ORIGINAL ARTICLE

Species Determination and Discrimination of Animal Blood: A Multi-Analytical Spectroscopic-Chemometrics Approach in Forensic Science

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ABSTRACT

Introduction: Non-destructive analysis of biological evidence has been paramount importance in the forensic investigation since it is an effective tool in establishing a standard that could be employed to differentiate ensuing destructive tests of bio-fluids upon sample division between the plaintiff and defendant. Species identification of bloodstain found at the crime scene is very crucial in routine forensic work as this can assist the initial investigation by incorporating or excluding stain that is not human and to identify its origin if animal blood is involved. **Methods:** In this research, identification and discrimination of various blood species collected from seven domestic animals namely chicken, cow, deer, duck, fish, goat, and pig were investigated using non-destructive analytical techniques; ATR-FTIR and visible spectroscopy coupled with principal component analysis and linear discriminant analysis (PCA-LDA) for classification purposes. **Results:** ATR-FTIR FTIR spectroscopic study demonstrated a higher rate of successful classification (≥ 90%) as compared to visible spectroscopic technique. **Conclusion:** ATR-FTIR spectroscopy has been an ideal, robust, and suitable tool for determining the blood species of domestic animals. The predictive model from PCA-LDA analysis can be utilised to produce higher classification rate for species determination from blood traces.

Keywords: ATR-FTIR, Visible, Blood species, Chemometrics, PCA-LDA

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INTRODUCTION

The victim or perpetrator can be distinguished by biological evidence collected from a crime scene. The carefully recovered DNA from biological evidence could be linked to known samples that might identify a perpetrator at the time of the crime. Such known samples are referred to as reference samples. Reference samples shall be obtained from victims except if those who may not want to comply with the request; a court order may be mandated in that sense. The laboratory would then have the questioned and reference biological evidence for comparative study (1).

Blood trace, among other biological evidence, is commonly found at crime scenes (2). Generally, a bloodstain can be characterised by three steps: visual evaluation, presumptive testing, and confirmatory testing (3). A forensic investigator's initial question when confronted with a stain that appears to blood on a scene of a crime would be, "Is it blood?". Then, the colour, shape, odours, and texture of it should be examined and recorded in the notebook. There are several methods of presumptive (chemical colourimetric) tests which can be utilised to indicate the existence of blood (4).

Presumptive tests focus solely on enzyme peroxidase reactivity of haemoglobin (5). Conventional methods used to identify presumptive blood are Kastle-Meyer (KM), Tetramethylbenzidine (TMB), Leucomalachite green (LMG), Hemastix tests (6), and other luminescent tests including luminol (7) and fluorescein (8) tests. The limitation of these tests that they have been potentially false-positive due to contaminant chemical reactions of bleach or different proteins of animals and vegetables (9). Once the investigator has obtained a positive result from the presumptive screening test whether the stain is blood, the next move is to figure out if that is human or non-human blood.

The precipitin ring test allows the detection of the protein present only in the blood of a human (10). Precipitin test is a traditional technique that requires an animal blood serum (anti-serum) which includes antibodies particular to human antigens, and hence if it agglutinates with

one another, it is human blood. This test has greater sensitivity for diluted blood, and the aged bloodstains can be positively identified (4). However, it may have some drawbacks. The test involves the consumption of large quantities of blood serum, and extreme caution must be exercised to prevent solutions mixing and destroying the ring (11).

Other several confirmatory blood tests are microscopic examinations, crystal tests, chromatographic assays, immunological analysis, and spectroscopic techniques (12). The easiest of microscopic examinations facilitates the blood cell determinations by observing the blood directly in a liquid state (13). Another most popular employed confirmatory screenings are Teichman and Takayama crystal tests (14). The Teichman crystal test depends on the hematin production when a dried bloodstain has been heated with the existence of glacial acetic acid and a halide (15). This test produces dark-brown rhombic crystals of hematin chloride and has been likely vulnerable to under and excessive heat (16). The Takayama crystal test has been focused on the hemochromogen production while heating a dried bloodstain under the presence of glucose and pyridine (15). A positive outcome of this test produces reddish-pink needle-shaped crystals of pyridine Hemochromogen (Pyridine ferroprotoporphyrin) (16,17). The disadvantages of these Takayama and Teichman tests that they would only determine when blood is present. However, they could not be used to specify which individual the blood belongs to or the blood type (17).

