Identification of invisible biological traces in forensic evidences by hyperspectral NIR imaging combined with chemometrics

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Abstract

The importance of detecting minute biological traces in forensic evidences feeds the continuous interest towards the development of new dedicated technologies both sensitive and reliable. The present study describes the opportunity to combine chemical properties derived from NIR signals with spatial features typical of RGB images by means of hyperspectral imaging (HSI). An analytical procedure based on HSI data collection and their multivariate processing followed by normalization of difference images (NDI) is proposed as a screening method to highlight otherwise invisible traces of biological fluids on different supports in view of their collection for DNA analysis. The pattern features identified inside the NDI provided insight into the nature of the biological trace, on the basis of the wavelength at which the stain is highlighted and irrespective of the support on which the stain is deposited. In particular, the procedure allowed to detect and distinguish traces (i.e., 10 and 20 µL volumes) of dehydrated blood, urine, and semen on glass, paper, cotton, denim and polyblend fabric. Beside the simulated specimen used to develop and test the protocol, its robustness was demonstrated also on real and unknown validation samples, confirming its feasibility in some real case studies. An interesting evolution of the proposed strategy is to lay the scientific foundations for the development of a handheld device directly applicable in field.

Keywords: hyperspectral imaging; near infrared spectroscopy, chemometrics; biological fluid; forensics analysis.

1. Introduction

The collection of biological fluids on the crime scene on which to conduct DNA profiling often represents the most important evidence for forensic investigators [1]. On the crime scene, a wide variety of biological fluids can be present on an even wider assortment of substrates, differing in terms of material and/or color. Frequently, these traces are not revealed by direct macroscopic examination (e.g., by naked eye) being either invisible or latent, possibly because of their small amount. In this context, the identification of latent biological traces can be remarkably complex, making a highlighting method necessary for their magnification [2]. This method has to be ideally non-destructive in order not to invalidate subsequent DNA analysis as well as bloodstain pattern evaluation [3].

To support the forensic analyst in locating and visualizing the questioned stain on an item forensic alternate light sources (ALS) are usually employed. ALS usage is based upon the low intensity intrinsic fluorescence emission (auto-fluorescence) that is observed when several biological compounds contained in biological fluids, such as prosthetic groups (e.g. heme group), aromatic amino acids, vitamins, etc., are enlightened with specific wavelengths (UV in the range 350-380 nm, visible in the range 400-460 nm, IR in the range 800-900 nm) [1].

In addition, simple optical tests that involves the use of forensic light sources (FLS) are available for forensic investigation [4]. This approach is based on a filtration system in which the samples are illuminated at specific wavelengths in a dark environment, allowing to distinguish biological fluids from the substrate by exploiting light interaction phenomena. Differently from ALS, FLS requires the use of reagents for maximizing differences between the fluid and the support, in terms of interaction with the radiation.

An important limitation of this strategy is given by the potential damage that ultraviolet wavelengths may cause to the DNA evidence in the forensic sample; this DNA damage can inhibit its typing during the polymerase chain reaction (PCR-STR)[5]. Other common tests for blood traces identification are the tetramethylbenzidine test [6] and the luminol test (LT) [7,8]. Particularly, the luminol test is based on a chemiluminescent light emission (about 425 nm) due to an oxidation-reduction reaction in which the blood hematin (heme prosthetic group containing ferric rather than ferrous iron, with the O_2 being replaced by the hydroxyl group) acts as a catalyst (peroxidase-like activity). Although LT is often defined as a non-destructive technique, which allows subsequent DNA analysis, recent studies have shown that under specific conditions it may undermine the subsequent DNA typing [9]. Another important drawback is that presumptive tests have been reported to possibly damage and degrade the DNA content. Several studies [10–12] have highlighted this effect when DNA analyses are performed several days after the application of presumptive tests reagents such as, for instance, luminol. This important drawback may produce misleading interpretation, especially when DNA mixtures, minor contributors, or touch DNA samples are under examination. Moreover, LT is designed to detect invisible blood traces, but it is not useful for other types of biological fluids. Both FLS and LT exhibit low selectivity, as they are susceptible to producing false positive results.

