NOFOR
NOVEL FORENSIC RESEARCH

DOI: 10.5455/NOFOR.2026.04.05

NOFOR. 2025;4(3):81-9

Original Article

Determination of gender from blood stains on different fabric types using a combined ATR-FT-IR spectroscopy and chemometric approach

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Received August 17, 2025; Accepted November 24, 2025; Available online December 15, 2025

Abstract

Aim: In forensic science, biological samples collected from crime scenes have a critical role in criminal profiling (such as determining gender, race, and age). Due to the time-consuming sample preparation processes required by traditional methods and their destructive nature, interest in ATR-FT-IR spectroscopy, which is based on molecular vibration and provides more rapid results, has increased in recent years. However, biological fluids at crime scenes are rarely found isolated; they are typically present on various surfaces such as fabric and glass. These surfaces affect the spectra of biological fluids and complicate the analysis. The aim of this study is to determine gender using ATR-FT-IR spectroscopy and chemometric methods from dried blood stains on different textile surfaces (cotton, denim, and polyester).

Materials and Methods: In this study, blood samples were collected from a total of 50 volunteers, including 25 women and 25 men. These samples were absorbed onto cotton, denim, and polyester fabrics and analyzed using ATR-FT-IR spectroscopy after a 24-hour drying period.

Results: Spectral analysis revealed variations in the characteristic band regions of the blood depending on the type of fabric. Principal Component Analysis (PCA) was applied to the obtained high-dimensional data set using the R package to reduce the number of dimensions; then, logistic regression analyses were performed using SPSS. Examination of the PCA loadings revealed that the spectral bands belonging to cholesterol and creatinine molecules have the potential to serve as discriminative biomarkers for gender differentiation.

Conclusions: The study results showed that logistic regression models built using only raw spectral data or simple variables had limited explanatory power for the gender-dependent variable. However, including the principal components obtained from PCA in the model had an effect that increased the model's explanatory power. When the substrate effect was evaluated, it was determined that blood samples on polyester fabric performed better in terms of accurate gender classification rates compared to cotton and denim surfaces. These findings highlight the importance of considering surface interactions when using spectroscopic methods in forensic serology.

Keywords: Chemometrics, ATR-FT-IR spectroscopy, gender determination, PCA, logistic regression

INTRODUCTION

Blood is one of the most ubiquitous and biologically significant forms of physical evidence encountered in forensic investigations. Accounting for approximately 8% of total human body weight, blood serves as a vital reservoir of both genetic and phenotypic information [1]. Physiologically, it is a highly complex biological fluid composed of an aqueous extracellular matrix, known as plasma, which contains a variety of dissolved proteins, inorganic salts, and water [2]. Suspended within this plasma are the distinct cellular components: erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets) [1]. From a forensic perspective, erythrocytes are of particular interest due to their high concentration of hemoglobin (Hb). This oxygen-transporting metalloprotein is structurally

CITATION
Kula Yesilot FB, Islek DS, Kiris E, et al. Determination of gender from blood stains on different fabric types using a combined ATR-FT-IR spectroscopy and chemometric approach. NOFOR. 2025;4(3):81-9. DOI: 10.5455/NOFOR.2026.04.05

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DOI: 10.5455/NOFOR.2026.04.05

composed of a porphyrin heme group and globin protein subunits. Historically, forensic serology has relied heavily on the peroxidase-like activity of this heme group; preliminary screening and subsequent confirmatory tests exploit the oxidation-reduction reactions of heme to indicate the presence of blood in suspected forensic stains [3,4].

Despite their widespread operational use, traditional serological and chemical assays present notable limitations. Foremost among these is the destructive nature of the chemical reagents, which can permanently alter or consume limited biological evidence. Additionally, these conventional methods often require extensive sample preparation and can be susceptible to environmental degradation or false-positive results [4]. To circumvent the destruction of critical legal evidence, forensic researchers have increasingly transitioned toward non-destructive, label-free analytical methodologies. Spectroscopic modalities, in particular, have emerged as powerful tools for the biochemical profiling of trace evidence. Techniques such as fluorescence spectroscopy [5] and Raman spectroscopy [6] have demonstrated significant utility in biological analysis. More recently, attenuated total reflection Fourier transform infrared (ATR-FT-IR) spectroscopy has been widely adopted due to its ability to rapidly and accurately identify the presence and biochemical origin of blood with minimal to no sample preparation [7,8].

While the isolated spectroscopic analysis of neat blood is well-documented, forensic realities dictate that biological evidence is rarely encountered in pristine conditions. Bloodstains at crime scenes are most frequently deposited onto porous, everyday materials such as cotton, denim, or polyester apparel. Consequently, the biochemical signature of the blood is heavily convoluted by the spectral signals of the underlying matrix. Overcoming this substrate interference requires a thorough understanding of the textiles themselves. Previous studies have successfully employed vibrational spectroscopic methods, including infrared (IR) spectroscopy [9,10] and ATR-FT-IR [11], for the rigorous chemical characterization and classification of diverse textile fibers. Furthermore, advanced research has begun to explore the differentiation of various body fluid stains directly on fabric substrates utilizing FT-IR coupled with multivariate analysis [12].

