

MULTI-SPECTROSCOPIC AND CHEMOMETRICS ANALYSIS FOR FORENSIC DETERMINATION OF BLOOD SPECIES

(Multi-Spektroskopi dan Analisis Kemometrik untuk Penentuan Spesies Darah dalam Forensik)

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Abstract

Blood trace is commonly found biological evidence at crime scenes. The main issues forensic investigators encounter upon retrieving blood samples is determining their origin. There is no statistical probability in the current examination methodology of potential blood evidence, and it is deemed subjective. Another method, involving DNA analysis, lengthy to perform and restricted due to its degradation and lack of samples. The main aim of this study was to evaluate the potential and suitability of tandem analysis using ATR-FTIR and UV/Vis spectroscopy as means for the characterization of blood samples in the context of forensic science. Conventional visual examination is insufficient to differentiate and classify spectra between blood from different animal species. Hence, to aid classification in each group, chemometrics analysis of the IR and UV/Vis spectral dataset was employed using the combined techniques of principal component analysis and linear discriminant analysis (PCA-LDA). PCA-LDA results demonstrated that blood spectra of various animal species differed in the compositions of blood protein i.e. amide A, I and II. The multivariate analysis based on PCA-LDA models indicated that ATR-FTIR and UV/Vis spectroscopy coupled with chemometrics provided excellent discrimination (94% correct classification) for the classification of blood samples from animal species. The non-destructive nature of vibrational and optical spectroscopic techniques and the success of chemometrics analysis demonstrated in this work have indeed offered a new dimension for the rapid identification of biomaterials of forensic relevance and essentially warrants further research.

Keywords: spectroscopy, chemometrics, forensic, blood species

Abstrak

Kesan darah merupakan bahan bukti yang biasa dijumpai di tempat kejadian jenayah. Isu utama yang ditempuhi oleh para penyiasat forensik selepas menjumpai sampel darah ialah menentukan asal sumbernya. Kaedah baru iaitu pemeriksaan terhadap bahan bukti darah tidak memberi keputusan yang mempunyai kebarangkalian statistik serta ia dianggap sebagai subjektif. Kaedah lain iaitu analisis DNA, memakan masa yang agak lama untuk dijalankan dan terhad disebabkan degradasi dan kuantiti sampel yang kurang. Tujuan utama kajian ini adalah untuk menilai potensi dan kesesuaian spektroskopi ATR-FTIR dan UV-Vis sebagai satu cara untuk pencirian sampel darah dalam konteks sains forensik. Pemeriksaan visual konvensional tidak mencukupi untuk membezakan dan pengelasan spektrum antara darah spesies haiwan yang berlainan. Justeru, bagi membantu pengklasifikasian dalam setiap kumpulan, analisis kemometrik terhadap data spektrum IR dan UV-Vis telah diaplikasikan dengan menggunakan gabungan teknik analisis prinsip komponen dan analisis diskriminan linear (PCA-LDA). Hasil keputusan PCA-LDA menunjukkan bahawa spektrum darah pelbagai spesies haiwan mempunyai perbezaan dalam komposisi protein darah iaitu amida A, I dan II. Analisis multivariat berdasarkan model PCA-LDA menunjukkan bahawa gabungan spektroskopi ATR-FTIR dan UV-Vis bersamaan kemometrik menghasilkan diskriminasi yang cemerlang (94% klasifikasi yang tepat) bagi pengklasifikasian sampel darah spesies haiwan. Teknik getaran spektroskopi yang tidak memusnahkan sampel dan kejayaan analisis kemometrik yang didemonstrasikan di dalam kajian ini menawarkan dimensi baru bagi pengenpastian bahan biologi dalam bidang forensik dengan lebih relevan dan memerlukan kajian yang lanjut.