The analytical spectroscopy techniques appear to be more appropriate for non-destructive analysis of bloodstain (18,19). This method comprises the detection of blood specimens from the distinctive pattern of spectral peaks. The nature of the spectroscopic approaches has been well-known for being simple, robust, quick, zero-solvents, cost-efficient and even easy to move and use in the forensic workplace as they are portable (20). Fourier-transform infrared (FTIR), Raman and Ultraviolet-visible (UV-Vis) spectroscopy are frequently employed as analytical approaches in body fluid detection as their non-destructive feature allows the samples to be examined repeatedly without causing any permanent damages to the sample.

Numerous studies on the detection of bloodstain using attenuated total reflection-fourier transform infrared (ATR- FTIR) spectroscopy were investigated (21-25). Bloodstains on various transparent fabrics were analysed to improve blood infrared detection limit on fabrics based on the blood concentration and fabrics sensitivity by ATR-FTIR. It was concluded that ATR-FTIR had an intense surface sensitivity when the substrate spectrum had been minimised (21).

Simulated case study by ATR-FTIR spectroscopy on different bodily fluids (menstrual and venous blood, breastmilk, saliva, and semen) was carried on five assorted substrates (cotton, glass, nylon, paper, and wood) (22). The different chemical elements (proteins, immunoglobulins, and other small molecules) of the bodily fluids, resulted in differences in their spectra. Range of peak was dependent on the fabric substrate permeability. The study has demonstrated the possibility to identify and distinguish bodily fluid discovered at a crime scene based on the distinctive peaks matching to a particular stain .

The applicability of analytical approaches could be further studied by chemometrics a technique of extracting applicable intelligence from a complex chemical spectral dataset (25). An experimental study has been conducted on the differentiation of human and non-human blood using Raman spectroscopy and demonstrated the application of Raman spectroscopy coupled with chemometric technique of partial least squares discriminant analysis (PLS-DA) for blood species differentiation (26). They analysed sixteen animal blood species and forty-nine human blood samples for their study. The PLS-DA model reported being a successful technique as the results show a 99% accuracy rate in random blood species classification.

A similar case study was performed by Lin et al. (27) on bloodstain species detection by ATR-FTIR spectroscopy combining with the multivariate statistical operation of PLS-DA method. They established two PLS-DA categorisation predictive models for three categories such as human, domestic fowl and mammals as well for species discrimination.

Hence, this study was aimed to investigate the applicability of analytical spectroscopic techniques, namely ATR-FTIR spectroscopy and visible spectroscopy in species determination and discrimination of blood traces. Blood samples from various domestic animal species were analysed in this study, while the human blood sample was used as a preliminary study for the parameter optimisation and validation purposes. The principal component analysis-linear discriminant analysis (PCA-LDA) classification models were developed to best enhance the differences in the spectral training dataset. Correct classification and differentiation of individual species of animal blood samples were targeted in this study for forensic discrimination.

MATERIALS AND METHODS

Sample selection, method, and preservation

Human Blood

For the pilot study, the human venous blood sample was employed to test the optimisation and validation method. The human blood was obtained from six consented volunteers by the phlebotomist. An aliquot of 3 mL whole blood was collected from each donor. The

freshly drawn blood samples were instantly transferred into vacutainer tubes that contained anticoagulant ethylenediaminetetraacetic acid (EDTA) and kept in the freezer at -20°C.

Animal Blood

In this current study, domestic animal blood specimens were chosen. The blood samples were obtained from seven animal species, namely chicken, cow, deer, duck, fish, goat, and pig. These chosen animal species were widely related in forensic crime since they can be (i) consumed by the human on a daily basis, (ii) notable to develop false-positive result when screened with presumptive blood tests, or (iii) involved in wildlife or hit and run crime.. Twelves blood samples were obtained from each animal species (n=12). Total 84 number of animal blood samples were collected. The fresh whole animal blood samples were collected from abattoir and stored in 10 ml tubes of EDTA preservative with labels at -20°C in a freezer prior to analysis. As for sampling size, a convenience sampling design method was employed in this study.

Instrumentation

This study employed ATR-FTIR spectrophotometer (Bruker Tensor 27, Germany) and UV-Visible spectrophotometer (Varian CARY 100 Bio UV-Vis, USA) for spectral measurements of blood traces. The spectra ranges were from 4000 cm-1 to 550 cm-1 and 800 nm to 350 nm for ATR-FTIR and visible spectroscopy analysis, respectively.

