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Overcoming obstacles: Analysis of blood and semen stains washed with different chemicals with ATR-FTIR

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ABSTRACT

Introduction: Blood and semen stains are the most common biological stains encountered at crime scenes. The washing of biological stains is a common application that perpetrators use to spoil the crime scene. With a structured experiment approach, this study aims to investigate the effects of washing with various chemicals on the ATR-FTIR detection of blood and semen stains on cotton.

Materials and methods: On cotton pieces, a total of 78 blood and 78 semen stains were applied, and each group of six stains was immersed or mechanically cleaned in water, 40% methanol, 5% sodium hypochlorite solution, 5% hypochlorous acid solution, 5 g/L soap dissolved pure water, and 5 g/L dishwashing detergent dissolved water. ATR-FTIR spectra gathered from all stains and analyzed with chemometric tools.

Results and discussion: According to performance parameters of developed models, PLS-DA is a powerful tool for discrimination of washing chemical for both washed blood and semen stains. Results from this study show that FTIR is promising for use in detecting blood and semen stains that have become invisible to the naked eye due to washing of the findings.

Conclusion: Our approach allows blood and semen to be detected on cotton pieces using FTIR combined with chemometrics, even though it is not visible to the naked eye. Washing chemicals also can be distinguished via FTIR spectra of stains.

1. Introduction

Biological stains appear as one of the main tools utilized to illuminate crime even in ancient times when crime scene investigation systematics were not yet established. Blood and semen stains, as serious indicators of forensic events, draw attention as the earliest and most studied evidences among all biological fluids [1–7].

The importance of biological stains has increased in parallel to the development in novel technologies regarding the analyzes of stains and the useful information provided by the results of these analyzes. Among these technologies, perhaps the most important and most prominent contribution has been the development of forensic DNA technologies. The series of studies, which led to a revolution in forensic sciences, in which the definition of "DNA fingerprints" was made by Sir Alec Jeffreys in 1985, and which is still accepted as the gold standard of forensic medical identification, increased the importance of biological stains and made tests that do not damage DNA more important [8–13]. FTIR emerges as a prominent method in the analysis of biological stains since it does not harm the sample, and although it is still in its infancy in terms of forensics, intensive studies have been carried out for its routine use in the future for several decades [13–18].

Chemometric methods are used with increasing frequency in studies conducted in both forensic sciences and health sciences, especially in studies based on spectroscopic examinations of biological stains in recent years [19–21]. The high resolution of the data obtained, the multitude of factors affecting the spectrum, especially

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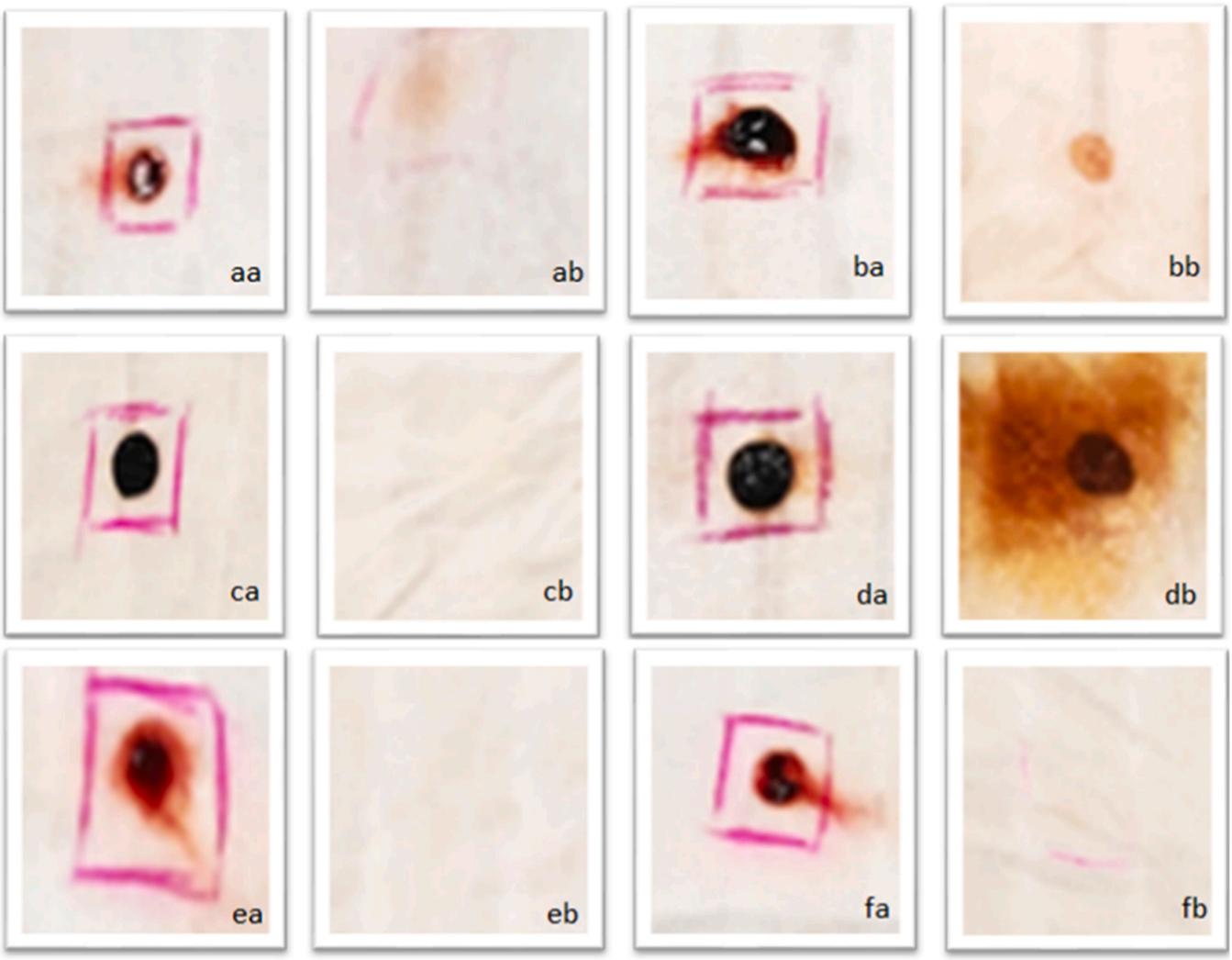

