature affected it.	[59]
		EPR spectroscopy	TSD estimation of blood stains for up to one year.	[17]
		Digital image analysis using a smartphone's camera + RF	Blood stain colour changes with time were quantified. Smartphone camera, temperature, humidity, light exposure, the addition of anticoagulant, and substrate colour affected the colour values.	[18]
		Hyperspectral imaging (230 W) + LDA	Estimation of the TSD of human and animal blood.	[60]
Construction materials	Tile	Raman micro-spectroscopy (406.7, 457.9, 488, 514.5, 647.1 and 785 nm, 10 s, 0.5–100% power) + chemometrics models	Careful wavelength selection can circumvent the problem of substrate interference.	[9]
	Wood	ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
	Metal Plywood	ATR FTIR spectroscopy + OPLS-DA	Differentiation of blood stains from three interferents.	[55]
	Bricks Metal	LIBS (1064 nm) + SVM	Differentiation of animal blood stains from analogue and TSD determination.	[61]

(*continued on next page*)

Table 3 (*continued*)

Substrate		Analytical methodology	Main information	Reference
	Tiles Gypsum board Plastic Slab	Digital image analysis using a smartphone's camera + RF	Blood stain colour changes with time were quantified. Smartphone camera, temperature, humidity, light exposure, the addition of anticoagulant, and substrate colour affected the colour values.	[18]
	Ceramics Metal	Atomic force microscopy (AFM)	Increase of red blood cells' cellular parameters. There are differences among the supports. Force x distance curves to calculate the TSD of blood stains.	[16]
	Linoleum polymer Tile	Raman spectroscopy (785 nm diode laser, 15 s, 2 mW) + LASSO	Age determination of blood stains on any surface.	[34]
Others	Silicon substrates coated with nickel	SERS (532.1 nm, 50 mW)	Identification of blood stains with higher sensitivity.	[25]
	Sample holder	NIR reflectance spectroscopy for pure blood. Vis-NIR reflectance spectroscopy for blood on cotton. PLSR.	Differentiation of blood and non-blood substances. Estimation of the TSD of one-month blood stains.	[56]

studied for six months. The magenta and saturation values correlated the most with TSD ($R^2 = 0.966$ and 0.911, respectively). To estimate the blood stains TSD, linear regression and RF models were created, which had a prediction error of 12%. The model was tested using 40 blood stains on filter paper, achieving 100% of prediction accuracy. However, the prediction accuracy was 83% when using mock casework stains (24 blood stains on paper, slab floor, plastic dish, sneakers, shirt, and handbag). Ambient conditions such as temperature, humidity, and light exposure affected the aging process of the samples. However, they saw that the use of anticoagulants (EDTA and heparin) did not affect the process. In contrast to heavily coloured materials, lightly coloured substrates did not interfere with the colour measurement [18]. HSI was also used for the study of blood stains. This technique combines visual and spectral information and can be used in the UV–Vis or IR regions [8]. Some devices are portable (like handhelds) but can be hampered by the background interference [3]. Li et al. [60] used a portable instrument for measuring visible wavelength HSI of blood stains for 30 days and then constructed a linear regression model to estimate their TSD.

In 2019, Wang et al. [61] used laser-induced breakdown spectroscopy (LIBS) to study the TSD of blood stains. Due to the improvements in the instrument's portability, LIBS is a real-time technique that can now be used *in situ* at the crime scenes. The laser ablates the sample which, on the one hand, allows the substance to be analysed without the effect of the substrate. On the other hand, the laser also destroys a small part of the sample and must therefore be considered a destructive technique. They employed equine blood and placed it on stone bricks, ceramic tiles, H62 copper, and 304 steel plates. To differentiate blood from potential interferents, red and rust red paints were used. The authors built an SVM-DA model to identify the blood, while the TSD of blood stains was estimated by the LIBS spectral line intensity ratios. The SVM-DA model was built with 75 spectra in the training set, while 25 spectra were used to test the model. The correct classification rate was 100%, but the analysis took several hours. RF was applied and the model achieved a correct classification rate of 96% in 20 s. For the TSD, the prediction accuracy was evaluated using intensity ratios for different substrates.

3.2. Menstrual blood

Menstrual blood is a complex biological fluid composed of blood and its characteristic proteins. As seen in Table 4, this fluid mixture has been mainly studied by ATR FTIR on several substrates. Along with semen and vaginal fluid, menstrual blood can be found in cases of sexual assault or rape. It is fundamental to classify and individualize peripheral and menstrual blood to prove that a crime of sexual nature was committed and by whom, especially when there are mixtures of menstrual blood and semen and when the samples are in underwear or intimate areas. Dating the menstrual blood samples can assist in the crime's investigation [21,39].

There were only nine papers analysing menstrual blood stains on different substrates [21,26,37,39,43,48–50,52]. Sikirzhytskaya et al. [26] were able to differentiate the menstrual from peripheral blood using NIR and Raman micro-spectroscopy coupled with chemometrics PLS-DA and SVM-DA modelling. When employing Raman spectra, both models identified the donor with a 100% sensitivity and specificity. Moreover, these two fluids were correctly differentiated from vaginal fluid using the SVM-DA model. Using fluorescence spectroscopy, Wójtowicz et al. [39] studied the aging process of menstrual blood for 9 h. Among the chemical compounds responsible for the fluorescence in the menstrual blood, tryptophan fluorescence decreased, NADH fluorescence increased steadily, and flavins increased their fluorescence from 2 h onwards. This study was followed up with the one conducted by Weber et al. [37] in which they analysed peripheral, menstrual blood, and vaginal fluid by fluorescence spectroscopy to estimate the aging of the fluids. The results obtained in the second study coincided with the results of the previous one.

Sharma et al. [48] used ATR FTIR to identify and differentiate the peripheral and menstrual blood from each other, menstrual blood from vaginal and seminal fluid, and from non-bodily fluid substances. They also studied the effect of the substrate. With the help of chemometrics, they differentiated peripheral and menstrual blood with high accuracy, independently of the substrate (glass, cotton, denim, plastic, wood, or sanitary napkins). They tested PCA, PLSR (in both models, menstrual and peripheral blood were divided in two groups), and LDA models (100% discrimination of menstrual blood, vaginal fluid, and seminal fluid). For the validation test, they used non-bodily fluid substances that could give false positives for menstrual blood. They also created PCA-LDA models that correctly classified all the samples (100% accuracy). Menstrual blood was easily identified on non-porous substrates, while on porous substrates the intensity of the peaks was weaker and some of them were overlapped with the substrate peaks. In 2021, Mistek-Morabito and Lednev [21] used ATR FTIR to distinguish among menstrual or peripheral blood, and vaginal fluid. Those fluids were studied after being scrapped from their corresponding substrates. With PCA, they saw that the vaginal fluid separated in a group from the peripheral and menstrual blood, but the model did not properly separate these two fluids. A PLS-DA model was created to successfully differentiate between both blood samples (99% of correct classification with 3/180 misclassified peripheral blood samples, and 150/150 well classified menstrual blood samples). For the external validation, 80 new spectra from the same donors (but newly measured) and 10 new spectra from new donors, were loaded into the PLS-DA model achieving a correct classification of all those new samples.