Infrared (IR) and Raman spectroscopies in the reflection mode are likely to represent particularly suitable techniques for the detection of biological traces directly in the original context and without damaging the DNA content. In fact, they are considered non-invasive and non-destructive,

allowing the examination of samples in their integrity and avoiding any pre-treatment step [13]. However, Raman spectroscopy has its own restrictions, limiting its applicability in the forensic field, since it cannot be used to test dark color substrates nor matrices undergoing internal fluorescence [14]. On the other hand, IR spectroscopy has already been used in forensic sciences for blood identification, both in the near-IR (NIR) and in the mid-IR (MIR) regions [15]. For the identification of other biological fluids, interesting results were obtained through the attenuated total reflectance Fourier-transform infrared (ATR FTIR) technique [16], although in this study the location of biological traces on white cotton was known *a priori*. The direct in-field applicability of FTIR spectra acquisition requires the previous identification of the specimen area where the biological trace is present or is likely to be present. However, this inherent drawback can be successfully addressed by introducing the spatial information during data acquisition, which is nowadays achievable by means of hyperspectral imaging (HSI). HSI is an innovative analytical strategy that combines conventional spectroscopy with imaging, thus obtaining both spatial and spectral information on the whole inspected area of the sample [3]. Until now, the HSI approach in the NIR region (HSI-NIR) has been applied for the detection of blood traces [15], while other biological fluids were not considered.

Aim of the present study is to develop an analytical protocol based on HSI-NIR data to be used for screening various common substrates with the aim of highlighting and identifying biological traces in the potential context of a crime scene. A key point of the present approach is a straightforward and reliable data processing for the extraction of the chemical information embodied within the spectra. In more detail, for an efficient visualization of stains, gray-scale images where obtained by codifying, for each pixel, the ratio between NIR reflectance at two wavelengths – one characteristic for the biological fluid and one characteristic for the background [17]. With a unique, rapid, and completely non-invasive procedure, it is possible to obtain prompt information on both the location and nature of the biological fluid encountered, while a considerable efficiency in terms of time/costs reduction is ensured and the occurrence of false positive errors is avoided. The acquisition of a NIR spectrum for each pixel allows to evaluate its chemical composition as a function of the variations observed at the spectral level, and its enactment into the two-dimensional space makes it possible to localize the characterizing compounds [18]. The analytical protocol was validated on unknown real and proficiency testing samples, in order to test its feasibility in real casework studies.

2. Materials and Methods

The experimental work was organized in three subsequent steps:

- ✓ evaluation of the characteristic NIR absorptions of each biological fluid;
- ✓ preparation of simulated samples by staining different substrates and materials with various biological fluids;
- ✓ validation of the proposed approach on several unknown samples.

Three biological fluids were investigated in the present study, among the ones more frequently encountered on the crime scene: blood, urine and semen. These fluids were provided by *Centro*

Regionale Antidoping e di Tossicologia "A. Bertinaria", Laboratorio di Genetica Forense (Orbassano, TO - Italy) after collection from healthy volunteers.
These biological fluids were deposited on four different substrates: white paper, dark cotton, white cotton and denim. All supports were obtained from commercially available materials, cut into individual squares (of about 5 cm side size) to simulate different forensic evidences. The same fluids were also deposited on a glass surface and analyzed (microscope slides).

2.1 Biological fluids on glass specimens

A drop of each biological fluid was placed onto a glass microscope slide and the HS image was collected after dehydration at room temperature for three days, with the aim of obtaining the characteristic NIR signal for blood, urine and semen. Glass was chosen as a blank support for its substantial transparency in the NIR region, permitting to collect the spectral signatures of the sole dehydrated fluid. A Spectralon® disk was used as the background, obtaining an acquisition in the transflectance mode.

2.2 Simulated samples: biological fluids on fabric specimens

The simulated samples were prepared by adding small volumes of each biological fluid (10 µL and 20 µL) on each support. Then, the samples were allowed to dry for one day at room temperature. To better mimic real operating conditions, samples were subsequently stored in paper bags for three days before the analysis.