Fig. 1. Appearance of bloodstain samples after washing (aa: sample immersed in distilled water, ab: mechanically cleaned sample in distilled water, ba: sample immersed in 40% methanol, bb: mechanically cleaned sample in 40% methanol, ca: sample immersed in 5% sodium hypochlorite, cb: mechanically cleaned sample in 5% sodium hypochlorite, da: sample immersed in 5% hypochlorous acid, db: mechanically cleaned sample in 5% hypochlorous acid, ea: sample immersed in 5 g/L soap, e: mechanically cleaned sample in 5 g/L soap, fa: sample immersed in 5 g/L dishwashing detergent, fb: mechanically cleaned sample in 5 g/L dishwashing detergent).

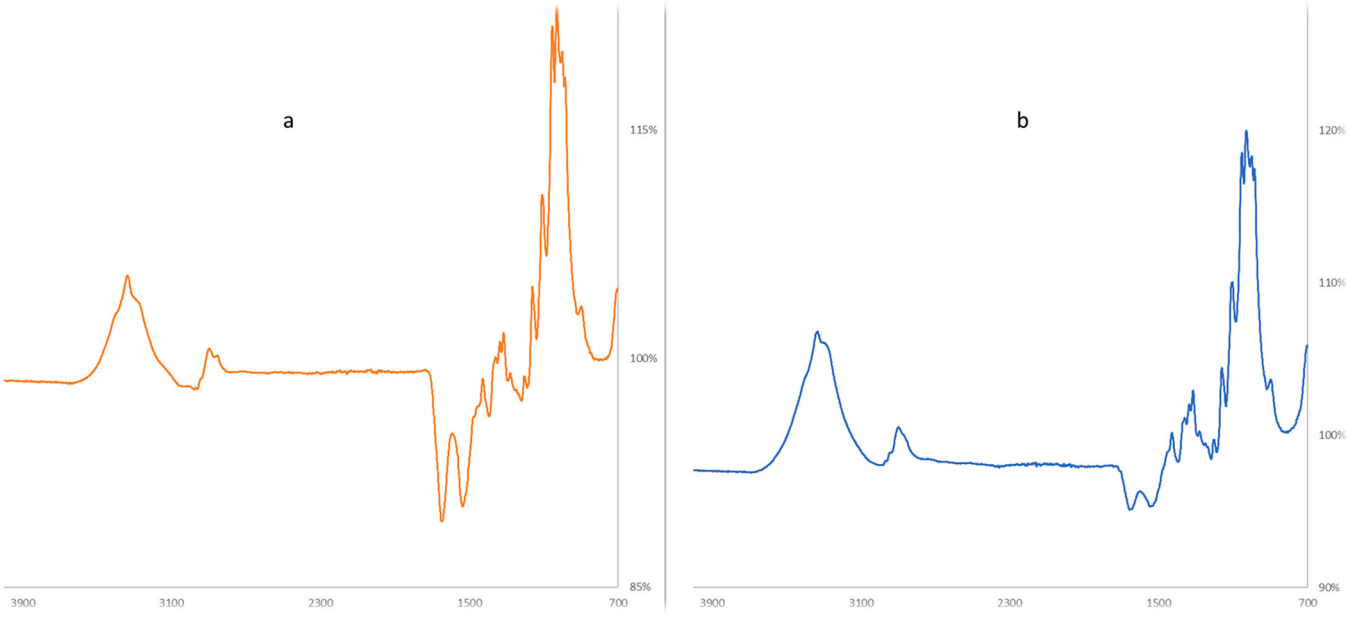

Fig. 2. Transmittance spectra of blood and semen samples (2a: blood, 2b: semen).