3.3. Semen

Semen is commonly found at sexual crime scenes, being its identification especially relevant in cases of sexual assault. Semen stains can give valuable information to prove that the crime was committed, and

Table 4
Menstrual blood stains on different substrates. The references are arranged according to the substrate employed to make the stains. Abbreviations as in Tables 1–3

Substrate		Analytical methodology	Main information	Reference
Aluminum		NIR Raman micro-spectroscopy (785 nm, 10 s, 10 mW) + PLS-DA + SVM-DA	Menstrual blood identification and differentiation from peripheral blood and vaginal fluid.	[26]
		Fluorescence spectroscopy	Aging study: changes in the fluorescent peaks are seen in the first 9 h since deposition.	[39]
		Fluorescence spectroscopy	Aging study: changes in fluorescence intensity of peripheral, menstrual blood, and vaginal fluid stains.	[37]
Glass		ATR FTIR spectroscopy + PCA + LDA + PLSR	Menstrual blood identification and differentiation from bodily and non-bodily fluids.	[48]
		ATR FTIR spectroscopy + PCA + LDA + PLSR	Menstrual blood was used to build a model. Semen identification and differentiation from bodily and non-bodily fluids.	[49]
		ATR FTIR spectroscopy + PCA + LDA	Menstrual blood was mixed with vaginal fluid to make stains. Vaginal fluid identification on different substrates and differentiation from mixtures and non-bodily fluids.	[50]
		ATR FTIR spectroscopy + PLS-DA	Differentiation of the peripheral and menstrual blood.	[21]
		ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
		ATR FTIR spectroscopy + PCA + LDA	Blood stains can be identified on a variety of substrates and after 15 days post-deposition and differentiated from other bodily and non-bodily fluids.	[52]
Fabrics	Cotton Denim Polyester	ATR FTIR spectroscopy + PCA + LDA + PLSR	Menstrual blood identification and differentiation from bodily and non-bodily fluids.	[48]
	Cotton Nylon	ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
Paper		ATR FTIR spectroscopy + PCA + LDA + PLSR	Menstrual blood identification and differentiation from bodily and non-bodily fluids.	[48]
		ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
Construction materials	Plastic Tile (floor) Wood	ATR FTIR spectroscopy + PCA + LDA + PLSR	Menstrual blood identification and differentiation from bodily and non-bodily fluids.	[48]
	Wood	ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
Others	Grass Sanitary napkins	ATR FTIR spectroscopy + PCA + LDA + PLSR	Menstrual blood identification and differentiation from bodily and non-bodily fluids.	[48]

DNA can be extracted to obtain individualization. It is paramount to distinguish semen in mixtures with other fluids, like blood [24,31], to differentiate it from animal semen and other substances [63] and to date the sample [64], as it can be used to link the suspect or victim to the crime.

As with blood, aluminium foil is one of the main substrates used to study semen stains. Table 5 shows the analysis of semen stains on aluminium foil according to a specific objective (identification, differentiation, and aging). Eight papers out of 23 focused on these stains on aluminium foil by Raman spectroscopy [4,5,7,24,29,31,33,65]. Feine et al. [7] tried to classify and individualize semen stains combining the use of a prostate-specific antigen (PSA) test and Raman micro-spectroscopy. They tested normal, diluted, and mixed stains. The PSA test was positive for semen and urine in normal conditions, diluted and mixed, but the Raman spectra allowed for the correct identification of the fluid/s. In 2017, Muro and Lednev [65] differentiated semen samples by the race of the donors with an SVM-DA model. The training model was built with 18 donors which were correctly classified with a 60% threshold. The external validation used seven donors which were also correctly classified. As with blood stains, this model could give rapid information about the people involved in the crime. Fikiet et al. [33] conducted a study in 2019 to see if azoospermatic semen could be identified as semen. Normal semen, sperm, azoospermatic semen, and seminal fluid were analysed (Fig. 6). For the identification model, samples of blood, oral fluid, and sweat were also analysed. The authors built an SVM-DA model capable of differentiating these four bodily fluids (99.6% of externally cross validation accuracy). This model included 38 spectra from azoospermatic semen and 18 spectra from seminal fluid, which allowed their correct classification as semen.

Casey et al. [4] published an article in 2020 describing the use of Raman spectroscopy in the identification of semen and its differentiation from environmental interferences. They also compared the semen spectra to the spectra of other bodily fluids: blood, oral, sweat, and vaginal fluid. They constructed a RF model that allowed the correct separation and classification of the bodily fluids *versus* the environmental interferences with a 100% accuracy.

Semen stains were studied on glass by Raman [66,67], ATR FTIR

Table 5
Semen stains on different substrates. The references are arranged according to the article's objective (identification, differentiation, and aging). Abbreviations: PSA: prostate-specific antigen. Other abbreviations as in Tables 1–3