Despite these advancements in substrate characterization and body fluid identification, a significant gap remains in the literature regarding the extraction of specific phenotypic characteristics from complex matrix-fluid mixtures. To date, no studies have comprehensively examined how the substrate effect influences the spectroscopic prediction of an individual donor's gender from a bloodstain. The extraction of such specific intelligence could provide crucial investigative leads in the absence of a DNA match.

Therefore, the aim of this study is to investigate the potential of ATR-FT-IR spectroscopy, combined with advanced chemometric data analysis, to accurately predict the gender of an individual from bloodstains deposited on common textiles, specifically targeting cotton, denim, and polyester fabrics. By evaluating and mitigating the substrate effect, this research seeks to expand the non-destructive forensic capabilities of vibrational spectroscopy in complex real-world scenarios.

MATERIAL AND METHOD

Sample Collection and Preparation

Textile sample collection and preparation

Cotton, polyester, and gray denim fabrics were collected and cut into 4x4 cm² samples. They were washed in cold water in a conventional washing machine on a delicate cycle without detergent and dried without human contact to prepare them for analysis. The dried samples were placed in sterile petri dishes (15 mm x 60 mm).

Blood sample collection

The experiments and protocols used in the study were conducted in agreement with the decisions and guidelines of the İstanbul University-Cerrahpaşa Faculty of Medicine Clinical Research Ethics Committee (Decision No: 158845, 12/03/2020) after the committee's approval was received. Blood samples (5 mL) were collected from 50 healthy volunteers (25 women, 25 men) aged 18 years or older using non-anticoagulized tubes by venous injection.

Modelling

Blood samples from both women and men, collected into tubes without anticoagulant using a micropipette, were applied to cotton, polyester, and denim fabrics at 30 µL each. The same procedure was performed three times for each fabric type per individual. The fabrics with applied blood were dried overnight at room temperature. Sample preparation is shown in Figure 1.

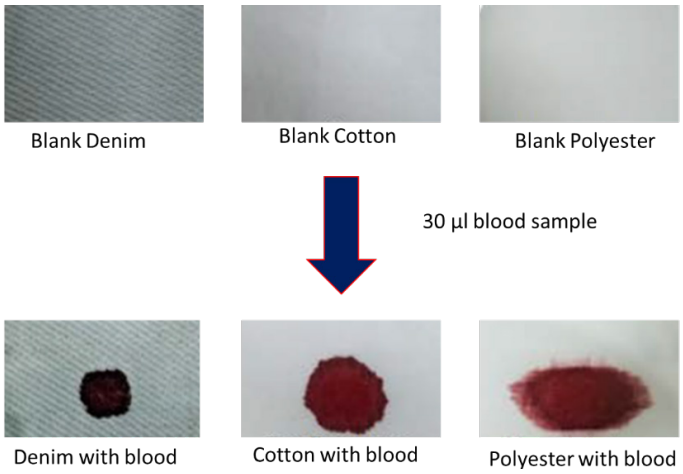

Figure 1. Sample preparation

ATR-FTIR Analysis

Dried blood stains on fabric were analyzed using an FT-IR spectrometer equipped with an ATR, DLaTGS detector (Bruker

DOI: 10.5455/NOFOR.2026.04.05

Tensor, Bremen, Germany) with diamond crystal. Spectra were obtained by performing 16 scans at 4 cm^{-1} resolution in the mid-IR range of 4000-400 cm^{-1} for each fabric type. Three replicates were performed for each volunteer on cotton, polyester, and denim fabric types. Before each spectrum was taken, the ATR crystal was cleaned with 70% ethanol, completely dried, and an air background was taken. Then, to minimize the effect of the fabric, an average spectrum was obtained by taking spectra from three areas of each fabric type without blood stains. Since blood does not spread homogeneously on the fabric, ten different measurements were taken from various regions of the blood-stained fabric to reduce the standard deviation between measurements, and an average spectrum was obtained.

Chemometric Analysis

Spectra were obtained and stored using OPUS 7.2 software (Bruker, Germany). Blank fabric spectra were automatically extracted from blood-spotted fabric spectra using OPUS 7.2 software. All chemometric procedures were performed using the R Package and SPSS (version 15.0) software package. Pre-processing was applied to the data. Subsequently, dimension reduction was performed using PCA. After variable selection, modeling was performed by applying logistic regression to the data.

RESULTS

The age range, height, weight, and BMI data of the participants are provided in Table 1.

Table 1. Volunteers' age, height, weight, and BMI values

Parameter	Man	Woman
Age range	22-58	23-55
Avg. of age	37.8 (±10.38)	36.3 (±11.01)
Avg. of height (m)	1.768 (±0.065)	1.629 (±0.073)
Avg. of weight (kg)	82 (±13.89)	63.32 (±12.65)
BMI	26.26 (Overweight)	23.89 (Normal)

The spectra of blank fabrics analyzed by ATR-FTIR for chemical characterization of fabrics are shown in Figure 2.