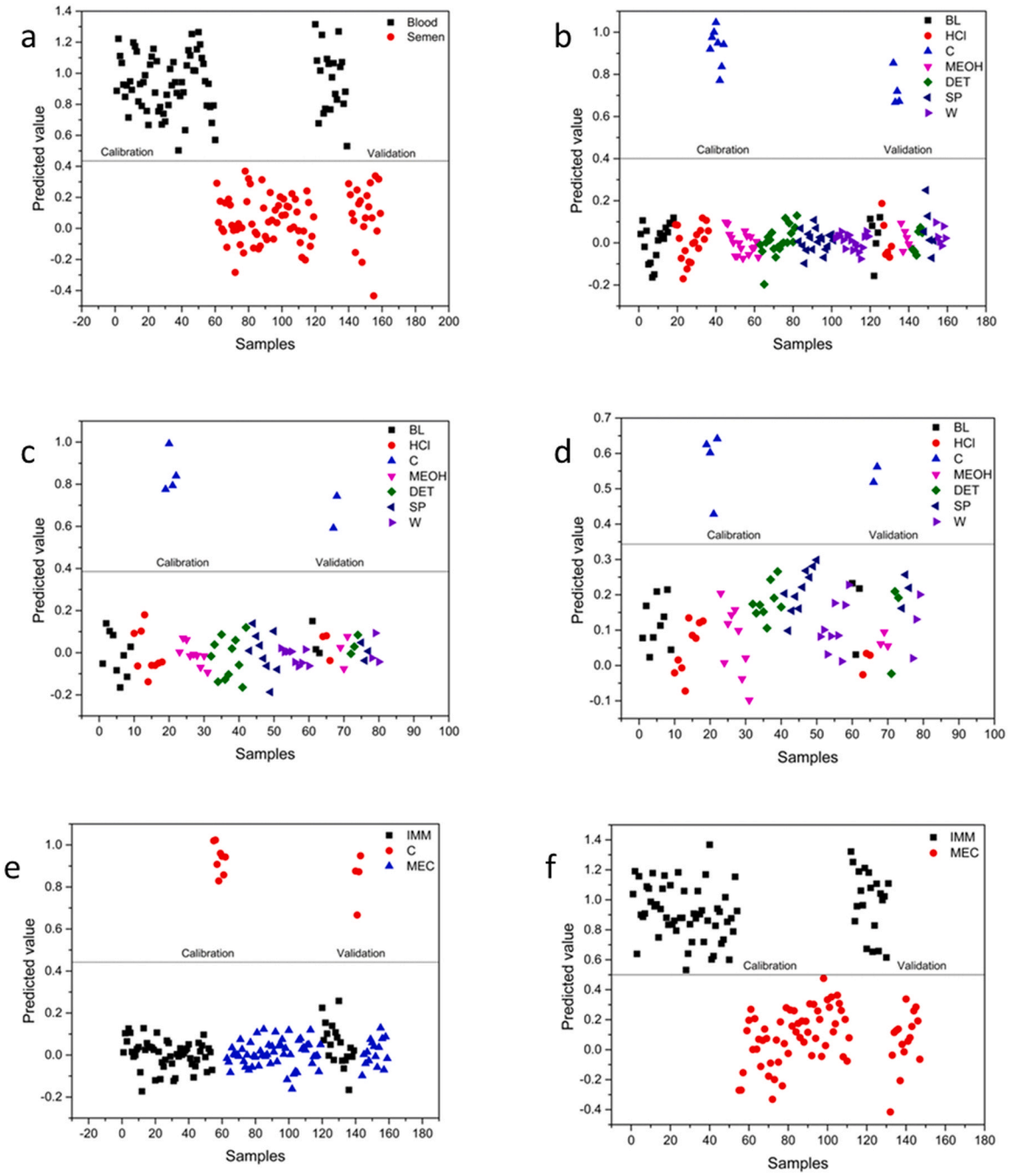

Fig. 3. Discrimination of blood, semen, washing method and cleaning chemicals (a: discrimination of blood and semen stains on fabric, b: discrimination of control and washed samples with all blood and semen data, c: discrimination of control and washed bloodstains, d: discrimination of control and washed semen stains, e: discrimination of control and washed washing types (immersion and mechanic), f: discrimination of immersion and mechanic washings. IMM, immersion; MEC, mechanic BL, bleach; HCl, hydrochloric acid; C, control; MEOH, methanol; DET, dishwashing detergent; SP, soap; W, water).

in biological samples, the effect of many factors such as noise and/or background spectrum caused by undesirable factors along with the data, make it necessary to use chemometric methods in combination with FTIR [22–25].