Substrate		Analytical methodology	Main information	Reference
Aluminum		Raman spectroscopy (785 nm, 10 s, 110 mW) + SVM-DA	Identification of seminal fluid and azoospermatic semen as semen.	[33]
		Raman spectroscopy (785 nm, 10 s, 130 mW) + RF	Identification and differentiation of semen from other bodily fluids and environmental interferences samples.	[4]
		Raman spectroscopy (785 nm, 10/30 s, 4/65/130 mW) + SVM-DA + PLS-DA	Identification and differentiation of bodily fluids.	[29]
		Raman spectroscopy (785 nm, 10 s, 40–80 mW) + SVM-DA + RF	Five bodily fluids were identified and differentiated from 24 environmental interferences.	[5]
		Raman micro-spectroscopy (633 nm, 60 s, 785 nm) + PSA test	Semen identification and differentiation from urine stains.	[7]
		Raman spectroscopy (780 nm, 20 s, 10 mW)	Bodily fluids were identified and differentiated from each other (not oral fluid). 8/9 mixture samples were identified.	[31]
		Raman spectroscopy (785 nm, 10 s, 10 mW) + SVM classification	Blood-semen mixtures could be identified and differentiated.	[24]
		Raman micro-spectroscopy (785 nm, 10 s, 65 mW) + GA + PLS-DA + SVM-DA	Differentiation of the race of the donors.	[65]
Glass		NIR Raman spectroscopy (785 nm, 10 s, 115 mW) + chemometrics	Identification of semen by its spectroscopic signature.	[66]
		Raman spectroscopy (785 nm, 10 s, 10–50% power) + PCA + PLS-DA	Identification of semen on a variety of substrates.	[67]
		ATR FTIR spectroscopy + PCA + LDA + PLSR	Menstrual blood identification and differentiation from bodily and non-bodily fluids.	[48]
		ATR FTIR spectroscopy + PCA + LDA + PLSR	Semen identification and differentiation from bodily and non-bodily fluids.	[49]
		ATR FTIR spectroscopy + PCA + LDA	Semen was mixed with vaginal fluid to make stains. Vaginal fluid identification on different substrates and differentiation from mixtures and non-bodily fluids.	[50]
		ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
		ATR FTIR spectroscopy + PLS-DA + LDA + Q-test	Discrimination of bodily fluids and exclusion of non-bodily fluids. Discrimination of aged bodily fluids.	[45]
		ATR FTIR spectroscopy + PCA + LDA	Blood stains can be identified on a variety of substrates and after 15 days post-deposition and differentiated from other bodily and non-bodily fluids.	[52]
		HSI-NIR + PCA	Blood, urine, and semen stains were detected even in real-life criminal samples.	[53]
		HSI-NIR + PCA + PLS	The TSD of blood, urine, and semen stains could be predicted.	[54]
Fabrics	Cotton 60% cotton/40% polyester Polyester	Raman spectroscopy (785 nm, 10 s, 10–50% power) + PCA + PLS-DA	Identification of semen on a variety of substrates.	[67]
	Cotton	ATR FTIR spectroscopy	Identification and characterization of bodily fluids and differentiation from non-bodily fluids.	[62]
	Cotton Denim	External reflection FTIR spectroscopy	Semen, vaginal fluid, and urine identified and differentiated on coloured and non-coloured fabrics.	[68]
	Cotton	ATR FTIR spectroscopy	Identification and differentiation of semen, vaginal fluid and urine and its mixtures on superabsorbent materials.	[69]
	Cotton Polyester Satin	HSI-NIR + chemometrics (MLR-ALS + PLS-DA + SVM-DA)	Different models as presumptive and confirmatory methods to identify and differentiate semen stains.	[63]
	Cotton Denim Polyester	Raman spectroscopy (780 nm, 20 s, 10 mW)	Bodily fluids were identified and differentiated from each other (not oral fluid). 8/9 mixture samples were identified.	[31]
	Cotton Denim Polyester	ATR FTIR spectroscopy + PCA + LDA + PLSR	Semen identification and differentiation from bodily and non-bodily fluids.	[49]
	Cotton Nylon	ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
	Cotton Denim	HSI-NIR + PCA	Blood, urine, and semen stains were detected even in real-life criminal samples.	[53]
	Cotton Polyester	HSI-NIR + PCA + PLS	The TSD of blood, urine, and semen stains could be predicted.	[54]
Paper		ATR FTIR spectroscopy + PCA + LDA + PLSR	Semen identification and differentiation from bodily and non-bodily fluids.	[49]
		ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
		HSI-NIR + PCA	Blood, urine, and semen stains were detected even in real-life criminal samples.	[53]
		Fluorescence spectroscopy + chemometrics	Age estimation of the semen stains.	[64]
Construction materials	Teflon	HSI-NIR + chemometrics (MLR-ALS + PLS-DA + SVM-DA)	Different models as presumptive and confirmatory methods to identify and differentiate semen stains.	[63]

(*continued on next page*)

Table 5 (*continued*)

Substrate		Analytical methodology	Main information	Reference
	Plastic Tiles Wood	ATR FTIR spectroscopy + PCA + LDA + PLSR	Semen identification and differentiation from bodily and non-bodily fluids.	[49]
	Wood	ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
	Plastic Tile	Fluorescence spectroscopy + chemometrics	Age estimation of the semen stains.	[64]
Others	Pig skin	Raman spectroscopy (785 nm, 10 s, 10–50% power) + PCA + PLS-DA	Identification of semen on a variety of substrates.	[67]
	Sanitary napkins Panty-liners Diapers	ATR FTIR spectroscopy	Identification and differentiation of semen, vaginal fluid and urine and its mixtures on superabsorbent materials.	[69]
	Skin Grass Condoms	ATR FTIR spectroscopy + PCA + LDA + PLSR	Semen identification and differentiation from bodily and non-bodily fluids.	[49]
	Thin layer chromatography plates	Fluorescence spectroscopy + chemometrics	Age estimation of the semen stains.	[64]

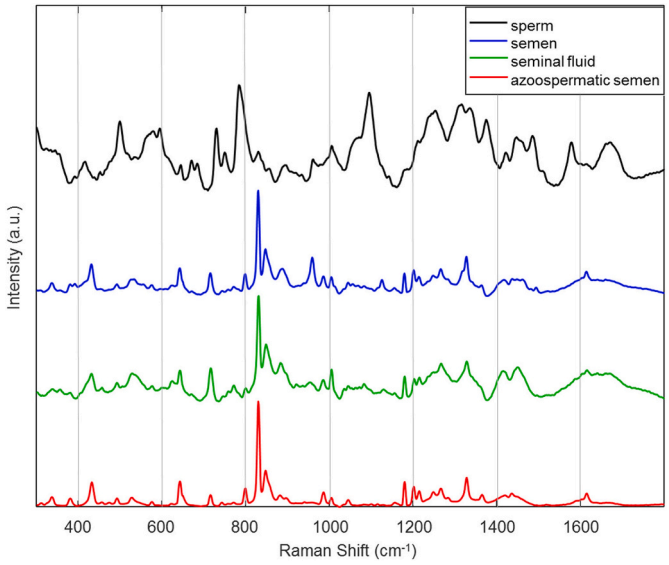

Fig. 6. Average spectrum of semen compared to the average spectra of seminal fluid, sperm, and azoospermatic semen. Reprinted from Ref. [33] M.A. Fikiet, I. K. Lednev, Raman spectroscopic method for semen identification: Azoospermia, Talanta. 194 (2019) 385–389 10.1016/j.talanta.2018.10.034, Copyright (2019), with permission from Elsevier.

spectroscopy [43,45,48–50,52], and HIS-NIR [53,54] (Table 5). Virkler and Lednev [66] published in 2009 the semen multidimensional spectroscopic signature, fitting the semen spectra and not the blood and oral fluid spectra. Therefore, they stated that the combination of the three PCs could be used as a unique spectroscopic signature to identify the presence of semen and distinguish it from other bodily fluids and other substances that can be present at a crime scene. Sharma and Singh [49] used ATR FTIR combined with chemometrics to classify semen from vaginal fluid, other bodily fluids (peripheral blood, menstrual blood, and oral fluid) and non-bodily fluid substances, which can be confused with semen. A PCA model was used to explore the data, and they saw that semen clustered away from vaginal fluid. Combining PCA with LDA, the model had 100% accuracy separating semen from vaginal fluid. Another PCA allowed the authors to see that semen, menstrual blood, peripheral blood, and oral fluid also seem to separate. These fluids were also separated by a PLSR model with 100% accuracy. An LDA model classified the four fluids with 98% of accuracy, while semen and non-semen substances were 100% separated by a PCA and correctly discriminated by an LDA model. Semen spectra characteristic bands could be seen in non-porous substrates in contrast to porous substrates. Semen-vaginal fluid mixtures were analysed but the authors were not able to observe the characteristic peaks of vaginal fluid as seminal fluid dominated the spectra.