2.3 Unknown samples: biological fluids on fabric swatches

The validation step of the proposed procedure was implemented by blindly analyzing samples whose nature and location of the biological fluid stain was unknown to the analysts. Three samples were considered, one for each biological fluid under study:

a. Black cotton underwear, provided by *Centro Regionale Antidoping e di Tossicologia "A. Bertinaria"* (Orbassano, TO, Italy), in the context of CTS circuit (Collaborative Testing Services) utilized for ISO 17025 proficiency tests (http://cts-forensics.com/);
b. Swatch of a wool trousers in the belt-buckle position;
c. Swatch of a wool trousers leg.

Samples b and c were taken from the same pair of trousers provided by *Reparto Carabinieri Investigazioni Scientifiche – RaCIS* (Rome, Italy) and collected from the crime scene of a murder; further details cannot be given for confidentiality issues.

2.4 Specimen examination by forensic alternate light sources (ALS)

Real specimen were initially examined to detect biological fluids (as in real casework) using a Crime-lite® ML2 ALS device (Foster + Freeman Ltd., Evesham, UK), a multi-wavelength lamp involving 8 high intensity white LEDs plus up to 4 additional narrowband LEDs including: UV, Violet, Blue, Blue/Green, Green, Orange & Infrared – wavelengths [19]. Proper bandpass viewing filters were applied depending on the wavelengths selected on the instrument to verify the presence of different body fluids.

In particular:

a. dried blood traces were checked for by irradiating the specimen with IR light in the 800-900 nm range;
b. dried urine traces were tested by UV light in the 350-380 nm range;
c. dried seminal fluid traces were tested by visible light in the 420-470 nm range.

2.5 Hyperspectral imaging

For all types of specimens (glass microscope slides, simulated fabric supports and unknown samples), HSI-NIR data were acquired by a push-broom system composed by a SWIR3 hyperspectral camera working in the 1000–2500 nm spectral range, at 5.6 nm resolution (Specim Ltd, Finland). The instrumental setting is characterized by three halogen lamps (35 W, 430 lm, 2900 K, each) as illumination sources and a horizontal line scanner (40 × 20 cm moving stage) on which samples are laid down. The system is controlled by the Lumo Scanner v. 2.6 software (Specim Ltd, Finland). Prior to each measurement, dark (closed shutter) and white (99% reflectance Spectralon® rod) images were automatically recorded and stored, and were used to compute the spectral reflectance value (R) for each pixel and wavelength. For the acquisition, the scan parameters were set as follow: frame rate equal to 50.00 Hz and exposure time equal to 9.00 ms; manual focus was tuned before the scan. The collected image data are organized in a three-dimensional data matrix, often called hypercube or, alternatively, spectral cube. The first two dimensions of the 3D array are vertical and horizontal spatial coordinates (in pixels), while the third dimension represents the wavelengths (spectral dimension).

2.6 Multivariate data processing

Due to the complexity of the information embodied within HSI-NIR data, a chemometric approach is required to identify patterns on the samples surface and, simultaneously, understand importance and inter-correlation of the spectral variables. According to the organization of the experimental part, data processing was performed in three steps. First of all, images of the dehydrated biological fluids dropped on glass were analyzed: principal component analysis (PCA) was performed for exploratory purposes [17]. NIR spectra were pre-processed by means of the standard normal variate transform (SNV), together with a Savitzky–Golay smoothing (15-datapoint window size, third-degree polynomial). From PCA outcomes allowing joint interpretation of loadings and spectral profiles of the stains, it had been possible to highlight the most informative wavelengths of the systems, yielding a characteristic spectral band for each biological fluid. Moreover, a spectral band characterizing each support was identified by analyzing the spectra collected in the clean areas. In order to make the procedure more robust with respect to small wavelength fluctuations, the average intensity value of a band composed by six contiguous wavelengths (instead of a single wavelength) for each fluid was taken into account. The selection of a reduced number of variables able to resume the information of the HSI-NIR images, allowed to propose a straightforward data processing for highlighting the stain and minimizing the background contribute; this approach is based on the calculation of a normalized difference image (NDI):

$$NDI = \frac{R_{\lambda s} - R_{\lambda f}}{R_{\lambda f}}$$

where $R_{\lambda s}$ is the reflectance value at the wavelength of the support and $R_{\lambda f}$ is the average reflectance value at the wavelength characteristic for each single dehydrated biological fluid. In this way, a monolayer grayscale image is obtained, which maximizes the difference between the stain and support, where the range is scaled between its maximum (forced to 255, white) and minimum (forced to 0, black) reflectance values. According to this representation, the stains are represented with light grey pixels standing out from a darker background.