Sample preparation

For ATR-FTIR analysis, the ATR diamond crystal stage was cleaned with acetone solution using a clean cotton gauze cloth. The single beam background scan was performed without any sample on the ATR crystal for spectral acquisition. Then, an aliquot of 5 µl liquid blood sample of each animal species was deposited homogeneously on the centre of ATR crystal. The EDTA preserved whole blood samples were analysed under direct FTIR spectra analysis. For each animal blood species, five replicate spectra were recorded for spectral reproducibility of the precision parameter.

In visible spectroscopy, the diluted whole blood (500 µl blood + 500 µl distilled water) was analysed by UV spectrophotometer. The blood samples were diluted because the absorption was very strong in the visible region. A clean cuvette containing distilled water has been used as a standard blank solution in the study. Similarly to ATR-FTIR, five spectra were collected from each animal species.

Prior to instrumental optimisation, all the blood samples allowed to thaw completely at room temperature. A clean cuvette and crystal stage in respective spectroscopic analysis is crucial to avoid any sample contaminations during the sample to sample analysis. Concerning the red blood cells (RBC) temperature and transportation duration, the EDTA-preserved whole blood samples were used as study samples rather than fresh whole blood samples. The EDTA-anticoagulant retains the stability of different haematological cells of RBC structure (28).

Spectral data processing and multivariate data analysis

Multivariate data analysis or chemometrics has been a great method to extract and classify chemical information of a data when sample mixtures were involved in an experimental procedure. The combination of spectroscopic studies with chemometrics was assessed for differentiation of blood samples from various species. The visual differentiation sensitivities in seven species of animal blood samples were analysed using OPUS 7.5 software for FTIR analysis while Varian Cary WinUV 4.1 version software for visible spectroscopic analysis.

Prior to chemometrics analysis, a data pre-treatment procedure was executed on spectral datasets followed by PCA and LDA using Minitab® 16 version statistical program. Standardisation, a process where the mean has subtracted and divided by the standard deviation. Twenty principal component scores (PCs) that contributed the most to total variance among animal blood spectra datasets were selected. These PCs scored were later applied as input data for LDA. PCA is generally utilised to compute a correlated variable into a smaller cluster of spectral data whereas LDA is applied to make a further comparison of these clusters by constructing 3-dimensional (3D) scatterplot models and ultimately characterising blood samples accordingly.

RESULTS

Parameter optimisation and validation for ATR-FTIR blood analysis

Optimisation of parameters and method validation for ATR-FTIR analysis was carried out using human blood. For the optimisation study method, it has been important to know the right resolution and number of background scan. The resolution of 2 cm^{-1}, 4 cm^{-1}, 6 cm^{-1} and 8 cm^{-1} were tested one by one with different background scan time.

The resolution of 4 cm^{-1} and 32 number of scans were chosen as the best ATR-FTIR parameters for liquid blood sample analysis. The benefit of this chosen parameter is lower the resolution, the lower spectral noise and shorter the time of spectral acquisitions (29). This is because the light intensity upon reaching the detector will be decreased, enhancing the relative value of spectral noises to be increased. Hence, it is unsuitable of resolution to be higher than required (30).

While for validation parameters, the sensitivity; limit of detection (LOD) was observed at 1:512 dilution and limit of quantification was at 1:256 (Figure 1). The

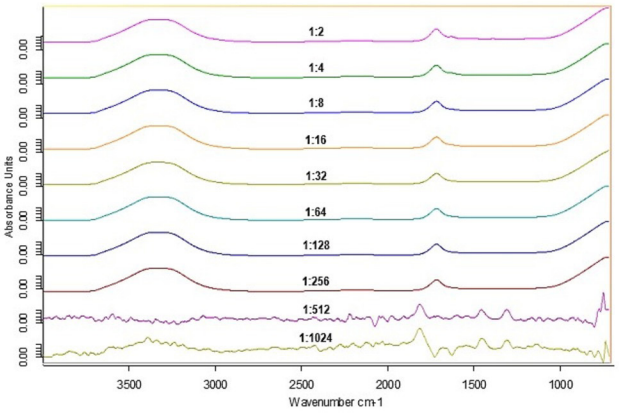

Figure 1: ATR-FTIR spectra of 10-fold dilutions of human blood from 1:2 to 1:1024

method has demonstrated better analytical precision of the repeatability and reproducibility in intra-day and inter-days tests (Figures 2 and 3).