Semen stains were also studied on other substrates (Table 5) like fabrics, paper, construction materials, and other [31,43,49,53,54,62–64,67–69]. McLaughlin and Lednev [67] published in 2015 an article where they studied and identified semen stains on glass, pig skin, denim, white cotton swab, light blue dyed 100% polyester, and a white blended fabric (60% cotton/40% polyester). With a PCA model, they separated semen and skin spectra with a 95% confidence interval. For homogeneous (glass, cotton) or moderately heterogeneous interferent substrates, automatic subtractions were used to identify semen characteristics. However, if the substrate was very heterogeneous (polyester, blended fabric), they used PLS modelling and manual subtraction. Semen-vaginal fluid mixtures were studied by Zapata et al. [68] in 2016 by external reflection FTIR spectroscopy. They identified and differentiated semen, vaginal fluid, and urine from each other and from non-bodily fluid substances that could give false positives or that were similar to the studied bodily fluids. Also, semen, vaginal fluid, and urine stains were analysed on white cotton or coloured fabrics. They created a PCA model and saw that the bodily fluids stains separated from each other when deposited on the substrate regardless of whether they were coloured or not. The authors created a Soft Independent Modelling by Class Analogy (SIMCA) model to correctly classify the bodily fluids stains and to exclude non-bodily fluids. In the case of semen/vaginal fluids stain mixtures, they were incorrectly classified as vaginal fluid, while semen was not detected. Gregório et al. [69] used ATR FTIR spectroscopy to identify semen, vaginal fluid, and urine in stains, and their mixtures on superabsorbent pads. Semen and vaginal fluid had similar spectra, while the urine spectrum was well differentiated. When the three fluids were mixed, urine was not detected, and vaginal and semen spectra could not be differentiated. In semen/urine mixtures the last one dominated the spectra, however, chemometrics was not applied. Silva et al. [63] used HSI-NIR and chemometrics to identify semen stains on different substrates such as cotton, polyester, and satin. Moreover, they also individualized human and animal semen (goat and horse semen) and other fluids that could give false positives such as lubricant and breast milk. Achetib et al. [64], in their paper from 2019, were the first to study the TSD of semen samples. To do so, they studied the aging kinetics of semen by fluorescence spectroscopy. Semen was first placed on thin layer chromatography plates and, after that, on four substrates: black and blue plastic, tissue, and white tile and studied for one month. The model was able to predict the age of semen stains with a mean absolute error of 1.7 days.

3.4. Vaginal fluid

Vaginal fluid is a complex fluid whose identification and differentiation from other bodily fluids is essential in sexual assault and rape crimes [70]. Table 6 lists works where vaginal fluid stains where studied. This fluid was mostly analysed with other bodily fluids [4,5,21,26,29,37,48,49,52,62,68,69], being scarce the papers focused on the study of vaginal fluid as the main fluid [50,70]. Raman, ATR FTIR, and fluorescence spectroscopy were the techniques of choice.

In 2011, Sikirzhytskaya et al. [70] published the multidimensional spectroscopic signature of vaginal fluid. The samples were analysed by Raman spectroscopy. The signature fitted well other vaginal fluid samples and did not fit the spectra of blood, semen, and oral fluid. The authors stated that this spectroscopic signature could be fitted to all the dry vaginal fluid samples and helped to differentiate vaginal fluid from other bodily fluids found at crime scenes. Sharma et al. [50] tried to identify and differentiate vaginal fluid on different substrates by ATR FTIR. Moreover, they tried to differentiate this fluid from non-bodily fluid substances along with bodily fluid mixtures. Aging was also studied. Vaginal fluid spectra were clearly observed on non-porous substrates, while some peaks were absent on porous substrates. The authors were not able to detect characteristic peaks of vaginal fluid when it was mixed with seminal fluid, menstrual blood, or peripheral blood. In contrast, the vaginal fluid peaks were observed in all aged samples. PCA showed a separation between vaginal fluid and non-vaginal fluid substances. Additionally, vaginal fluid was successfully differentiated from non-vaginal fluid substances with an LDA model (100% accuracy).

3.5. Urine

Table 7 shows that urine stains have been analysed in a total of nine papers on aluminium foil [6,7,31], on glass [45,53,54], and on fabrics or other substrates [53,54,62,68,69]. Vyas et al. [6] in 2020 published a paper, where they updated the SVM-DA model created by Muro, Doty et al. [29] in 2016 by including the urine spectra on such model. The urine samples were analysed using Raman spectroscopy. The non-negative PCA showed significance donor to donor variation, where four PCs explained 98% of the spectral variance. The previous SVM-DA model was updated with urine spectra. When it was coupled with a genetic algorithm (GA), the model achieved 99% classification accuracy using cross-validation (3549/3570 spectra was correctly classified) and 100% accuracy using external predictions (861/861 spectra) [6].

3.6. Oral fluid

Oral fluid is the combination of saliva (secreted by salivary glands), mucins, mucosal transudate, and gingival fluid. It is a transparent, non-coloured fluid produced inside the mouth. Saliva and oral fluid are not the same [71]. As seen in Table 8, oral fluid stains were analysed using Raman spectroscopy or ATR FTIR spectroscopy on different substrates [4,5,29,31,33,43,45,49,52,62,72–75]. In 2016, Muro et al. [73] created a model that used Raman spectra to differentiate the donor sex of various oral fluid samples. They created a SVM-DA model that correctly classified 86% of the spectra using a calibration set and another 86% of the spectra using an external validation set. At the donor level, using a 67% threshold, the SVM-DA model reached 94% of accuracy (45/48 of the calibration donors) and 92% accuracy using the external validation (11/12 donors). Schlagetter et al. [31] studied oral fluid samples along with blood, semen, and urine by Raman spectroscopy on aluminium foil and on fabrics, but they were not able to identify the oral fluid by its characteristic's peaks. More recently, Al-Hetlani et al. [74] tried to differentiate smokers from non-smokers by analysing their oral fluid samples. The stains were created on glass slides covered with aluminium foil and were analysed with Raman spectroscopy. The spectral data was used to make ANN models coupled with GA. They achieved 100% sensitivity and specificity as they correctly classified all the test samples

Table 6
Vaginal fluid stains on different substrates. The references are arranged according to the substrate employed to make the stains. Abbreviations as in Tables 1–3