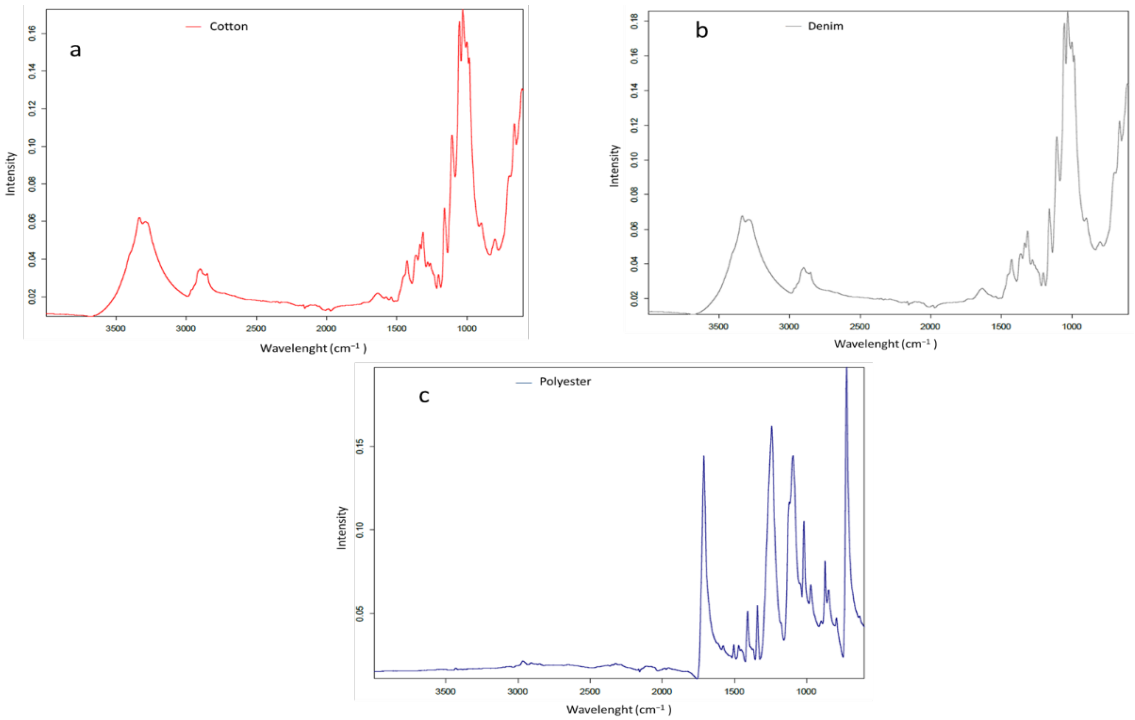

Figure 2. Spectra of a) cotton, b) denim, and c) polyester fabrics without bloodstains

Cotton and denim fabrics showed similar spectra, with the intense and broad band at 3550-3100 cm^{-1} attributed to O-H stretching in cellulose, while the medium and broad band at 2900-2800 cm^{-1} was attributed to C-H stretching. In the 1430-1300 cm^{-1} region: 1429 cm^{-1} indicates C-H plane vibration, 1368 cm^{-1} represents C-H bending, and 1316 cm^{-1} denotes C-H out-of-plane vibration. The sharp peaks at 1160 and 1108 cm^{-1} of the ring structure forming cellulose result from asymmetric C-O-C bridge stretching. 1057 cm^{-1} shows the asymmetric in-plane ring stretching of the cellulose molecule, while the wavenumbers 1030 and 1000 cm^{-1} show C-O stretching [13].

When the IR spectrum of polyester fabric is examined, the peak observed at a wavelength of 1715 cm^{-1} due to C=O stretching is found to be distinctive. The medium intensity C-H stretching observed in the 2966 cm^{-1} band shows the presence of an alkane group in the IR spectrum. The peak observed at 1241 cm^{-1} is associated with vibrational movements related to

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the aromatic ester functional group. The 1331 and 1021 cm^{-1} bands correspond to the carboxylic ester functional groups in the polyethylene terephthalate (PET) molecule [14]. The peak at the 720 cm^{-1} band corresponds to benzene derivatives in the IR spectrum. Additionally, since the polyester utilized in the experiment yielded spectral signals at 846, 973, and 1340 cm^{-1}, it is concluded that the PET is in the trans-conformation.

In this study, blood samples obtained from man and woman participants were applied to three different fabric types, and the resulting dried bloodstains were analyzed via ATR-FTIR spectroscopy. The IR spectra of the dried bloodstains (woman-man) on cotton fabric, along with the unstained cotton fabric control, are presented in Figure 3.