The crime scene can be manipulated or changed by the perpetrator or by other people, either intentionally or unintentionally. The washing of biological stains is a common application that perpetrators use to spoil the crime scene. Washing can be in the form of immersion, mechanical cleaning by hand or washing with a laundry machine, and various cleaning chemicals can be used in these processes. The impact of washing biological stains on presumptive tests, confirmatory tests, and genetic profiling has been evaluated in published studies. However, in most of these publications, the intensity of the washing procedures applied and the complexity of the composition of used chemicals seem to compromise the reproducibility of the experiments [26–34].

This study aims to examine the results of immersion and manual mechanical cleaning methods on the detection of blood and semen stains on cotton via ATR-FTIR with a standardized experiment model. To the best of our knowledge this is the first study in the literature to use a large variety of chemical cleaners and to strictly control the experimental conditions so far.

Table 1
PLS-DA discrimination performance parameters.

	Blood and semen	Control and washed	Control and washing type (immersion, mechanic)	Immersion and mechanic washing
LV	4	9[a] 6[b] 8[c]	10	7
REMSEC	0.1731	0.0927[a] 0.1865[b] 0.0685[c]	0.065709	0.2062
REMSECV	0.2161	0.1336[a] 0.2047[b] 0.1122[c]	0.129965	0.3498
STR (%)	100	100[a] 100[b] 100[c]	100	100
SPR (%)	100	100[a] 100[b] 100[c]	100	100
TPR (%)	1	1[a] 1[b] 1[c]	1	1
FNR (%)	0	0[a] 0[b] 0[c]	0	0
EFR (%)	100	100[a] 100[b] 100[c]	100	100

[a] blood;
[b] semen;
[c] blood and semen; LV, latent variable; RMSEC, root mean squares error calibration; RMSECV, root mean squares error cross validation; STR, selectivity rate; SPR, specificity rate; TPR, true positive rate; FNR, false negative rate; EFR, efficiency rate.

2. Materials and methods

2.1. Chemicals and reagents

Methanol for lab analysis gradient grade for LC were purchased from Merck (Darmstadt, Germany). Sodium hypochlorite (40%) and hypochlorous acid (5%) solutions were obtained from Seba Kimya (Istanbul, Turkey). Pure soap granules and liquid dishwashing detergent were purchased from Procter&Gamble (Istanbul, Turkey). Detergent was not containing enzymes. The ingredients of dishwashing detergent are as follows: 5–15% anionic surfactant, < 5% non-ionic surfactant, methylisolythiazolinone, phenoxyethanol, perfume, geraniol, limonene.

2.2. Blood and semen collection

Venous blood samples were collected from three adult males and three adult females, each 10 cc in amount, with their written consents. Complete blood count was performed on the samples taken, and it was observed that the values were in the normal range. After semen samples were taken from three male subjects who had also written consent and had a three-day sexual abstinence, cell count and pH analysis were performed in the semen. In semen samples, pH, sperm count, and morphology were determined within the normal range. Anticoagulants or any other preservative were not added into the samples to avoid interference, and to simulate crime scene properly. Since anticoagulant substances were not added to the blood samples, the stain was transferred immediately after it was taken. Semen samples were kept at room temperature for 20 min, liquefied and homogenized, and then stain transfer was started.

2.3. Preparation of biological stains

20 µl of blood and 20 µl of semen were dripped onto separate pieces of cotton cloth with an area of 2 cm^2. Although it is possible to interfere with the spectrum of biological stains due to its organic structure, cotton fabric was preferred because it is widely used in underwear and therefore it is frequently encountered in forensic examinations. After a total of 78 blood and 78 semen stains were transferred, the samples were left to dry for 3 h under constant conditions in a cabinet providing 10000 lumens of white light, 23 °C temperature and 40% Rh. After deposition, the samples were separated so that each group consisted of 6 stains. After this stage, the same procedures were applied to both blood and semen stains.