3. Results and Discussion

3.1 Biological fluids on glass specimens

The evaluation of the absorption patterns of dehydrated fluids on glass represented the first crucial experimental step to gain in-depth understanding of the chemical information embodied within HSI-NIR data, including the selection of the most informative wavelengths for the subsequent data processing. It was performed by combining the spectral profiles interpretation with the outcomes of PCA. Figure 1 shows the results of data processing performed on a matrix containing 25 spectra for each stain, chosen randomly inside the stains, and the mean spectra of each support, calculated from 100 random spectra of non-contaminated areas. As shown in Figure 1a, the spectral features of each dehydrated fluid are characteristic and differ substantially from each other and the supports in many regions along the NIR spectrum. On the other hand, an interesting similarity is noticeable among the different supports, due to the common chemical nature, relative to cellulose matrix. The similar pattern for the various substrates is confirmed by the score plot (Figure 1b), in which an individual group of points (corresponding to pixels in the hyperspectral image), ascribable to all the non-contaminated supports, is well defined at negative values of PC1 scores (50.28% of explained variance) and positive values of PC2 scores (37.36% of explained variance). In the same orthogonal space, explaining about 90% of the total variance, clear separation among the various stains is evident, with blood at positive score values of PC1, and urine and semen at negative values for both PC1 and PC2. Better separation is observed in the score plot between the substrate and both blood and urine than between the substrate and semen; likewise higher contrast is expected for the first two matrices in the real casework. The marked differentiation in the PC scores observed in Figure 1 can be attributed to the fact that the spectral signatures of the dehydrated fluids were recorded on a support transparent in the NIR region and compared with the signatures of clean fabric supports. A less evident separation is obtained when spectra of biological fluid deposited on a fabric tissue are processed by PCA together with the ones of the same tissue without stains (*data not shown*). The position of the data-points for substrates and biological traces in the score plot, combined with the loadings distribution along the wavelength scale (Figure 1c), allowed to make an effective selection of the most characteristic wavelengths for each system under study.

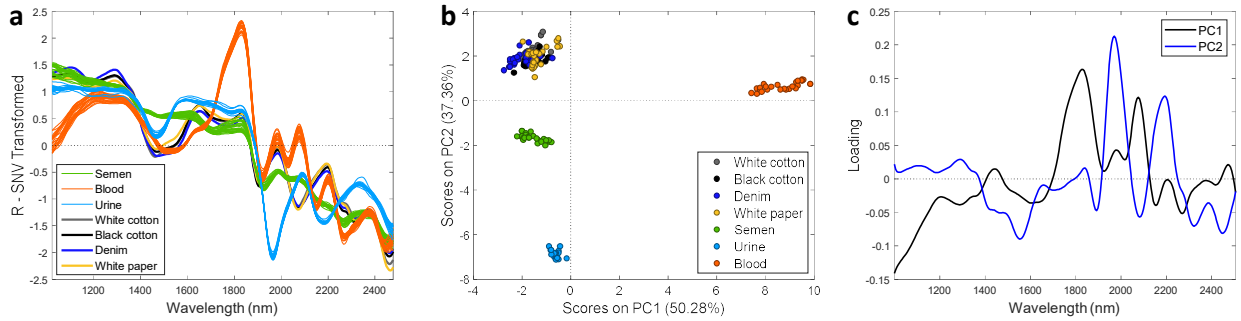

Figure 1: Biological fluids on glass specimens. a) spectral profile of fluids and supports; b) score plot; c) loading plot.