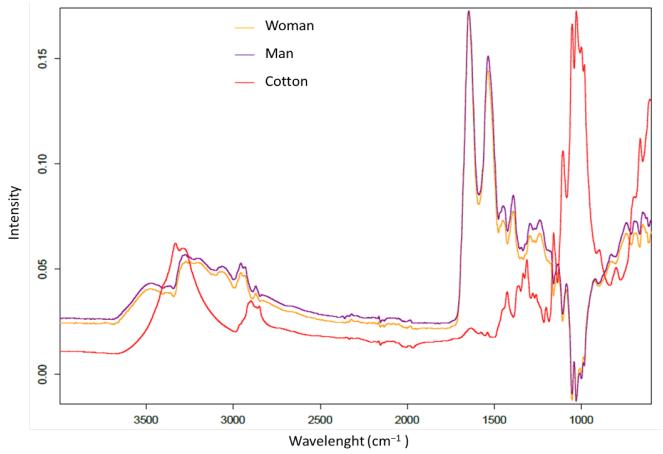

Figure 3. Dried blood stains on cotton fabric (woman-man) and the IR spectrum of cotton fabric without blood stains

Spectrum in Figure 3 shows that the broad bands observed in the 3600-3000 cm^{-1} region are N-H stretching vibrations characteristic of the amide functional groups in the structure of proteins, hemoglobin, and urea components found in blood. The C-H stretching vibrations specific to lipid molecules were detected as bands in the 3000-2800 cm^{-1} wavenumber region [15,16]. The Amide I area of the erythrocyte membrane proteins that form the characteristic structure of blood is distinct in the spectrum, producing strong peaks in the 1700-1620 cm^{-1} region [17]. Additionally, Amide II peaks formed as a result of the interaction between N−H in-plane bending and C−N stretching modes were observed in the 1450–1600 cm^{-1} region; this finding has been considered an important spectral difference that would distinguish dried blood stains from the spectrum of cotton fabric [17,18]. Likewise, two characteristic peaks corresponding to the Amide III area in the 1400-1200 cm^{-1} region were identified, and these peaks were observed in both the woman and man gender spectrums [17,18]. In the spectral fingerprint area between 1200 and 950 cm^{-1}, bands associated with the glucose molecule were observed [19,20].

Figure 4 shows the spectra of blood samples applied to denim fabric. The spectra were examined and no significant difference was found between the average spectra of dried blood stains from woman and man participants.

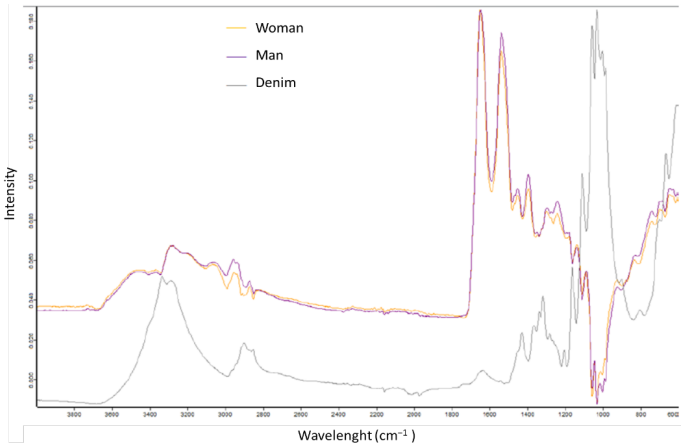

Figure 4. Dried blood stains on denim fabric (woman-man) and IR spectrum of denim fabric without blood stains

Following the spectral analysis of dried bloodstains on polyester fabric the presence of Amide I and Amide II bands— characteristic markers of blood—was identified in the IR spectrum as shown in Figure 5. However, due to spectral interference arising from the chemical structure of the polyester fabric, it was observed that the IR regions and frequency values associated with blood components exhibited variations compared to the spectra obtained from cotton and denim fabrics

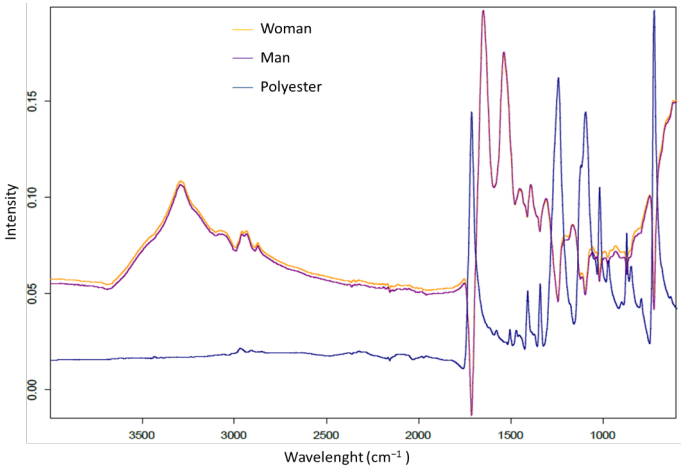

Figure 5. Dried blood stains on polyester fabric (woman-man) and IR spectrum of polyester fabric without blood stains

Chemometric methods were applied to distinguish between the genders of bloodstains and to compare the classification differences between fabrics, due to the fact that there is no visually noticeable difference in the spectrum of dried bloodstains on fabrics for both women and men.