Substrate	Analytical methodology	Main information	Reference
Aluminum	Raman spectroscopy (785 nm, 10 s, 115 mW) + chemometrics	Spectroscopic signature of vaginal fluid.	[70]
	NIR Raman micro-spectroscopy (785 nm, 10 s, 10 mW) + PLS-DA + SVM-DA	Menstrual blood identification and differentiation from peripheral blood and vaginal fluid.	[26]
	Raman spectroscopy (785 nm, 10/30 s, 4/65/130 mW) + SVM-DA + PLS-DA	Identification and differentiation of bodily fluids.	[29]
	Raman spectroscopy (785 nm, 10 s, 40–80 mW) + SVM-DA + RF	Five bodily fluids were identified and differentiated from 24 environmental interferences.	[5]
	Raman spectroscopy (785 nm, 10 s, 130 mW) + RF	Vaginal fluid was used to build the model. Identification and differentiation of semen from other bodily fluids and environmental interferences samples.	[4]
	Fluorescence spectroscopy	Changes in fluorescence intensity of peripheral, menstrual blood, and vaginal fluid stains.	[37]
Glass	ATR FTIR spectroscopy + PCA + LDA + PLSR	Menstrual blood identification and differentiation from bodily and non-bodily fluids.	[48]
	ATR FTIR spectroscopy + PCA + LDA + PLSR	Vaginal fluid was mixed with semen to make stains. Semen identification and differentiation from bodily and non-bodily fluids.	[49]
	ATR FTIR spectroscopy + PLS-DA	Differentiation of peripheral and menstrual blood.	[21]
	ATR FTIR spectroscopy + PCA + LDA	Vaginal fluid identification on different substrates and differentiation from mixtures and non-bodily fluids.	[50]
	ATR FTIR spectroscopy + PCA + LDA	Blood stains can be identified on a variety of substrates and after 15 days post-deposition and differentiated from other bodily and non-bodily fluids.	[52]

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Table 6 (*continued*)

Substrate		Analytical methodology	Main information	Reference
Fabrics	Cotton	ATR FTIR spectroscopy	Identification and characterization of bodily fluids and differentiation from non-bodily fluids.	[62]
	Cotton Denim	External reflection FTIR spectroscopy	Semen, vaginal fluid, and urine identified and differentiated on coloured and non-coloured fabrics.	[68]
	Cotton	ATR FTIR spectroscopy	Identification and differentiation of semen, vaginal fluid and urine and its mixtures on superabsorbent materials.	[69]
	Cotton Denim Polyester	ATR FTIR spectroscopy + PCA + LDA	Vaginal fluid identification on different substrates and differentiation from mixtures and non-bodily fluids.	[50]
Paper				
Construction materials	Plastic Tiles Wood			
Others	Sanitary napkins Panty-liners	ATR FTIR spectroscopy	Identification and differentiation of semen, vaginal fluid and urine and its mixtures on superabsorbent materials.	[69]

(5 donors). These models are promising and have great potential for being used by forensic investigators, as they can "learn" and differentiate among samples of the same nature with small differences.

Virkler and Lednev [72] published in 2010 the multidimensional spectroscopic signature of oral fluid. Oral fluid stain samples were analysed by NIR and Raman spectroscopy. The oral fluid signature fitted well the other oral fluid samples but not the blood or semen spectra. In this work, the authors stated that the combination of the three PCs could be used as a unique spectroscopic signature to identify the presence of oral fluid and potentially distinguish it from other bodily fluids and substances of artificial nature found at a crime scene.

Cano-Trujillo et al. [75] analysed human and canine oral fluid stains with ATR FTIR and chemometrics with the aim to identify and discriminate oral fluid stains by their origin. The stains were created in nine different porous substrates: office, kitchen and tissue paper, corduroy, white towel, pink cotton, yellow cotton, denim, and polyester fabrics. Using OPLS-DA models, the oral fluid stains were discriminated by the species of the donor independently of the porous substrate they were deposited on. The study demonstrates the ability of this technique to detect small changes in the same fluid of two different species and to discriminate them by a combination with chemometric models. As different companion animals may be involved in the crime scene, this method has potential to be used in forensic investigations to discriminate human biological fluids from fluids of animal origin.

3.7. Sweat

Sweat samples found in crime scenes can provide very useful information, such as the use of drugs by the suspect or the victim [76]. Only five out of the six papers studying sweat mention the analyses of sweat stains on aluminium foil as the substrate [4,5,29,33,76], while in the remaining paper, the substrate chosen was glass [45]. Moreover, the stains on aluminium foil were analysed by Raman spectroscopy, while

Table 7
Urine stains on different substrates. The references are arranged according to the substrate employed to make the stains. Abbreviations as in Tables 1–3

Substrate		Analytical methodology	Main information	Reference
Aluminum		Raman spectrometry (785 nm, 10 s, 130 mW) + GA + SVM-DA	The bodily fluid classification [29] was updated with the urine spectra.	[6]
		Raman micro-spectroscopy (633 nm 60s/785 nm) + PSA test	Semen identification and differentiation from urine stains.	[7]
		Raman spectroscopy (780 nm, 20 s, 10 mW)	Bodily fluids were identified and differentiated from each other (not oral fluid). 8/9 mixture samples were identified.	[31]
Glass		HSI-NIR + PCA	Blood, urine, and semen stains were detected even in real-life criminal samples.	[53]
		ATR FTIR spectroscopy + PLS-DA + LDA + Q-test	Discrimination of bodily fluids and exclusion of non-bodily fluids. Discrimination of aged bodily fluids.	[45]
		HSI-NIR + PCA + PLS	The TSD of blood, urine, and semen stains could be predicted.	[54]
Fabrics	Cotton	ATR FTIR spectroscopy	Identification and characterization of bodily fluids and differentiation from non-bodily fluids.	[62]
	Cotton Denim	External reflection FTIR spectroscopy	Semen, vaginal fluid, and urine identified and differentiated on coloured and non-coloured fabrics.	[68]
	Cotton	ATR FTIR spectroscopy	Identification and differentiation of semen, vaginal fluid and urine and its mixtures on superabsorbent materials.	[69]
	Cotton Denim Polyester	Raman spectroscopy (780 nm, 20 s, 10 mW)	Bodily fluids were identified and differentiated from each other (not oral fluid). 8/9 mixture samples were identified.	[31]
	Cotton Denim	HSI-NIR + PCA	Blood, urine, and semen stains were detected even in real-life criminal samples.	[53]
	Cotton Polyester	HSI-NIR + PCA + PLS	The TSD of blood, urine, and semen stains could be predicted.	[54]
Paper		HSI-NIR + PCA	Blood, urine, and semen stains were detected even in real-life criminal samples.	[53]
Others	Sanitary napkins Panty-liners Diapers	ATR FTIR spectroscopy	Identification and differentiation of semen, vaginal fluid and urine and its mixtures on superabsorbent materials.	[69]

Table 8
Oral fluid stains on different substrates. The references are arranged according to the substrate employed to make the stains. Abbreviations as in Tables 1–3