2.4. Experimental groups and washing procedures

A blood and a semen stain group were separated as the control group and kept until analysis without any action. A group of blood and semen stains were immersed in 200 cc of cleaning liquid for one hour, another group was subjected to mechanical cleaning in the cleaning liquid, and then kept in the liquid for one hour. Mechanical cleaning was provided by rubbing with the same type of fabric under a pressure of 60–80 N/cm^2 on the stain for five minutes. Purified water, 40% methanol, 5% sodium hypochlorite solution, 5% hypochlorous acid solution, 5 g/L soap dissolved pure water and 5 g/L dishwashing detergent dissolved water were used as the cleaning liquid.

After the immersion and mechanical cleaning processes, the samples were kept in constant conditions for 12 h in a cabinet providing 10000 lumens of white light, 23 °C temperature and 40% Rh, and then analyzed.

2.5. Instrumentation

Perkin Elmer Spectrum 400 FT-IR / FT-NIR spectrometer with Universal ATR Sampling Accessory unit was utilized in this study. The sample spectra were obtained using Spectrum 10.5.4 (Perkin Elmer Inc., Norwalk, CT, USA) software. Sample spectra were obtained using Spectrum 10.5.4 (Perkin Elmer Inc., Norwalk, CT, USA) software with 4 scan numbers at a resolution of 4 cm^{-1} at a spectral range of 4000–650 cm^{-1} at constant pressure. Three spectra were obtained from different parts of each sample and the average of these spectra was used. In order to eliminate the effect of fabric and chemical influences on spectra, the background spectrum was taken from an empty area of the fabric without stain, and this obtained spectrum was automatically extracted from the stained fabric spectrum with the aid of software. To prevent cross contamination between samples, before and after each sample analysis, the surface on which the sample was placed was wiped with methanol and allowed to dry.

2.6. Ethical approval

This research has been approved by Hacettepe University Committee of Research Ethics with project number GO 17/508 and decision number 17/508–20.

2.7. Data analysis

For chemometric analysis of FTIR data MATLAB software (Mathworks Inc. Natick, MA) and PLS_Toolbox version 8.2 (Eigenvector Research, Inc. Manson, WA) were used. Partial least squares discriminant analysis (PLS-DA) was used for discrimination of the FTIR data with training and test data sets at 3:1 ratio. PLS-DA were utilized discrimination of (i) blood and semen (all spectrums); (ii) control samples and washed samples; (iii) control and washed samples for semen stains; (iv) control and washed samples for bloodstains; (v) type of washing processes (immersion and mechanic); (vi) what