Kata kunci: spektroskopi, kemometrik, forensik, spesies darah

Introduction

In a forensic examination, biological evidence has been essential in distinguishing a victim or perpetrator, and even in resolving a criminal investigation [1]. Biological evidence was originated from the human and non-human body. Blood has been the most known biological evidence found in a crime scene [2]. Blood identification has broadly been evaluated in three main stages: visual assessment, presumptive and confirmatory screening, and lastly species identification [3]. The presumptive and confirmatory tests often lead to potential false positives [4]. Determining the blood source has been fundamental throughout the forensic practice, as this could streamline the original inquiry by integrating or excluding non-human stains [5]. Besides, blood traces further assisted in crime scene reconstruction, as bloodstain patterns could provide information to the homicide, such as the suspect's locations during the blood splatter, the source of the blood traces, or the level of effect that the bloodspots were created [6].

DNA profiling is the primary goal for blood trace identification, which requires to extract DNA of blood specimen. Moreover, this method has been expensive, time-consuming and needs the reactants to activate a chemical reaction that results in DNA destruction of a blood specimen [7]. The vibrational spectroscopic study of blood trace is a non-destructive method that tends to be more suitable. The method involves distinguishing the blood samples from the unique spectral peak structure [8]. Attenuated total reflectance - Fourier-transform infrared (ATR-FTIR), Raman spectroscopy and Ultraviolet-visible (UV-Vis) spectroscopy were widely applied in scientific methods of biological fluid determination.

ATR-FTIR spectroscopy has been extensively applied in other research fields for the study of biochemical functions in biomedical, chemistry, forensic science, and pharmaceutical. The utilization of ATR-FTIR has been in demand for its well-known nature for being robust, zero or little usage of solvents, cost-effective and importantly time-saving as were been little or no sample preparation [9] was needed for the analysis. This nature allows the sample to be analyzed in the different forms either in solid, liquid or adsorbed upon a surface [10]. In addition, FTIR spectroscopy was also employed to analyze trace evidence that discovered in the scene of a crime, for examples, vehicle paints [11-12], fake currency [13-14], hair analysis [15-16], and questioned document examination [17-18]. Such a non-destructive characteristic enables multiple uses of the sample without creating no lasting disruption to the DNA biomolecules.

A comprehensive, robust and controlled research case of different human biological fluids involving breast milk, semen, saliva, and blood (menstrual and venous) were tested by ATR-FTIR spectroscopy on various materials such as fabrics, wood and paper. The diverse chemical functions namely immunoglobulins, proteins and, other micro-molecules of the biological fluids differ in spectral variations. The peak frequency relied on the porosity of the fabric substrates. This finding illustrated that the body fluid found at the crime scene can be determined and differentiated depending on the unique peaks variations that match a specific stain [19]. In liquid samples even on materials, the identification and detection of bodily fluids has proven how this method has been significant to forensic practice. Another study was conducted using UV-Vis and ATR-FTIR spectroscopy to estimate the post-mortem interval (PMI), which has been a significant role in a forensic application using blood plasma of male rabbit based on their varied spectral regions like amino acids, proteins, lactate and, fatty acids [20].

The use of Raman spectroscopy in species differentiation was also explored by Mclaughlin et al. [21, 22]. However, his work implied which the bloodstains of human and non-human could not be efficiently identified and differentiated by Raman's technique based on the spectral analysis. Thus, this allows the significance of vibrational spectroscopic methods to be further investigated using chemometrics, a method used to derive accurate data from a specific chemical spectral dataset [23]. This implication was supported by Kaminska et al. [24] in their study that showed by using principal component analysis (PCA), over 98% accuracy in the ABO blood classification can be achieved. Furthermore, ATR-FTIR coupled to chemometrics was proven to be an effective procedure to discriminate, predict and categories human and animal blood samples in species discrimination study [25].

Thus, in this study, ATR-FTIR and UV-Vis spectroscopy were utilized for blood species determination. Blood specimens of different species namely chicken, cow, deer, duck, fish, goat, and pig were analyzed. In order to improve the distinction of the spectral training dataset, the principal component analysis-linear discriminant analysis (PCA-LDA) of the statistical tool was established as predictive models. Accurate categorization and discrimination of different animal blood species were the primary aims of this research.