Five spectra of blood were collected from each animal species. Total 840 spectra were obtained using both ATR-FTIR and visible spectroscopy which have been further discriminated and classified using advanced chemometric techniques.

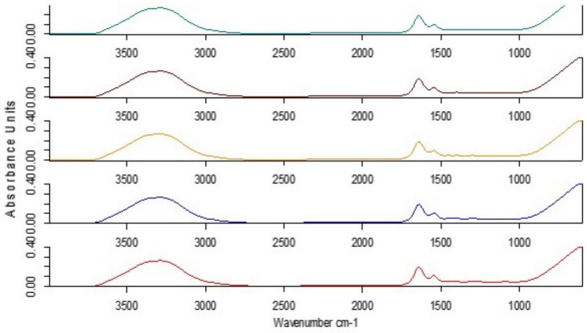

Figure 2: ATR-FTIR spectra of human blood sample (Intra-days Test)

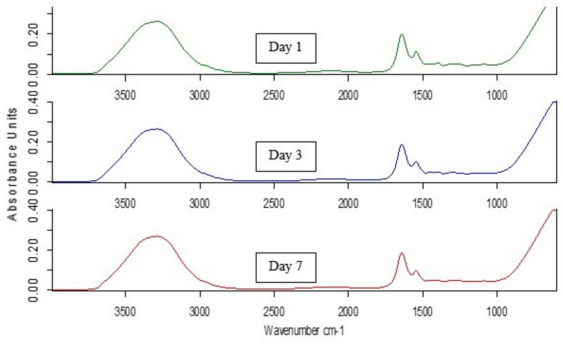

Figure 3: ATR-FTIR spectra of human blood sample on Day 1, 3 and 7 (Inter-days Test)

The distinction of blood species by ATR-FTIR spectroscopy

Interestingly, of all 420 spectra collected from animal blood samples, 94% (395/420) of animal blood spectra were grouped accurately to their respective species. 100% (60/60) of duck spectra were correctly classified to their group. Among deer and fish species spectra, about 97% (58/60) of deer and fish spectra were grouped correctly with only two of deer spectra were mistaken for pig species, and the two of fish spectra were misclassified as chicken and goat species. As for cow species, 96% (58/60) were categorised perfectly. Two of cow spectra were misidentified as chicken and goat species. About 95% (57/60) of chicken spectra were classified accurately with two misidentifications for cow species and one as a goat species. 92% (55/60) of pig spectra were grouped accordingly to their species whereas five spectra were misclassified as deer species. 83% (50/60) of goat spectra were correctly classified with ten spectra were mistaken for pig blood samples.

The distinction of blood species by visible spectroscopy

The rate of accuracy in the classification of the animal spectral dataset by visible spectroscopy was reduced as compared to that of using ATR-FTIR. About 88% (370/420) of animal blood spectra were accurately categorised to their species. All spectra of chicken and duck species were correctly classified (100%) to their respective groups. As for pig species, about 97% (58/60) of spectra were identified correctly. Two of pig spectra were mistaken as deer species. For fish species spectra, 91.7% (55/60) were correctly classified while the five fish spectra were misclassified as goat species. About 78% (47/60) of goat spectra and 77% (46/60) of deer spectra were grouped accordingly. Thirteen of goat species were misclassified as cow, deer, and pig, while 14 of deer species were mistaken as pig spectra. 73% (44/60) of cow species was identified correctly to their species, but the remaining spectra were misclassified either as chicken, pig, goat, or deer.

The PCA-LDA of ATR-FTIR spectral dataset for animal blood samples

The score plot of two principal components; principal component one (PC1) and principal component two (PC2) is illustrated in Figure 4. PC1 and PC2 contributed 68.9 % and 21.5% of the total variance in the datasets, respectively. The cumulative percentage of the total variance of PC1 and PC2 was 90.5%. It was observed that PCA was not discriminative enough for a larger spectral dataset. Consequently, the blood spectra were scattered and not clustered in their respective groups. The overlaps between animal species were also observed, which formed one larger cluster. Since the differentiation was not subtle, the scores for twenty principal components extracted from animal blood spectral datasets were further analysed using LDA as PCA-LDA predictive tools to characterise them in a more conclusive manner. PCA-LDA has successfully classified the blood spectra to their