Substrate	Analytical methodology	Main information	Reference
Aluminum	Raman spectroscopy (785 nm, 10 s, 110 mW) + SVM-DA	Oral fluid was used to build the SVM-DA model. Identification of seminal fluid and azoospermatic semen as semen.	[33]
	Raman spectroscopy (785 nm, 10/30 s, 4/65/130 mW) + SVM-DA + PLS-DA	Identification and differentiation of bodily fluids.	[29]
	Raman spectroscopy (780 nm, 20 s, 10 mW)	Bodily fluids were identified and differentiated from each other (not oral fluid). 8/9 mixture samples were identified.	[31]
	Raman spectroscopy (785 nm, 30 s) + ANN + GA	Smokers' oral fluid can be differentiated from non-smokers' oral fluid	[74]
	Raman spectroscopy (785 nm, 10 s, 40–80 mW) + SVM-DA + RF	Five bodily fluids were identified and differentiated from 24 environmental interferences.	[5]
	Raman spectroscopy (785 nm, 10 s, 130 mW) + RF	Oral fluid was used to build the model. Identification and differentiation of semen from other bodily fluids and environmental interferences samples.	[4]
	Raman spectroscopy (785 nm, 30 s, 130 mW) + SVM-DA	Identification of the sex of oral fluid donors correctly.	[73]
Glass	NIR Raman spectroscopy (785 nm, 10 s, 115 mW) + chemometrics	Oral fluid spectroscopic signature.	[72]
	ATR FTIR spectroscopy + PCA + LDA + PLSR	Oral fluid was used to build a model. Semen identification and differentiation from bodily and non-bodily fluids.	[49]
	ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
	ATR FTIR spectroscopy + PLS-DA + LDA + Q-test	Discrimination of bodily fluids and exclusion of non-bodily fluids. Discrimination of aged bodily fluids.	[45]
	ATR FTIR spectroscopy + PCA + LDA	Blood stains can be identified on a variety of substrates and after 15 days post-deposition and differentiated from other bodily and non-bodily fluids.	[52]
Fabrics	Cotton Denim Polyester	Raman spectroscopy (780 nm, 20 s, 10 mW)	Bodily fluids were identified and differentiated from each other (not oral fluid). 8/9 mixture samples were identified.	[31]
	Cotton	ATR FTIR spectroscopy	Identification and characterization of bodily fluids and differentiation from non-bodily fluids.	[62]
	Cotton Nylon	ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]
	Corduroy Cotton Denim Polyester	ATR FTIR spectroscopy + OPLS-DA	Discrimination of human and canine oral fluid stains.	[75]
Paper		ATR FTIR spectroscopy	Identification and characterization of bodily fluids and differentiation from non-bodily fluids.	[62]
		ATR FTIR spectroscopy + OPLS-DA	Discrimination of human and canine oral fluid stains.	[75]
Construction materials	Wood	ATR FTIR spectroscopy	Detection and differentiation of venous and menstrual blood, semen, oral fluid, and breast milk.	[43]

the sample on glass was analysed using ATR FTIR. In 2012, Sikirzhytsky et al. [76] published a paper describing the multidimensional spectroscopic signature of sweat to use it to potentially identify unknown samples as sweat.

3.8. Breast milk

Only two of the studies collected in this review analysed breast milk stains [43,63]. It is not a fluid usually encountered on a crime scene, but it could be relevant in a forensic investigation when found. Silva et al. [63] used HSI-NIR to classify and individualize semen stains and other fluids that could give false positives: lubricant and breast milk. The stains were placed on cotton, polyester, and satin. To the best of our knowledge, there is a lack of studies about breast milk identification and its multidimensional signature is not described yet. Quinn and Elkins [43] used ATR FTIR to analyse the breast milk stains on glass and other four substrates: cotton, nylon, wood, and paper. The breast milk was also deposited onto plastic and, after drying it, the corresponding layer was placed directly on the diamond crystal. The authors admitted that their paper was the first report by ATR FTIR spectroscopy for the differentiation of menstrual blood and breast milk from other bodily fluids.

3.9. Other bodily fluid stains

In 2011, Elkins [62] used ATR FTIR to analyse 12 samples of

biological materials: blood, semen, earwax, faeces, fingernails, fingerprints, hair, nasal mucus, vaginal mucus, oral fluid, tears, and urine. These materials were placed directly on the ATR diamond, on cotton and on paper, and then analysed and compared to non-biological substances. Each material had its own characteristic bands, and this could be used to differentiate them from each other. In blood/oral fluid and blood/urine mixtures, bands from both fluids can be seen in the spectrum. The author indicated that her paper was the first report where IR spectroscopy was employed to evaluate tears and nasal mucus [62].

4. Discussion

The first significant outcome of this review is the evidence of the widespread interest in scientific research about biological fluid stains, because they can be essential for the investigation of many crimes. Among all the considered fluids, blood and semen are the most studied ones. Nonetheless, it is imperative to analyse all bodily fluid stains, especially vaginal fluid, urine, oral fluid, or sweat, as it is necessary to classify and individualize them when they are found at a crime scene.

One of the main advantages of spectroscopic techniques is that they can be used at the scene, requiring no sample preparation and very small amounts of sample [3,57]. As Weber and Lednev [2] also indicated, major advances are being made in the analysis of a wide variety of samples of forensic interest with spectroscopic techniques. Such facts are bringing these analytical methods closer to their application in the crime scene investigations.

The bodily fluid stains appear deposited on a variety of substrates that must be taken into consideration when studying the behaviour of the fluids on them [1–3] and when creating models that allow the investigators to classify and individualize them. This was proved by McLaughlin and Lednev [67] who obtained different semen's signals depending on the porosity of the substrate. Sharma et al. [48–50,52] used several types of substrates for the analysis of blood, menstrual blood, semen, and vaginal fluid. They found differences in the intensity and number of the fluid's bands that could be seen depending on the nature of the substrate. Gregório et al. [69] proved that not only the nature of the substrate is important, but also the nature of the fluid, as semen was more retained in the upper layers of the pads when compared to urine. These are some examples of why substrates, apart from aluminium or glass, need to be researched in future experiments, to mimic real life case scenarios, where stains can be found on very different and varied surfaces.

Not only the substrates are important, the ambient conditions the stains are exposed to also need to be considered, because they can modify some of the stains' characteristics after their deposition. Most of the studies kept the samples in laboratory conditions [4,6,26,27,30,74]; however, these conditions do not represent the conditions in which samples are found in a crime scene. Not only temperature and humidity must be considered. Other aspects, as indicated by Sikirzhytskaya et al. [10], can generate changes in the fluid stains that prevent their correct identification by traditional or spectroscopic techniques. Such factor can be contamination with sand, dust or soil, and the incidence of sunlight or microbial contamination [3]. Hanson et al. [58,59] proved that the aging of blood stains depends on the temperature and humidity conditions, and that the process could be influenced by the exposure to direct sunlight. Thanakiatkrai et al. [18] also observed that the blood's colour changes were influenced by the temperature, the humidity, and the exposure to sunlight. Models that showed promising results with samples in controlled substrates and conditions should be extended and validated with samples that could be encountered in real-life situations. In this way, once the methodology has been optimized, those models could be applied on criminal investigations.