Normalization was applied to the data obtained from the ATR-FTIR spectroscopic analysis of bloodstains deposited on denim, cotton, and polyester fabrics. Dimensionality reduction was performed on the pre-processed data using the PCA technique. According to the PCA results, a total of 150 principal components were identified. The first five principal components (PC1: 96.10392%; PC2: 0.09826654%; PC3: 0.03785720%; PC4:

DOI: 10.5455/NOFOR.2026.04.05

0.02379158%; PC5: 0.01493602%) account for 97.852034% of the total variance, and the modeling was conducted using these top five components. The factor loadings plot for the first five principal components is presented in Figure 6.

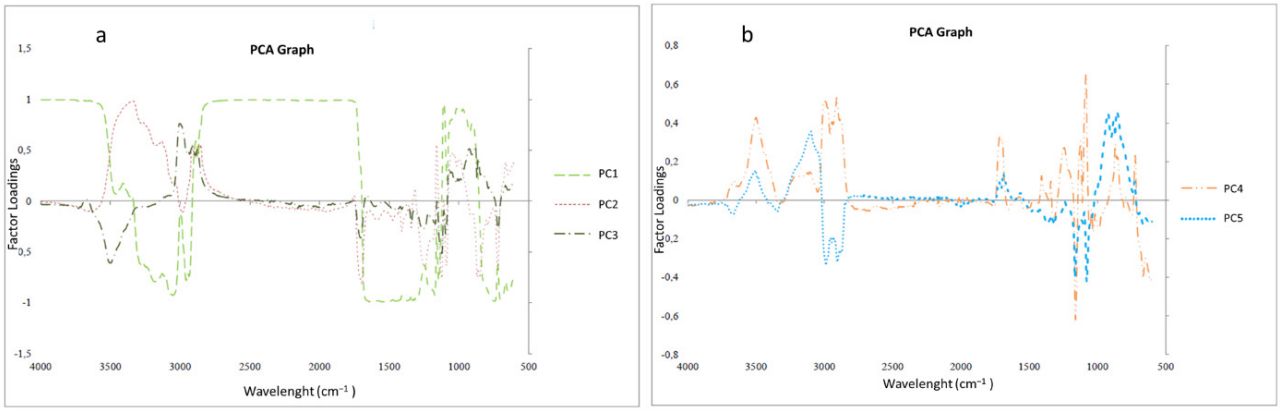

Figure 6. Factor loadings graph of the data set to which PCA was applied: a) PC1, PC2, and PC3, and b) PC4 and PC5

PC1 exhibits a significant correlation with the protein-derived Amide A region (3400-3300 cm^{-1}). In this compound, distinct peaks corresponding to the presence of creatinine (1107 cm^{-1}) [15] and DNA (1200–1000 cm^{-1}) [21] were observed. While the loading in the 2699-1711 cm^{-1} range was evaluated as resulting from the ATR crystal. PC2 was associated with lipid and carbohydrate structures in cholesterol and glucose. Positive correlations specific to cholesterol (2916 cm^{-1}), Amide III (1317 cm^{-1}), and glucose (1055–984 cm^{-1}) molecules were identified. In PC3, which serves as a discriminative factor for gender determination, lipid-derived C-H stretchings (3000–2800 cm^{-1}), the Amide I (1659 cm^{-1}) region, and DNA signals (1070–858 cm^{-1}) become prominent [15,16]. While PC4 reflected the C=O stretching of lipids (1750–1700 cm^{-1}) and the fabric background effect, PC5 exhibited a positive correlation with protein/hemoglobin-derived N-H and cellulose-derived O-H stretchings (3600–3000 cm^{-1})

According to the PCA results, five principal components were identified and displayed on the PCs with distribution graphs of the man and woman's data sets according to gender. The graphs of the women's and men's data sets on the components are shown in Figure 7.

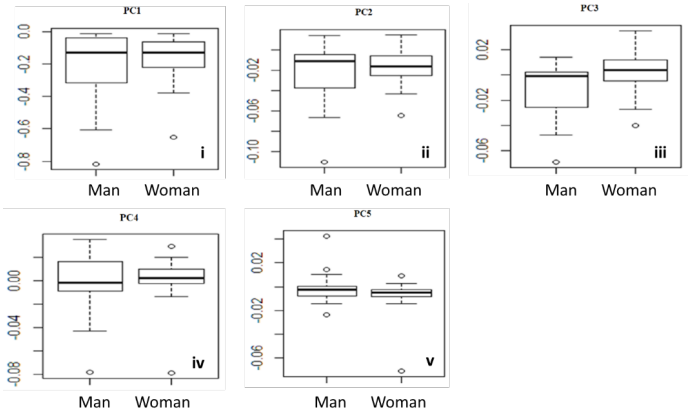

Figure 7. Boxplot graphs of the woman and man data sets on the i) PC1, ii) PC2, iii) PC3, iv) PC4, and v) PC5 components

The graphs for the principal components PC1, PC2, PC3, and PC4 were examined, and it was observed that the data set for man participants showed a higher variance compared to women and that outliers were located further from the center of the distribution. It was found that the data related to gender groups significantly collided in these components; this points to the fact that it is statistically difficult to make a clear distinction between genders based on the relevant principal components. However, when examining the distributions on the PC5 component, it was found that the woman and man's data sets had a more similar and homogenous structure compared to the other components, but outliers were also maintained in both groups.