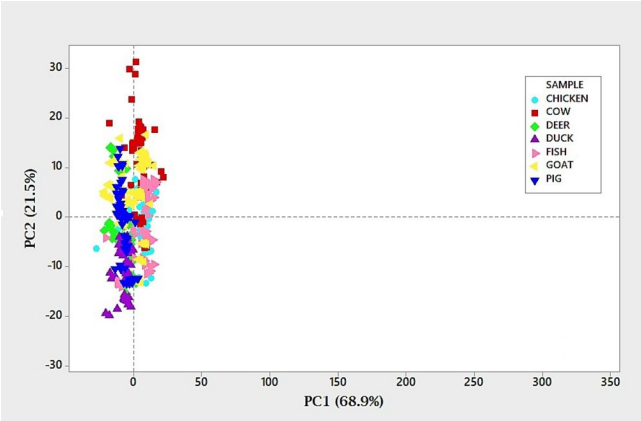

Figure 4: Score plot of PC1 vs PC2 for animal blood samples analysed using ATR-FTIR spectroscopy

species with 94% correct classification. Figure 5 displays the 3D discriminant functions (DF) scatterplot of animal blood samples analysed using ATR-FTIR spectroscopy.

PCA-LDA of the visible spectral dataset for animal blood samples

The score plot of two principal components; principal component one (PC1) and principal component two (PC2) is shown in Figure 6. PC1 and PC2 contributed 85.1 % and 13.1% of the total variance in the datasets, respectively. The cumulative percentage of the total variance of PC1 and PC2 was 98.2%. Although the cumulative percentage of the total variance was higher, yet the animal blood spectra cannot be distinguished distinctively among the seven animal species as they were observed to be scattered and overlapped with one another. Since discrimination was not evident, the score of twenty principal components of animal blood extracted from visible spectral datasets were further analysed using PCA-LDA predictive models to categorise them distinctly. Among all analysed samples, 88% were correctly classified using PCA-LDA. Figure 7 shows the 3D discriminant functions (DF) scatterplot of animal blood samples collected using visible spectroscopy.

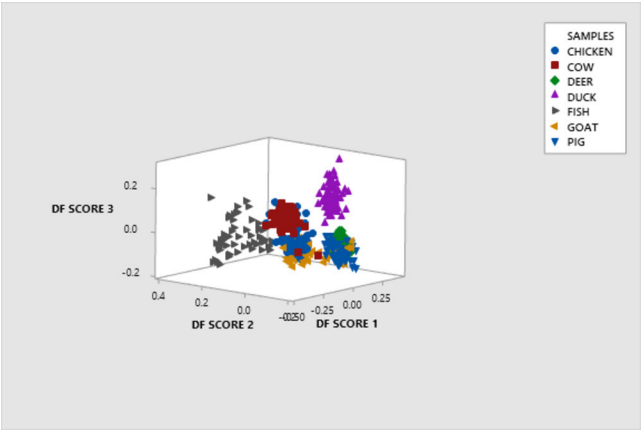

Figure 5: 3D scatterplot of DF scores for animal blood samples analysed using ATR-FTIR spectroscopy

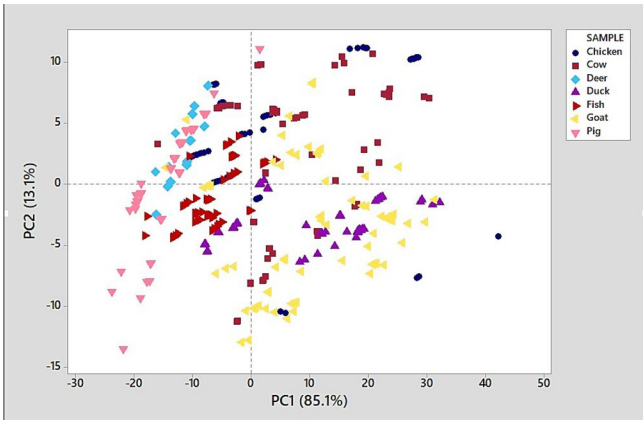

Figure 6: Score plot of PC1 vs PC2 for animal blood samples analysed using visible spectroscopy