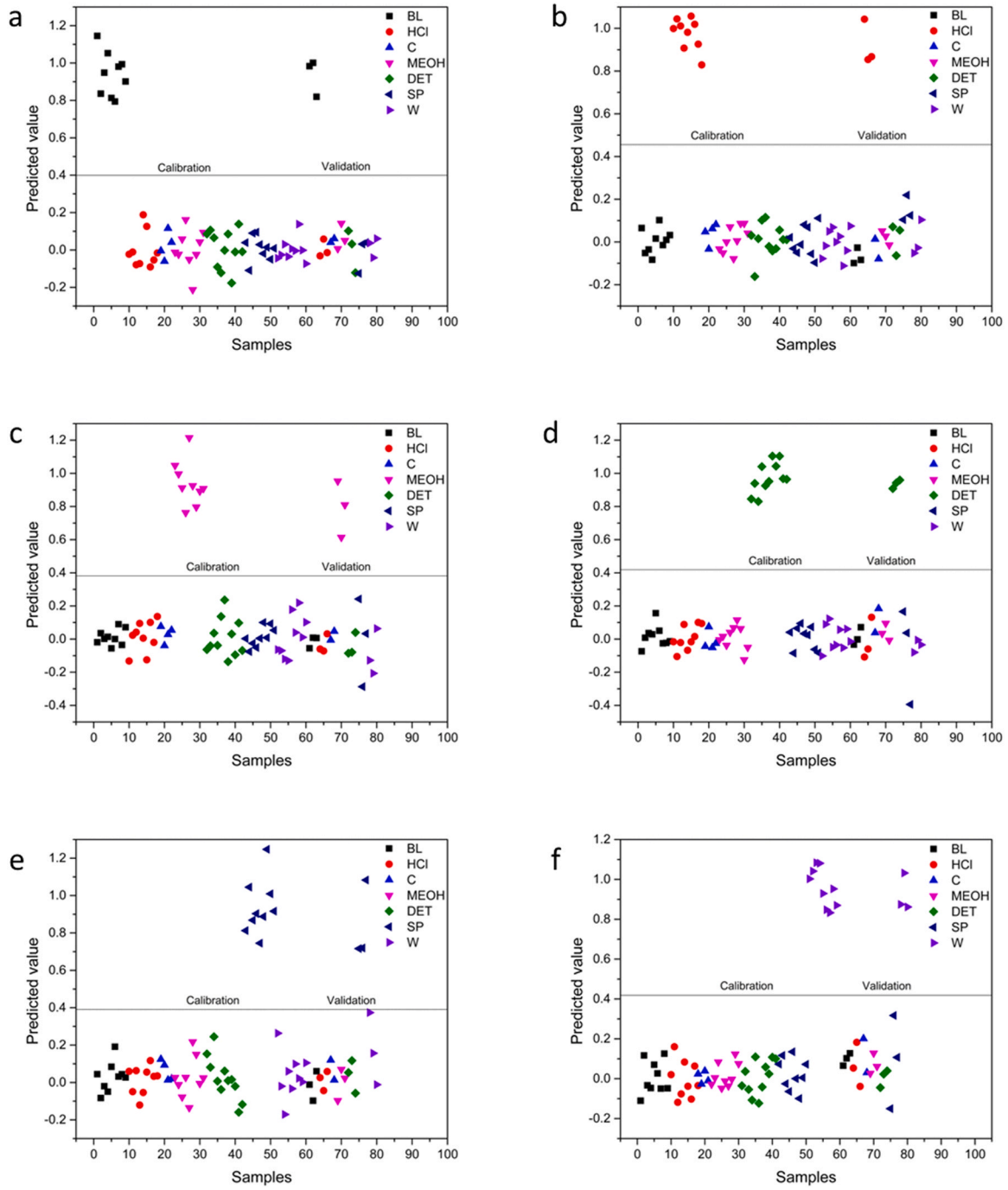

Fig. 4. Discrimination of washing chemicals on bloodstain (BL, bleach; HCl, hydrochloric acid; C, control; MEOH, methanol; DET, dishwashing detergent; SP, soap; W, water).

chemical is used for washing of blood and semen stain (blood and semen samples were evaluated separately and together).

In addition, to determinate performance of PLS-DA discrimination, sensitivity rate (STR, %), specificity rate (SPR, %) and model efficiency rate (EFR, %) were calculated using true positive rate (TPR), false negative rate (FNR), true negative rate (TNR), false positive rate value (FPR).

$$STR = TPR / (TPR + FNR) \quad (1)$$

$$SPR = TNR / (TNR + FPR) \quad (2)$$

$$EFR = 100 - (FPR + FNR) \quad (3)$$

3. Results

After washing, all semen samples in all groups became indistinguishable to the naked eye. While the blood samples were still visible after immersion, it was observed that the samples exposed to bleach, soap and dishwashing detergent became indistinguishable after mechanical cleaning (Fig. 1).

3.1. FTIR spectra of blood and semen

The average transmittance spectra of the obtained blood and semen control samples are shown in Fig. 2.

Table 2
Performance parameters of PLS-DA discrimination of washing chemicals.

	WASHING CHEMICALS					
	BL	**HCl**	**MEOH**	**DET**	**SP**	**W**
LV	9[a]	9[a]	9[a]	9[a]	9[a]	9[a]
	6[b]	6[b]	6[b]	6[b]	6[b]	6[b]
	8[c]	8[c]	8[c]	8[c]	8[c]	8[c]
REMSEC	0.0905[a]	0.0650[a]	0.0975[a]	0.0721[a]	0.1077[a]	0.0796[a]
	0.0984[b]	0.1044[b]	0.0783[b]	0.1099[b]	0.1396[b]	0.0876[b]
	0.0745[c]	0.0680[c]	0.0656[c]	0.0808[c]	0.1103[c]	0.0671[c]
REMSECV	0.1142[a]	0.0956[a]	0.1343[a]	0.1265[a]	0.1352[a]	0.1189[a]
	0.1274[b]	0.1322[b]	0.1143[b]	0.1282[b]	0.1622[b]	0.1139[b]
	0.1005[c]	0.0889[c]	0.0942[c]	0.1011[c]	0.1255[c]	0.0978[c]
STR (%)	100[a, b,c]	100[a, b,c]	100[a, b,c]	100[a, b,c]	100[a, b,c]	100[a, b,c]
SPR (%)	100[a, b,c]	100[a, b,c]	100[a, b,c]	100[a, b,c]	100[a, b,c]	100[a, b,c]
TPR (%)	1[a]	1[a]	1[a]	1[a]	1[a]	1[a]
	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]
	1[c]	1[c]	1[c]	1[c]	1[c]	1[c]
FNR (%)	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]
	0[c]	0[c]	0[c]	0[c]	0[c]	0[c]
EFR (%)	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]
	100[b]	100[b]	100[b]	100[b]	100[b]	100[b]
	100[c]	100[c]	100[c]	100[c]	100[c]	100[c]

[a] blood;
[b] semen;
[c] blood and semen; LV, latent variable; RMSEC, root mean squares error calibration; RMSECV, root mean squares error cross validation; STR, selectivity rate; SPR, specificity rate; TPR, true positive rate; FNR, false negative rate; EFR, efficiency rate. BL, blench; HCl, hydrochloric acid; MEOH, methanol; DET, dishwashing detergent; SP, soap; W, water.