Another aspect to contemplate, as also recognized by Zapata [68], is the number and characteristics of the fluid donors. Although, in general, the number of biological sample donors in the articles is low, the methods developed by the authors can accurately identify the bodily fluid samples. In addition, in most articles, the fluids are purchased or donated by healthy individuals. It is possible that the characteristics of the fluids may change depending on the presence or absence of a disease, diet, exercise, habits such as smoking, alcoholism or drug use, etc [2,3]. Nichols and Lednev [36] analysed blood stains from different subjects with blood-affecting diseases and found that the model previously created by Muro et al. [29] for the discrimination of biological fluids was able to classify these samples as normal blood. On the other hand, Al-Hetlani et al. [74] discriminated oral fluid stains from smoking versus non-smoking donors combining Raman spectroscopy and ANN models. The ability to distinguish the donors' sex [73] or race [28,65] was already proved, so all these differences that can be detected using spectroscopy with chemometrics should be taken into account when creating models and validating them for their use in real criminal investigations. In addition, determining the origin of the sample as human or animal may also be relevant depending on the context. This is important because companion animals such as dogs or cats are found in a multitude of scenarios and could be involved in the crime scene. The first species differentiation study was carried out by Virkler and Lednev [41] in 2009, in which they used Raman spectroscopy coupled with chemometrics for the analysis of blood samples. In 2017, Fujihara et al. [57] used portable Raman spectroscopy to differentiate human and non-human blood. Mistek-Morabito and Lednev [22], Kumar et al. [47], and Wang et al. [51] used ATR FTIR coupled with different chemometric models to analyse and discriminate human and animal blood. From a forensic point of view, these differences should be studied in all biological fluids, not just the most common ones, in addition to potential interfering substances. Because of this, in 2023 Cano-Trujillo et al. [75] analysed human and canine oral fluid stains with ATR FTIR and chemometrics.

Some of the already studied techniques and models still need to be further tested and properly integrated in forensic procedures to provide reliable results useful for forensic investigators and acceptable by the judicial system as evidence. One example is the Muro et al. [29] method that used Raman spectroscopy coupled with a SVM-DA model which was able to classify and predict samples from five bodily fluids (peripheral blood, semen, oral fluid, sweat, and vaginal fluid) with 100% accuracy. This model was updated with urine samples by Vyas et al. [6] while maintaining the prediction accuracy in 100%. Another model that could be used in real cases could be Rosenblatt et al.'s [5] model, which combines Raman spectroscopy with RF models and correctly differentiated biological samples from interfering substances. Takamura et al. [45] developed a model that combined ATR FTIR spectroscopy with a PLS-DA model, a dichotomous classification tree, and Q-statistics and was able to correctly discriminate bodily fluids (fresh or aged) from non-bodily fluids.

Most of the reviewed articles present proof-of-concept studies that are not followed up and need to be validated using a larger number and diversity of donors, as well as using real samples that can be analysed in collaboration with forensic investigators. A good example would be the work done by Malegori et al. [53] who used real samples to validate their proposed method for the detection and identification of fluid stains on different tissues using HSI-NIR. The challenge is the implementation of those models on-site for forensic investigations. Portable devices, like the one used by Fujihara et al. [57] (Raman spectroscopy) or by Li et al. [60] (HSI-NIR) should also be deeply studied because of their advantages to be used at crime scenes for *in situ* analysis. This would provide faster information to the analyst, who may not be an expert in the field, which in turn, would support timely criminal investigation.

Few researchers are developing novel techniques and models to process biological samples at crime scenes such as smartphones to identify and date blood samples [18]. However, due to the potential of the vibrational spectroscopy, most of the researchers are using Raman spectroscopy, where Lednev's group stands out, which established the multispectral signature of blood, semen, vaginal fluid, oral fluid, and sweat [23,66,70,72,76], as well as other methods for the identification,

differentiation, or dating of biological fluids. The other techniques of choice are FTIR [21,22,43–45,47–52,55,62,69,75] and IR approaches combining spectroscopy and imagining HIS-NIR [53,54,60,63] for this purpose.

5. Conclusions and future perspectives

This review showed the advantages of combining spectroscopic analyses with chemometric approaches to differentiate, identify, and/or date bodily fluid stains. At the same time, they also help to find subtle characteristics that cannot be found with other traditional methods.

Despite its advantages, there are still many aspects to be studied. Before their application in real case scenarios, the methods here discussed need further development. Most of the studies used bodily fluids donated by adult healthy donors. Bodily fluids from donors with different types of characteristics, conditions, diseases, disorders, or habits (*e.g.*, age, sex, race, diabetes, diet, smoking, alcoholism, etc.) need to be considered. Also, the number of donors is usually low, which is not representative of the whole population. Therefore, a key issue is the preparation of representative samples which can be used as legitimate evidence of a crime.

Blood and semen are the most studied bodily fluid stains; thus, it is imperative to continue and expand the analysis of other relevant bodily fluid stains in forensics, such as those from urine, oral fluid, or vaginal fluid that can be commonly found at crime scenes. In addition to their identification, it is very important to be able to obtain knowledge on the effect of time, human intervention (*e.g.,* cleaning), context, transference, persistence of the stains, etc. In the case of blood stains, there are few studies that have focused on researching the TSD as main objective. Related to the context, temperature and humidity, the incidence of sunlight, or the microbial contamination, may influence the degradation of the bodily fluid stains. Most of the papers found present proof-of-concept studies, which need to be followed-up for their future implementation.

Portable devices allow to analyse the samples *in situ* at the crime scene, but a deep study need to be carried out as the conditions may differ from the table-top instruments. Many on-site approaches listed in this review deserve a special interest for their use on-site, allowing collection of traces to be further characterized in laboratories with complementary techniques traditionally used in proteomics such as mass spectrometry. In this sense, the development of on-site fast and automated techniques will increase the knowledge (including spectral databases) and will strongly support the methodologies already used by Law Enforcement Agencies in caseworks.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Fernando E. Ortega-Ojeda's. He is a researcher in the fields of chemistry, spectroscopy, and physical chemistry properties of forensic (police and military) and non-forensic products and substances. His research includes R&D, data analytics, and mining regarding physical chemical analysis (meat and soap), chemical and non-chemical analysis, big data, Chemometrics (Multivariate Data Analysis), Spectral analysis (Multi- and Hyperspectral Data and Image), and Bio-photon emission detection. He also program MATLAB algorithms and apps for data analysis, artificial intelligence, object recognition, machine learning, and matching of spectroscopic and non-spectroscopic data, etc.