In this study, PCA was primarily employed for the exploratory evaluation of the data structure and to identify the specific wavenumbers that account for the dominant variance within the ATR-FT-IR spectra, rather than to achieve direct class separation. Accordingly, PCA loading plots served as the principal analytical tool to elucidate the contributions of individual spectral variables to the overall variance. Although PCA score plots were systematically examined, no distinct clustering or spatial separation between the male and female blood samples was observed. This lack of differentiation is further corroborated by the results of the logistic regression analysis. As a supervised classification method, logistic regression also failed to yield a statistically significant separation between the two groups. This concurrent outcome strongly suggests that any gender-dependent spectral variations within this particular dataset are neither highly pronounced nor linearly separable. Consequently, it is methodologically consistent that an unsupervised technique such as PCA would similarly be unable to produce a meaningful class distinction under these parameters.

In this study, logistic regression models were established using scores obtained from PCA in order to predict gender in blood samples obtained from denim, cotton, and polyester fabric surfaces. For each fabric type and the general dataset, gender

DOI: 10.5455/NOFOR.2026.04.05

was defined as the dependent variable, and the first five principal components (PC1-PC5) were defined as independent variables. The logistic regression method without a constant term was preferred in the analyses. The comparison of R^2, overall significance, and classification success of the logistic regression models is given in Table 2, and the comparison of variable coefficients and significance levels for the logistic regression models is given in Table 3.

Table 2. Comparison of R^2 values, overall significance, and classification accuracy across logistic regression models

Model	-2 Log likelihood	Cox & Snell R2	Nagelkerke R2	Chi-squared (χ2)	SD	p (Sig.)	Correct classification rate
General model	62.343	.130	.174	6.181	8	.627	58.0
Cotton	63.851	.104	.138	4.801	8	.779	64.0
Denim	62.343	.130	.174	12.255	8	.140	56.0
Polyester	62.501	.127	.170	6.947	8	.542	68.0

Table 3. Comparison of coefficients and significance levels for logistic regression models

Model	Variables	B	S.E.	Wald	SD	p	Exp(B)=ODDS
General model	PC1	.265	.245	1.167	1	.280	1.304
	PC2	-.426	.619	.475	1	.491	.653
	PC3	.233	.244	.912	1	.340	1.263
	PC4	.070	.490	.021	1	.886	1.073
	PC5	.546	.358	2.328	1	.127	1.727
Cotton	PC1	.021	.015	2.160	1	.142	1.022
	PC2	.010	.049	.043	1	.836	1.010
	PC3	.056	.067	.688	1	.407	1.058
	PC4	.049	.062	.617	1	.432	1.050
	PC5	-.095	.083	1.321	1	.250	.909
Denim	PC1	.014	.009	2.263	1	.132	1.014
	PC2	.006	.052	.014	1	.907	1.006
	PC3	.035	.031	1.331	1	.249	1.036
	PC4	-.039	.086	.209	1	.647	.961
	PC5	.051	.095	.286	1	.593	1.052
Polyester	PC1	.009	.019	.213	1	.645	1.009
	PC2	-.042	.035	1.468	1	.226	.959
	PC3	.134	.072	3.421	1	**.064**	1.143
	PC4	.017	.102	.027	1	.869	1.017
	PC5	.146	.103	2.029	1	.154	1.158

Cotton:Upon examining the goodness-of-fit values for the model developed for cotton fabrics, it was observed that the five principal components explained 10.4% of the variance in the gender variable according to the Cox & Snell R^2, and 14.0% according to the Nagelkerke R^2. The overall significance test, conducted to evaluate the collective significance of the model coefficients, indicated that the model was not statistically significant ($p > 0.05$). This finding is consistent with the acceptance of the null hypothesis (H_0), demonstrating that the principal components do not have a determinative effect on gender prediction on cotton fabric surfaces. Regarding classification performance, the Correct Classification Rate (CCR) was calculated at 64.0%. The lack of statistical significance suggests that this rate stems from random assignment and indicates low predictive reliability. Evaluation of individual variable contributions revealed that the p-values for all parameters exceeded the significance threshold ($p > 0.05$). Furthermore, although the Odds Ratios (Exp(B)) suggested that components other than PC5 exert a positive effect on the gender variable, these effects were determined to be statistically non-significant.

Denim: In the model constructed for denim fabric surfaces, the explanatory power of the independent variables on the dependent variable was found to be 13.0% according to Cox & Snell R^2 and 17.4% according to Nagelkerke R^2. Similar to the cotton fabric model, the p-value in the global model significance test exceeded the significance threshold ($p > 0.05$), leading to the conclusion that the model is entirely non-significant. The Correct Classification Rate (CCR) calculated for denim fabric remained at 56.0%. This rate indicates that the model's predictive performance is indistinguishable from random chance and does not provide a reliable classification. In the analysis of individual

DOI: 10.5455/NOFOR.2026.04.05

variables, all parameters were found to be non-significant; based on the Odds Ratios, it was determined that all components except for PC4 exerted a positive effect.