3.2. PLS-DA analysis

In this study, we used PLSA-DA method for discrimination of blood and semen on fabric (all washed and non-washed stains), control and washed samples, types of washing and chemical cleaners utilized for washing. All washed and non-washed blood and semen stains were successfully discriminated, as shown in Fig. 3a, using PLS-DA with 100% sensitivity, specificity, and model efficiency. FTIR spectra of all blood and semen stains were pre-processed with 2nd Derivative (order: 2, window: 7 pt, incl only, tails: polyinterp).

PLS-DA graph of control and washed stain samples was given in Fig. 3b, which includes blood and semen stains. Control and washed blood and semen discrimination were shown in Fig. 3c and d, separately. PLS-DA model in Fig. 3b, spectrums were pre-processed with 2nd Derivative (order: 2, window: 9 pt, incl only, tails: polyinterp) and Autoscale. It can be seen in Table 1. Classification model was accurately developed with SPR, STR and EFR of 100%.

In Fig. 3e, control samples and washed samples were successfully discriminated according to washing types while Fig. 3f shows differentiation of immersion and mechanic washing. Highly accurate models with 100% of STR, SPR and EFR were obtained discrimination of washing methods.

Finally, washing chemical that used for cleaning of blood and semen stains were discriminated. Discrimination of washing chemicals on bloodstain were shown in Fig. 4 with 100% sensitivity, specificity, and model efficiency (Table 2). In Fig. 5, semen stains were classified according to washing chemicals. 2nd Derivative (order: 2, window: 7 pt, incl only, tails: polyinterp) was used as preprocess for both blood and semen spectral data.

4. Discussion

In the literature on biological stain analysis, FTIR is becoming more and more prevalent. Despite the positive outcomes, before using FTIR analyzes on a regular basis, studies are still required to look into the reproducibility of the results and how they apply to various situations.

It is known that the cotton substrate affects the FTIR spectra of biological stains, including the bio-fingerprint region [17,20,35–37]. Extracting the sample by scraping or liquid extraction from the substrate is one of the methods has been tried in the literature previously as a successful way of removing the effect of the substrate on the spectra [38–40]. However, in this case, the ability of FTIR to perform unlimited analysis with very few samples without damaging the sample is restricted. Zapata et al. [36], in their study dealing with the identification of biological stains on fabric with FTIR, stated that it is impossible to evaluate the 900–1500 cm^{-1} region of the bio-fingerprint area due to the interference of cotton peaks, but sufficient information can be obtained for identification from the 1500–1800 cm^{-1} area, which also includes Amide I and II peaks. Similar interference was observed in our study for both blood and semen. Since the background spectrum was extracted by the software in our study, major cotton peaks, especially the 1032 cm^{-1} and 3330 cm^{-1} peaks, which are the two most intense peaks defined in the literature [41], were seen as reverse peaks in both the 900–1500 cm^{-1} region and the 2800–3600 cm^{-1} region of the spectra. In parallel with our results, a similar reverse peak formation was observed in another study previously conducted in the literature that extracted the background spectrum of cotton from sample spectra [42]. The peaks corresponding to the wavenumbers of 966 and 1236 cm^{-1}, which are defined in the literature as DNA-related peaks, and also 1059 cm^{-1}, which is assigned to prostate specific antigen become incapable of providing information due to the interference of cotton fibers (Fig. 2) [15,43]. This may pose an extra difficulty in semen stain analyses, since DNA-related peaks also gives information whether the sample contains spermatozoa.

Washing of the samples prevents visual detection, as well as affecting the results of many presumptive tests, however forensic genetic profiling can be still possible [28,29,31,33,34,44–47]. In a number of studies, automated machines were used as the washing method [11,28,29,34]. In some of them, including few studies using FTIR as the analytical method, manual-washing was used, but since the method standards were not fully explained, it is not possible to make a comparison with our study [38,48]. As a result of washing with our method, it can be observed that blood and semen can be detected on washed cotton fabrics via FTIR combined with chemometrics.