Materials and Methods

Materials / Solutions

EDTA tubes (BD Vacutainer® 367863, USA), test-tube racks (Thermo Scientific™ 59700030, USA), disposable 5 mL plastic pipettes (Sigma-Aldrich Z740301, USA) gloves (CleanGuard, Malaysia), ice-packs (V-Cool, China), cooler bag (V-Cool, China) and cotton gauze cloth (McKesson, China) were used in this research. Distilled water and acetone (Sigma-Aldrich W332607, USA) were used as cleaning solvents. These materials and solvents were obtained from Unit Pengurusan Makmal Sains (UPMS), School of Health Science, USM, Kelantan.

Samples

Domestic animal bloods were chosen as samples for analysis in this study namely chicken, cow, deer, duck, fish, goat and pig. The total number of blood samples analyzed was 93.

Sample collection, storage and preservation

Fresh animal blood samples were obtained from the slaughterhouses and stored in 10 mL tubes with anti-coagulant ethylenediaminetetraacetic acid (EDTA) for preservation and stored at -20°C in the freezer.

Instrumentation and sample analysis

ATR-FTIR spectrophotometer (Bruker Tensor 27, Germany) and UV-Vis spectrophotometer (Varian CARY 100 Bio UV-Vis, USA) were employed to collect the spectra of blood samples. For the FTIR analysis, the spectra were collected in the ranges from 4000 to 550 cm^{-1} with a 4 cm^{-1} resolution. For UV-VIS analysis, spectra were collected within 800 to 350 nm. Prior the analysis, all blood samples were thawed completely at a room temperature. A clean cotton gauze cloth with acetone were used to clean the ATR ZnSE crystal stage and for UV-Vis, a clean cuvette contained distilled water was used as blank sample. The cuvette was ensured to be cleaned from any fingerprints or dirt. All samples were analyzed in replicates (five times) for each animal species.

Spectral data pre-treatment and chemometric analysis

The spectral dataset was obtained from the both ATR-FTIR and UV-Vis analyses and the data were saved in Excel formats of .CSV as well as .XLS. The raw spectral data then imported into Minitab version 16 software (Minitab Incorporated, State College, USA) to perform chemometrics analysis; principal component analysis-discriminant analysis (PCA-DA). The scores for 20 principal components were extracted from the original spectral datasets which contributed the most to the total variance. These reduced spectral datasets were later included in the discriminant analysis (DA). The spectra data points were truncated in the range of 1900-1500 cm^{-1} (IR) and 600-400 nm (UV-Vis) for chemometrics analysis. Data pre-processing technique namely standardization was carried out on data set to minimize error due to larger variance by subtracting the mean and dividing standard deviation prior to PCA-DA.

Results and Discussion

Figures 1 and 2 illustrate the stacked plot of average ATR-FTIR and visible spectra of blood analyzed from various animal species. The overall ATR-FTIR and visible spectra were visually similar, but few differences can still be observed that related to the strength of peak intensity. The spectral peaks intensity can be sectioned into two regions, broad bands (wide and smoother) and narrow bands (smaller and sharp as dagger) [26]. In ATR-FTIR analysis, for instance, chicken, cow, deer and goat have an intense peak ranged from 1546 cm^{-1} to 1552 cm^{-1} while duck and fish spectra have lower peak intensity which contributed to a vital information about presence of secondary structures of protein known as parallel β -sheet of functional group. Güler et al. [27] described that allotting detail structural configuration to specific protein was challenging because of the functional groups number that lead to peak intensity. In addition, broader peaks were observed at wide range of wavenumbers from 3286 to 3302 cm^{-1} while narrower peaks can be seen at 1638 to 1464 cm^{-1} .