In Table 1, the four selected wavelengths are reported, together with the wavelength typical for wool (keratin), subsequently used in real sample interpretation (see Section 3.8). The absorption band at 1865 nm corresponding to the support is relative to the overtone of OH stretching, due to the presence of cellulose in both cotton fabrics and paper. For dehydrated biological fluids, a single wavelength was not adequately representative of their inter-individual variability. Therefore, a range of six wavelengths was chosen and the mean of the related reflectance values was calculated. For urine, the most informative band was centered at 1950 nm, due to the absorption of amines and urea. Semen and blood spectra were characterized by similar features corresponding to the absorption of protein amide bonds. The absorption was weaker in semen and stronger in blood at the two characteristic wavelengths of 2150 nm and 2280 nm, respectively. Notably, 1950 nm corresponds to a positive PC2 value in Figure 1c, while 2280 nm to a negative PC1 value, since the reflectance is high when the concentration of the characteristic feature (urea and amide bonds, respectively) is low [20].

Table 1: Band attribution for fluids and support in the NIR region.

Variable numbers	**Central wavelength (nm)**	**Biological trace**	**Band attribution** (Jerry Workman Jr. 2012; [22])
155	1865	Cellulose support (background)	Overtone of O-H stretching
183	2020	Wool support (background)	Combination band of N-H bending second overtone + C=O stretching / N-H in plane bending / C-N stretching of keratin.
170 (168:173)	1950	Urine	Combination band of N-H bending and stretching (amines and urea).
207 (205:210)	2150	Semen	Second overtone of $CONH_2$ stretching and bending (amide bands) of α-helix from peptide structures (weaker).
230 (228:233)	2280	Blood	Second overtone of $CONH_2$ stretching and bending (amide bands) of α-helix from peptide structures (stronger).

3.2 Simulated samples: biological fluids on fabric specimens

The analysis of the simulated samples proved crucial for understanding the influence of the supports on the stain absorption during HSI-NIR acquisition. The NDI calculation made it possible to distinguish the chemical information relative to the dehydrated biological fluids with respect to the support, allowing to detect the stain independently from the type of material on which it was deposited. In Figure 2, a portfolio resuming the NDI for all the simulated samples is presented. In detail, the images allow to identify the stain for dehydrated blood (Figure 2a), urine (Figure 2b) and semen (Figure 2c) at both deposition volumes (10 µL and 20 µL) on black cotton, denim, white cotton, and white paper. As expected, a smaller volume deposition created a less intense stain with respect to the larger one. Especially for semen, the stain from the 10 µL drop deposition was detected with some difficulty, in particular on black cotton and denim. Nevertheless, the HSI-NIR proved capable to detect each simulated evidence on all the substrates considered, in spite of the scarce deposition volumes involved.

To investigate selectivity of the selected spectral bands, hyperspectral images of each biological stain were processed by NDI computation using the spectral bands chosen for the other biological fluids. As an example, the case of urine stain images, processed using spectral bands of semen, blood and urine is shown in Figure 1S (Supplementary Material). As it can be seen, the band actually selected for urine provides the most clear identification of stains, with the highest contrast in the related NDI image (Figure 1S.b), while NDI images obtained with the other bands do not reveal the stains or provide a less evident identification (Figures 1S.a and 1S.c).