Considering that hydrochloric acid and methanol facilitate the formation of methemoglobin in different ways and therefore cause the stain to remain visible for a longer time and cause differences in

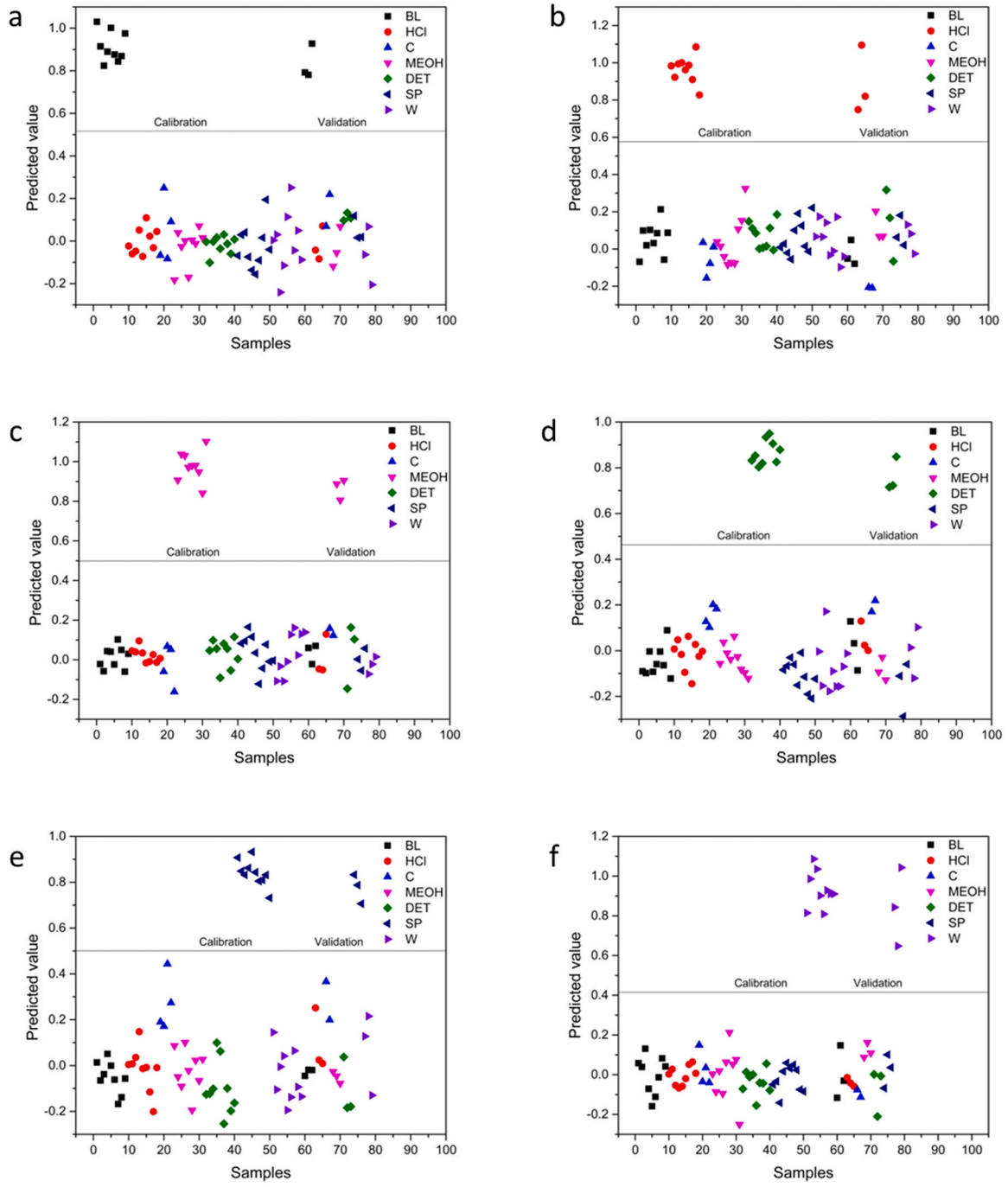

Fig. 5. Discrimination of washing chemicals on semen stain (BL, bleach; HCl, hydrochloric acid; C, control; MEOH, methanol; DET, dishwashing detergent; SP, soap; W, water).

test results, it is necessary to study other factors that increase methemoglobin formation, including effects of washing and deposition temperature on FTIR spectra [28,32,49].

5. Conclusions

In studies combined with chemometric methods and in which environmental conditions are controlled during the experiment, the discrimination power of different properties of biological fluids with FTIR can reach up to 100%, as in our study [40,50–52]. According to performance parameters of developed models, it can be seen that PLS-DA method is a powerful tool for discrimination of washing chemical for both washed blood and semen stains. Results from this study show that FTIR is promising for use in detecting blood and semen stains that have become invisible to the naked eye due to washing of the findings. Since FTIR forensic stain analysis is still in its infancy, there is a need for many studies in which all factors are examined separately with standardized experiments.

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.

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