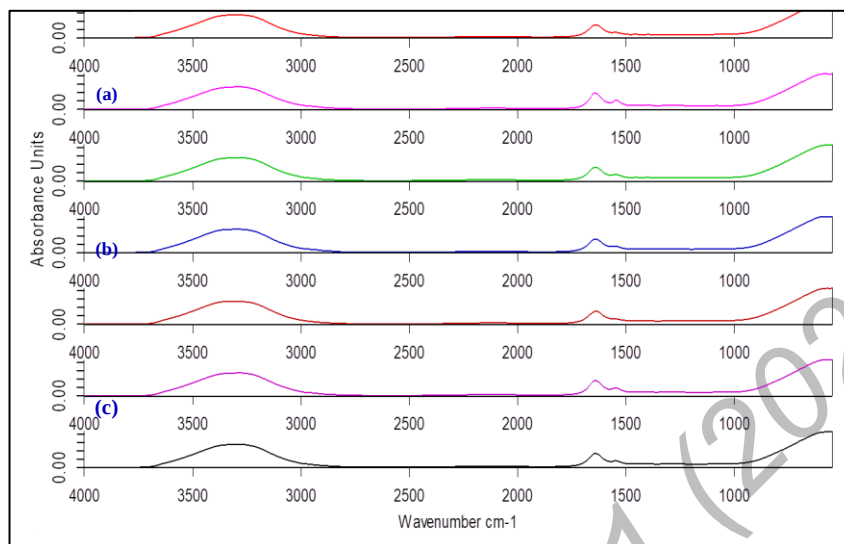


Figure 1. ATR-FTIR spectra of blood from a) chicken, b) cow, c) deer, d) duck, e) fish, f) goat and g) pig

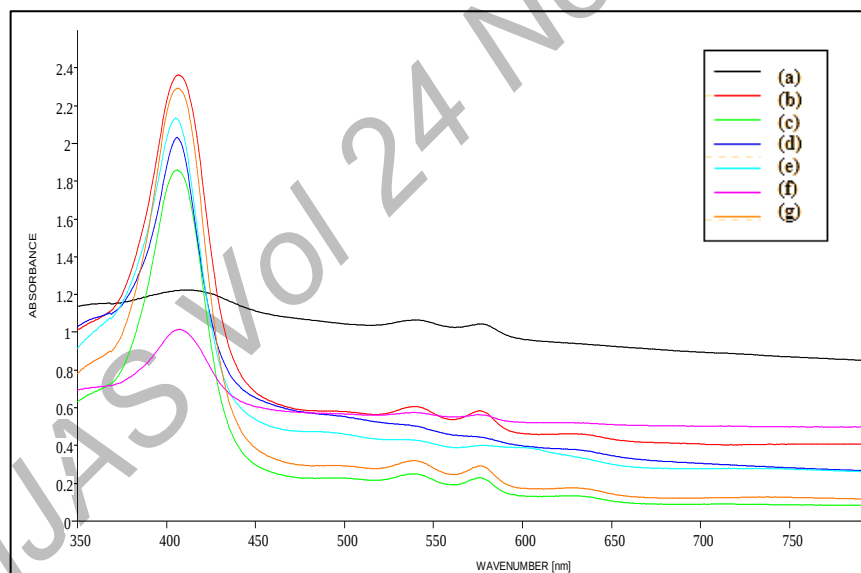


Figure 2. Visible spectra of blood from a) chicken, b) cow, c) deer, d) duck, e) fish, f) goat and g) pig

In visible spectral analysis, the narrower bands intensity of all blood species was predominantly ranged from 410 to 415 nm due to the maximum absorption rate by the samples. The broader bands were observed from 500 to 600 nm ranges. Bahrudeen et al. [28] reported that the stronger band intensity, the more or high specific wavelength being absorbed. Although, all the species showed similar absorption peaks that appeared at same location and same frequency or wavenumber in the both ATR-FTIR and visible spectra, the spectral peak intensity diverged around the spectra and can be classified as strong, medium and weak peaks as demonstrated by Coates [29]. Usually, a medium or weak peak intensity could be identified as smaller peaks with lower or minimal rate of absorption contrasted to strong peaks. Mistek and Lednev [30] reported that in their study, this may be since differences in blood

composition of these species come only from the quantitative changes within the same components, not deviations of components between species. De Wael et al. [31] also reported that visual distinction of these spectra was not possible. The difference between these species referred primarily to the composition of blood such as concentration of haemoglobin, glucose, ascorbic acid and various enzymes and hormones.