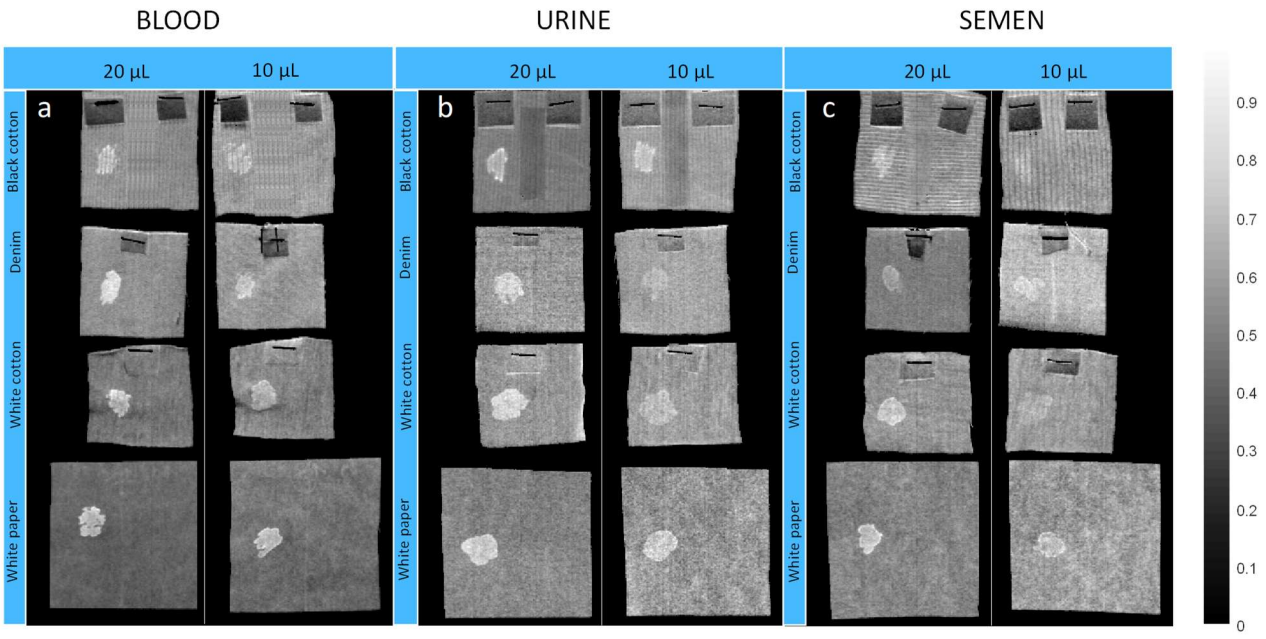

Figure 2: Portfolio of all the simulated samples – three biological fluids on four supports at two deposition volumes.

3.3 Unknown samples: biological fluids on fabric swatches

For the validation step, several unknown samples provided by *Reparto Carabinieri Investigazioni Scientifiche (RaCIS)* of Rome were analyzed by HSI-NIR and processed by the calculation of NDI. These samples were deliberately chosen because they carried only a low amount of biological fluid or small stains, so that the robustness of the proposed approach could be deeply tested.

Consequently, it is expected to observe a lower contrast of the stains to be revealed with respect to the simulated samples discussed in the previous paragraph. All the possible NDI were computed and a hypothesis about the nature of the fluid was proposed, according to the wavelength at which the stain was detected. Among these samples, two exemplifying cases are presented, one for blood and one for urine stains, both on wool trousers. A third unknown specimen of cotton underwear arising from the CTS circuit for quality control and inter-laboratory comparison is also presented, which turned out to be contaminated by semen. Notably, for the first and second cases, the specific NIR profile collected from the uncontaminated wool support suggested us to use the characteristic wavelength of 2020 nm for the computation of the NDI, in order to maximize the difference between the clean support and the stain.

In Figure 3, the comparison between RGB images, ALS images and NDI of the three selected samples is shown, with focus on the area in which the stains were detected. In Figure 3a, tiny drops of blood are visible in the RGB image on both the black wool and the red stripe of the trousers. Thanks to ASL images, the drops were clearly located and visualized by increasing the contrast with the textile support. Even better evidence of the presence and location of these droplets is provided by the black-and-white NDI image. In contrast, no stains of urine are detectable in the RGB and ASL images of the black wool trousers, whereas the presence of four urine droplets is clearly detectable in the corresponding NDI image (Figure 3b).

The case reported in Figure 3c is even more striking: the RGB image does not exhibit any visible trace, while a faint shape is barely visible with the use of visible light in the 420-470 nm range (ASL image). Conversely, a neat stain of semen is visible in the NDI image on the upper-right part of the slip near the rubber-band. In this case, it can be assumed that had traditional operations of blind sampling being used, they would probably have failed to collect the biological sample, precluding the subsequent DNA analysis.