He has a large experience as teacher at university level (Food Science and Biotechnology, Maths, Statistics, and IT), and supervising research projects. He does have some international experience (*e.g.*, Sweden, Germany, and Finland) working for several important research institutions and industries (*e.g.*, AstraZeneca, Nestlé R&D, Tetrapak, and Nabisco). He has a short experience while trying to solve specific production problems (Bayer, EMSUR Macdonell, and Maxam).His great work flexibility and self-directed learning abilities help him to speak five languages (Spanish, English, Swedish, some Portuguese and Norwegian), thus, gaining excellent communications skills. He uses several types of scientific software for data analytics, mining, and big data (MS Power Business Intelligence, SQL Server + Business Intelligence, The Unscrambler, SIMCA, MATLAB, SPSS Data Modeler, Orange Biolab, etc.), and general science computing (Origin Pro, SPSS, Statgraphics, Smartdraw, EndNote, ChemBioOffice, etc.), database software, XAMPP, virtual environments, cloud storage, translation, office suites, paper handling, graphics and multimedia editing, Internet searching, and many more general, IT and scientific resources. He is an advanced Windows administrator, with a basic experience in Ubuntu Linux and Mac OS environments.

Cristina Cano-Trujillo's. She graduated from the University of Alcalá in 2019 obtaining her degree in Sanitary Biology. In 2020, she got her master's degree in Biochemistry, Molecular Biology, and Biomedicine from the Complutense University of Madrid. She started her PhD in Forensic Sciences from University of Alcalá in March 2021, as a member of the Research in Chemical and Forensic Sciences group (CINQUIFOR) and the University Institute for Research in Police Sciences (IUICP). She obtained a "Formación de Personal Investigador" predoctoral grant from the University of Alcalá. Her thesis is part of the European project Real-tIme on-site forenSic tracE qualificatioN project (RISEN). She teaches Chemistry for the Biology degree and Principles of Physics and Biophysics for the Pharmacy degree, both at the University of Alcalá. She also participates in different dissemination activities, such as the Researchers' Night or the Week of Science. She also has participated in different national and international congresses, as well as speaker in conferences and courses.

Francesco Romolo's. Forensic scientist with more than twenty-five years of experience in crime scene investigation and chemical analysis related to major criminal cases in Belgium, Italy, Switzerland, United Kingdom.

Court reporting forensic scientist on several forensic subjects, including arsons, documents, drugs of abuse, explosions, explosives, gunshot residue (GSR) with more than twenty-five years of experience.

Principal Investigator carrying out research projects about security and forensic science funded by national or international institutions, including International Atomic Energy Agency (IAEA), European Union, NATO-Russia cooperation.

Providing adult professional education seminars for judges, lawyers, specialists of armed forces, police forces and fire brigades.

Subcontractor for international organisation about subjects such as regulations in security and ISO 17025.

Gemma Montalvo's. Her career has been mainly developed at the University of Alcalá (UAH), where she graduated (1993) and obtained the title of European Doctor in Chemical Sciences (1999). She has been Associate Professor (1999), Full Professor (2005), and Full Professor of Physical Chemistry since 2001. Her postdoctoral stay was at the Department of Physical Chemistry of the University of Lund (Sweden).

In 2011, she joined the Research in Chemical and Forensic Sciences group (CINQUIFOR) and the University Institute for Research in Police Sciences (IUICP), initiating the research line on drugs of abuse, and participating in the already established research on explosives and incendiary devices, in the development of methodologies for vibrational spectroscopic techniques: Raman and Fourier Transform Infrared spectroscopy.

She is co-author of international publications, most of them Q1; she participates with presentations in national/international conferences; and she is or has been a collaborator in multidisciplinary research projects of diverse funding, some of them European: one on identification of explosives and explosive residues used in improvised explosive devices, another of the Marie Curie Initial Training Networks programme in which she has been coordinator for the UAH (of which she is a member of the steering committee and was responsible for scientific dissemination), and the RISEN project, in which she represents and coordinates the UAH participation among other European institutions. She is also a reviewer of manuscripts in scientific journals indexed in JCR.

She has participated in several teaching innovation projects at the UAH. She is the coordinator of the first year of the Pharmacy degree and of several subjects. She is currently a member of the IUICP Council, of the Pharmacy Faculty Board and of the Teaching Commission of the Pharmacy degree (UAH), and of the editorial board of the journal Alimentos, Ciencia e Ingeniería (indexed in Latindex). She has been a member of two scientific committees (VIII and IX Ibero-American Congress of Science Education) and of tribunals for teaching posts, doctoral theses and final degree and master's degree projects.

Since 2014 she is coordinator of the Framework Agreement for Scientific and Technological Cooperation between the UAH and the UPTC.

Carmen García-Ruiz's. She graduated from the University of Jaén in 1997 and obtained her PhD in Chemical Sciences at the University of Alcalá in 2001. In 2002, she did a postdoctoral stay at the Free University of Amsterdam with a "Marie Curie" grant. In 2004, she returned to Spain, to the University of Alcalá, through the "Ramón y Cajal" programme. In 2009, she obtained a position as full professor at the University of Alcalá. During this first research stage, she developed methodologies for the determination of proteins, organic compounds, and chiral compounds with separation and spectrometric techniques.

She began her research career in Forensic Sciences with the creation of the University Institute for Research in Police Sciences (IUICP) in 2007. The first national project (2009–2012) she led was aimed at investigating the detection of nitrocellulose in explosives such as dynamite. Subsequently, she coordinated two European projects in a crime-fighting call (2012–2015) focused on increasing the analytical tools of police forces to fight improvised explosive devices (IEDs). In 2017, she opened an initiative aimed at raising financial support through crowdfunding to include research focused on detecting personal states and how they are affected under chemical submission. She contributes to training in Chemical Sciences through her involvement in the bachelor's degree in chemistry and the master's degree in research in science, and in Forensic Sciences through her participation in the official bachelor's, master's and doctorate degrees in Forensic Sciences at the University of Alcalá. As a researcher committed to transfer and dissemination, she transmits the knowledge achieved to forensic institutions and companies and disseminates it through radio, press (local, national, and international publications) and television. In addition, she participates in scientific dissemination activities such as the European Researchers' Night and Science Week. In November 2020, her first book "Introduction to Forensic Chemistry" was published. In it, she describes all the analytical methods available to Forensic Sciences to deal with the determination, identification, quantification, and comparison of substances of multiple natures in the contexts of fires, explosions, inks and documents, trace materials (fibres, paints, glass, etc.), drugs and psychoactive substances, their metabolites, pharmaceuticals, gunshot residues, and many more. The book reflects on the importance of the transdisciplinarity implicit in Forensic Chemistry and describes two current topics where it is necessary to go beyond the examination, analysis, and interpretation of analytical results.