All the peaks in the ATR-FTIR spectra represented functional groups. A strong peak observed around 3319 cm^{-1} corresponded to amide-A band which is due to stretching vibration of N-H bond [32]. This amide-A band peak represented the component in the blood. It can be attributed to several possible components that were found in blood, including carbohydrates and lipids [33]. The two major intense band groups were focused at 1650 cm^{-1} and 1533 cm^{-1} within the FTIR range of blood. The Amide I and Amide II were correlating to them. Both groups represented secondary protein structures. Amide I group exhibited due to the formation of symmetry of C = O and stretching vibrations of hydrogen bonds. This group of Amide I provided the most information regarding the protein secondary structure. Whereas, Amide II was assigned to stretching of C-N as well as bending modes of NH and CH₂. The absorption regions Amide I and Amide II have also been affiliated with secondary subunit structures including α -helix, β -sheet, and protein coil. In Figure 2, the peaks characteristics of haemoglobin in visible spectra were observed at around 410, 539, and 577 nm.

Since a mere visual examination of spectra was insufficient to distinguish between them and hence the need for multivariate analysis was required. Despite having similar spectral features, the differences in relative intensities of the peaks/bands in bone spectra from different species exist. These variations can be interpreted using a chemometrics method. PCA was initially performed on the combined ATR-FTIR and visible spectral datasets after the standardization. The first twenty PCs explained most of the total variance in the spectral data and these were extracted to provide PC scores for the LDA input variables. Spectral datasets were analyzed by two common chemometric technique namely PCA and LDA. PCA known for data dimensionality reduction technique which extracted small number of data components from a larger training datasets also looks for pattern or cluster while LDA a computed linear combination of variables was used to determine direction of spectral space and to create models to classify the samples in classes [15].

The PCA score plot for the first two principal components (PC1 vs PC2) is shown in Figure 3. First and second principal components represents 43.3% and 24.0%, respectively of the total variance in the data (cumulatively 67.3%). The overlap between the animal groups was evident which resulted in no distinct cluster to distinguish them. Nevertheless, PCA was found not to be suitable technique in categorization problems as illustrated in Figure 3 because it cannot function well for class information of determining the spectral features extracted of a new dataset [34]. Hence, a supervised technique of PCA-LDA as predictive model to classify them. LDA has been renowned technique that observed a linear expression mode which spectral feature clusters have been most divisible later the transformation [28,35]. Figure 4 depicts the 3-dimensional discriminant function plot. Remarkably, results demonstrated 94% correct classification was achieved among animal species. This result was coherent with Efron's testimonial to employ 90% confidence intervals rather than 99% confidence intervals as the intervals work more best when they are less vivid coefficients [36]. The classification results are tabulated in Table 1. All bloods from duck and fish species were correctly classified to their respective groups. Meanwhile, correct classifications were achieved ranging between 84% to 97% for other animal species. The findings have proven that the combination of multi-spectroscopic analysis and PCA-LDA are effective and valid in species classification.