Polyester: In the model constructed for polyester fabrics, the coefficients of determination were calculated as 12.7% for Cox & Snell R^2 and 17.0% for Nagelkerke R^2. In the overall significance test, the H_0 hypothesis, which states that 'the independent variables for polyester fabric are insufficient to explain the gender variable,' was accepted; consequently, the model was found to lack statistically significant predictive power ($p > 0.05$). Although the polyester fabric model achieved the highest Correct Classification Rate at 68.0% compared to the other fabric types, this performance lacks statistical significance due to the overall non-significance of the model. None of the individual parameters were found to be significant ($p > 0.05$), and it was determined that all components except for PC2 exerted a positive effect.

General Model and Evaluation: In the general model, which incorporated the entire dataset regardless of fabric type, the explanatory power of the five principal components for the gender variable was determined to be 13.0% via Cox & Snell R^2 and 17.4% via Nagelkerke R^2. Following the overall significance test ($p > 0.05$), it was observed that the independent variables were insufficient for gender prediction, leading to the acceptance of the null hypothesis (H_0). The Correct Classification Rate (CCR) for the general model was 58.0%, suggesting that gender assignment was essentially stochastic and that the method lacks sufficient reliability for forensic applications. Upon individual examination of the model parameters, all p-values were found to be greater than 0.05, indicating non-significance. Nevertheless, based on the Odds Ratios (Exp(B)) it was noted that the PC1, PC3, PC4, and PC5 components exerted a positive effect on the model.

DISCUSSION

This study aims to estimate gender from dried bloodstains on cotton, denim, and polyester fabric surfaces, which are frequently encountered in forensic cases, using ATR-FT-IR spectroscopy and chemometric methods. The study was designed to use blood samples without anticoagulants on selected fabric types, considering worldwide manufacturing volumes and everyday usage prevalence [22,23] to simulate crime scene conditions. Analyses were performed on dried samples [24] and on data where the fabric spectrum was removed as background to eliminate the masking effect of water molecules.

Based on the spectral data studied, it was observed that the physical structure of the fabrics (whether porous or non-porous) directly affects blood absorption and, consequently, the intensity of the spectral bands. In cotton and denim fabrics with a porous structure, the absorption of blood by the fibers reduced the blood concentration remaining on the surface, causing the band intensities in the ATR spectrum to decrease. In contrast, on polyester fabric—characterized by its hydrophobic and non-porous structure—the tendency of blood to exhibit bead formation [25] facilitated the development of a denser blood film. Consequently, this phenomenon allowed for the highest absorption intensity to be obtained from this specific fabric type.

Across all fabric types, the Amide I (1700–1620 cm^{-1}), Amide II (1450–1600 cm^{-1}), and Amide III (1400–1200 cm^{-1}) bands, which signify the protein structure in blood, were distinctly observed. These findings are consistent with similar studies in the literature [7,22]. However, the band observed at approximately 3292 cm^{-1} in polyester fabric, attributed to the Amide A region, could not be detected in cotton and denim fabrics. As noted by DeJong et al. (2015), this is evaluated to result from the masking of the Amide A region by the intense absorption of cellulosic hydroxyl (O-H) bonds inherent in the structure of cotton fabrics [26]. Similarly, while the weak peak at 1112 cm^{-1} in the polyester spectrum indicates the presence of hemoglobin the dominant signals of the substrate in the other fabrics hindered the detection of these specific details [27].

Following the PCA performed to reduce the dimensionality of the spectral data and to explain the variance, it was determined that the first five principal components (PCs) accounted for 97.85% of the total variance. Examination of the PC factor loadings revealed spectral traces of potential biomarkers—including creatinine, cholesterol, glucose, and lipids—that could elucidate sexual dimorphism. The peaks observed in PC1 and PC2 within the 1230–1100 cm^{-1} range, specifically at 1107 cm^{-1} are considered to be associated with the creatinine molecule, which has been reported in the literature to be higher in males than in females [28,29]. Additionally, lipid/cholesterol bands in the 3000–2800 cm^{-1} region contributed to the variance. For PC3, the loadings are explained by gender-dependent physiological differences in glucose and lipid metabolism [30,31] and age-related changes in cholesterol [32]. Given the mean ages of the participants in the study group (Male: 37.8, Female: 36.3), cholesterol levels were expected to be a discriminating factor. However, despite these expected biochemical differences, an analysis of the resulting PC score plots showed that the male and female datasets did not exhibit distinct clustering and largely overlapped. As reported in studies by Takamura et al. and Mistek et al., this situation can be attributed to the inherent heterogeneity and individual variations of biological samples (blood, urine) masking the mean differences between genders [4,33].