From examination of these real casework specimens, it clearly appears that NDI images processed from hyperspectral acquisitions proved capable of revealing the presence of biological traces in all three circumstances, even when RGB and ALS images failed. It can be noticed that high total reflection effects may occasionally generate bright areas in the processed images (Figure 3), possibly interpreted as positive traces in the early stage of investigation. However, these patterns can be easily identified as false positive due to the peculiar shape of the corresponding areas – which follows the fabric folds – and because they disappear when either the incidence angle of the irradiation source is changed or the folds are flattened.

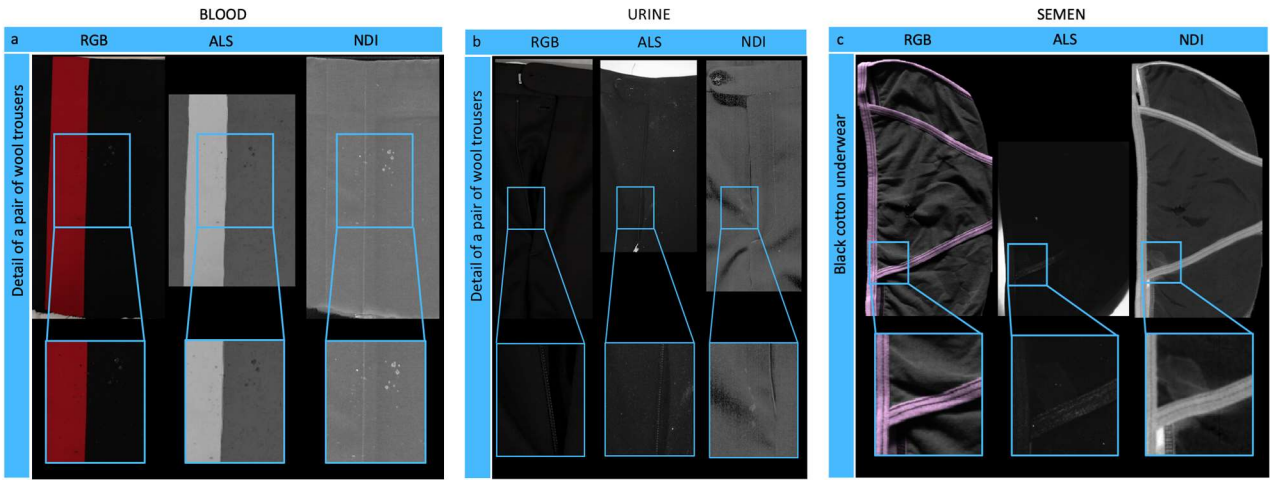

Figure 3: Portfolio of all the unknown samples – one evidence for each biological fluid.

4. Conclusions

The recent advancements in DNA extraction and amplification allow to obtain extensive DNA profiling from extremely small amounts of biological material. Whenever the location of the biological material on the forensic evidence is easily deducible (for example, on a cigarette butt or on a cup), it can commonly be recovered without actually seeing it. However, in most cases of forensic interest, the extreme sensitivity of DNA analysis cannot be exploited if the biological trace is not clearly localized. The present study offers a suitable strategy to detect faint or even invisible biological traces on forensic specimen, taking advantage of the combined spatial and spectral information provided by hyperspectral imaging and data mining. Moreover, the characteristic wavelength at which the stain is highlighted in the NDI allows to identify its nature. While most of the experiments were conducted on cloth substrates of natural origin (cotton, wool), it is conceivable that other background materials (polymeric, inorganic, *etc*.) may offer identical opportunities.

Several investigation steps have still to be accomplished before the presented procedure can be proposed for wide acceptance and applicability, including the verification of the aging effect of the biological traces on different substrates and their image persistence as long as the crime scene inspection is delayed with respect to offense time. Another important improvement to be possibly implemented is to transfer the present technology into a handheld device directly applicable in field. This development would allow to scan the surfaces and substrates not suitable to be transferred to the laboratory (floor, wall, furniture). Most importantly, a portable device would directly and immediately identify and characterize an unknown spot during the inspection of the crime scene, avoiding to collect useless specimen and to wait for authorizations and laboratory results. The strategy proposed in the present study lay the scientific foundations for such a likely feasible development.

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