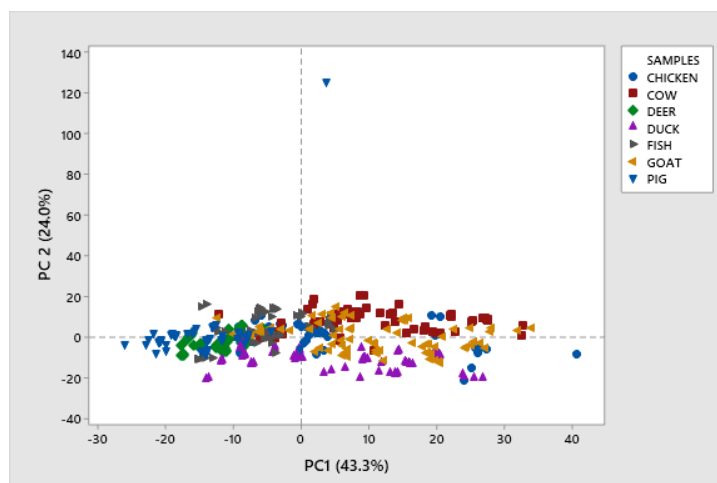


Figure 3. Score plot of PC1 and PC2 for animal blood samples

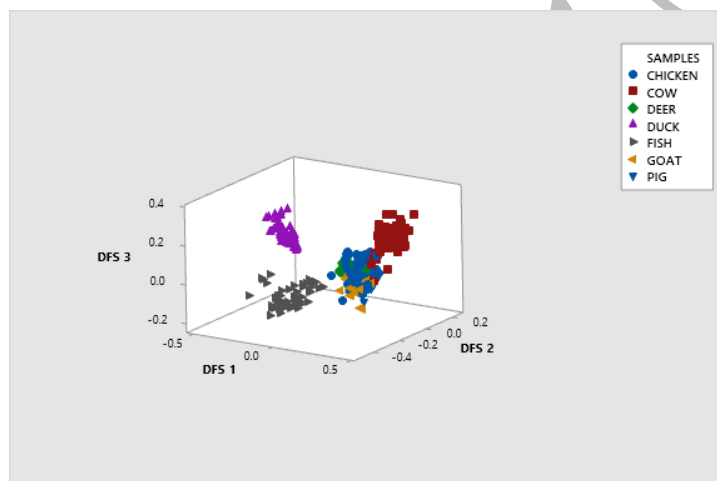


Figure 4. 3D discriminant function (DF) plot for animal blood samples

Table 1. Classification summary of blood species by PCA-LDA

Put into Group	True Group						
	Chicken	Cow	Deer	Duck	Fish	Goat	Pig
Chicken	57	0	0	0	0	4	0
Cow	0	68	0	0	0	0	0
Deer	0	0	57	0	0	5	7
Duck	0	0	0	70	0	0	0
Fish	0	0	0	0	60	0	0
Goat	3	2	0	0	0	67	0
Pig	0	0	3	0	0	4	58
Total N	60	70	60	70	60	80	65
N Correct	57	68	57	70	60	67	58
Proportion	0.950	0.971	0.950	1.000	1.000	0.838	0.892

N = Number of datasets, N = 465, N correct = 437, Total Proportion Correct (%) = 0.940

Conclusion

The primary objective of present study was to investigate the potential and application of multi-spectroscopic and chemometric analysis in forensic blood trace identification for different blood species. The results of this study have highlighted that multivariate analysis of PCA-LDA is an ideal and great tool of blood species identification and differentiation than PCA alone. This has been proven as the findings of PCA-LDA combination versus PCA alone were distinguished, the multivariate combination provides higher successful classification rate which was over 94% accuracy. In summary, the introduction of chemometrics to the vibrational multi-spectroscopic research study was an eminent way of extracting and gathering important records of spectral datasets. In chemometrics, the PCA-LDA technique has been shown to establish a successful classification rate for different animal blood species, although similarities in visual spectral comparison were noted in all spectra with reference to the peak position and pattern. The PCA-LDA models established in this research can be utilized to characterize spectra of unknown samples into a predicted group in forensic casework. Since, the current study has considered a limited number of blood species samples, therefore, further work should be conducted to represent a larger number of samples from various animal blood species and breeds as well as wildlife animals for larger database. In addition, human blood samples from different races and gender can be further investigated and explored for predictive classification model.

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