In the logistic regression models constructed using the components derived from PCA, it was observed that polyester fabric exhibited a lower '-2 Log Likelihood' value and the highest correct classification rate (68%) compared to cotton and denim fabrics. Although this partial success is attributed to the fact that polyester causes less masking of the spectral data, global significance tests revealed that the p-values for all models remained above the threshold for statistical significance ($p > 0.05$). The results demonstrate that the PCA-based variable selection and the subsequent logistic regression models do not possess sufficient discriminative power for gender prediction under the current sample set and experimental conditions. While an examination of the Odds Ratios indicated positive directional effects for certain components (specifically PC1, PC3, and PC4), these effects were determined to be statistically non-significant

DOI: 10.5455/NOFOR.2026.04.05

The failure to achieve the high accuracy rates targeted in this study stems from several experimental and instrumental constraints;

1. The application of blood to the fabric via the dripping method and the subsequent "coffee ring" effect during the drying process led to a non-homogeneous distribution within the stain. As noted by McCutcheon et al., while dip-coating techniques provide more uniform surfaces, the dripping method was preferred in this study to maintain ecological validity relative to crime scene realities. This approach resulted in high variance among spectra acquired from different regions of the same stain [34].
2. The lack of controlled temperature and humidity during the drying process may have induced degradation in blood proteins and macromolecules, potentially leading to spectral shifts [35,36]
3. The constant pressure applied by the ATR accessory altered the crystal-to-sample contact and, consequently, the penetration depth across fabrics with different thicknesses and weaving characteristics (e.g., denim vs. polyester). This variation complicated the standardization of spectral intensities [11]
4. In this study, PCA—which maximizes variance—was utilized for variable selection. However, to enhance classification success, the use of more advanced "Variable Selection" techniques that perform goal-oriented wavelength selection, such as Genetic Algorithms (GA), is recommended for future studies to eliminate noise and improve model performance [4,37]

Despite the limited classification success achieved in this study due to the spectral masking challenges posed by blood-fabric interactions, recent research in the literature demonstrates that ATR-FTIR spectroscopy and chemometric methods hold high potential for sex determination in forensic sciences. For instance, studies conducted on hair samples have proven that the integrated use of ATR-FTIR spectroscopy and chemometric analyses is highly effective in distinguishing between male and female hair. Similarly, high accuracy rates in sex classification and prediction within a forensic context have been achieved through the chemometric analysis of spectral data obtained from fingernail clippings. Unlike blood or complex body fluids, the combined use of machine learning and ATR-FTIR on solid biological samples with less matrix interference, such as human skin, also offers a novel and successful perspective for gender determination. These findings support the notion that if the substrate masking effect encountered in our study is overcome, or if more advanced machine learning algorithms are employed beyond PCA, ATR-FTIR could achieve similar success in predicting sex from biological traces on textiles [38-41].

CONCLUSION

This study investigated the feasibility of sex determination from dried bloodstains on cotton, denim, and polyester textile surfaces—commonly encountered at forensic crime scenes—utilizing ATR-FTIR spectroscopy and chemometric methods (PCA and Logistic Regression). The findings indicated that the logistic regression models constructed on PCA scores did not produce statistically significant results ($p > 0.05$), and the sex classification accuracies (ranging from 56% to 68%) did not reach the reliability levels required by forensic standards. When comparing fabric types, polyester, which allows blood to remain on the surface due to its hydrophobic nature, provided more distinct spectral data and relatively higher classification success compared to the cellulosic and absorbent cotton and denim fabrics. However, the dominant masking effect of substrate (fabric) signals on the blood spectrum limited the clear differentiation of sex-specific biomarkers (e.g., creatinine, cholesterol) across all surfaces. Furthermore, it was concluded that the heterogeneous distribution of blood during the drying process and physical variables in the sample preparation phase adversely affected model performance by increasing spectral variation. Consequently, under the current experimental conditions, sex determination from blood on textiles via ATR-FTIR spectroscopy has not yet reached sufficient maturity for practical applications. While this study demonstrates the exploratory potential of ATR-FT-IR spectroscopy for this application, the findings must be interpreted in light of certain limitations. Specifically, 50 individuals is relatively limited when considering the extensive natural biological variability present in whole blood. Consequently, subsequent studies utilizing expanded sample sizes are essential to confirm these results and improve the robustness of the predictive models. In future studies, the use of more sensitive sampling techniques to minimize fabric interference and the integration of advanced chemometric algorithms that perform goal-oriented variable selection, such as Genetic Algorithms (GA), may enhance the discriminative power of the method.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical Approval
Approved by the İstanbul University-Cerrahpaşa Faculty of Medicine Clinical Research Ethics Committee (03/12/2020-158845); all participants provided informed consent.

Acknowledgements
The authors would like to thank Murat Yayla and Gülten Rayimoğlu from Istanbul University–Cerrahpaşa, Institute of Forensic Sciences and Legal Medicine, for their support during the blood sample collection process from volunteers. The authors also acknowledge Prof. Dr. Arzu Altın Yavuz and Asst. Prof. Dr. Barış Ergül from Eskişehir Osmangazi University for their support and contributions to the statistical analysis of the study.

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DOI: 10.5455/NOFOR.2026.04.05

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