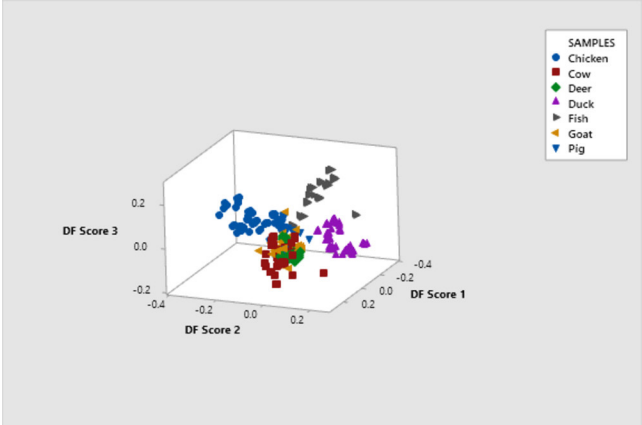

Figure 7: 3D scatterplot of DF scores for animal blood samples analysed using visible spectroscopy

DISCUSSION

Seven blood species were studied using the combination of a multi-analytical spectroscopic approach (ATR-FTIR and visible spectroscopy), and chemometrics since a simple visual examination of spectra was insufficient to differentiate them individually. Therefore, the demand for multivariate analysis was necessitated. Regardless of featuring similar spectral characteristics, the variations from band or peak intensities of the blood spectra from distinct animal species exist. These deviations can be represented by a chemometrics technique.

Total twenty PCs were added in LDA for categorisation, and six discriminant functions were produced for the analysis. The performance index data of ATR-FTIR and visible spectroscopic analyses were 94% and 88%, respectively. Espinoza et al. (31) suggested in their study that the performance index above 90% was reliable and robust for classification.

Some classification errors between different species were observed. All those misidentifications might be induced by the complying causes: (i) the blood composition of the misclassified species are almost

alike that the FTIR and visible spectra among them are identical; (ii) the individual blood samples of FTIR and visible spectra datasets are unbalanced for spectral analysis; (iii) when developing the method, there is a variance in the data fitting. These may be due to the random training process that affects the model. Thus, maintaining a consistent data acquisition process, data preprocessing, and parameter model optimisation are crucial for good classification performance (32). Apart from these, a significant amount of blood samples was efficiently identified with great accuracy.

ATR-FTIR and visible spectral features of animal blood samples

The infrared spectra of blood samples comprise of three major peaks, including two minor peaks. The first major peaks for animal blood species are observed in the ranges of 3325 cm-1 to 3286 cm-1, which are assigned to the stretching vibration of hydroxyls (O-H) group (33). The second and third dominant peaks in animal blood species are noted from 1642 cm-1 to 1639 cm-1 and from 1559 cm-1 to 1545 cm-1, corresponding to Amide I and Amide II proteins, respectively (18,22). While, the other two minor peaks at 1463 cm-1 to 1454 cm-1 and 1416 cm-1 to 1399 cm-1 are assigned to scissoring mode of CH2 and symmetric bending vibration of methyl (CH3) groups, respectively (33).

There are one major and two smaller peaks of absorption are observed in visible spectra of blood. The prominent haemoglobin-linked peak is observed at wavelengths of 415 nm to 405 nm. The absorption at those wavelengths was associated with a greater number concentration of free haemoglobin in the animal blood samples (34, 35). The medium absorption peak at 577 nm has been correlated with proteins in the animal blood samples associated with the amino acids such as tyrosine and tryptophan (36). The other protein peak is noted at a wavelength of 540 nm, corresponding to the amide backbone (37).

Zailer et al. (38) have reported that humans and several vertebral bloods also have common characteristics, including those of the three blood cell types namely RBC, white blood cells (WBC) and platelets. The more similar the animal species with humans, the greater will be the blood overlap. The variations in the blood composition levels such as albumin, amino acids, glucose, proteins, lipoproteins, and peptides can distinguish human from animal blood species. Human and animal species of RBC are majorly composed of haemoglobin components, an iron carrying metalloprotein, in which certain animals apply various binders depending on iron or copper (hemocyanin) for oxygen transportation (39). All these compounds affect the FTIR and visible blood spectral fingerprint regions in this study.

As mentioned in a previous study, a 100% accurate classification was achieved for blood species discrimination using proton nuclear magnetic resonance spectroscopy (1H NMR) combined with multivariate analysis of PCA. However, this 1H NMR spectroscopy method is limited to a small number of study samples that the blood species of elephant and bison were not included in the PCA for discrimination (38,40). Thus, the human blood was only discriminated from cats and dogs blood species. Unlike, in the present study, a larger number of animal blood samples are used in species classification.

Previous researchers have proven 100% precision rate in blood species differentiation by combining Raman spectroscopy with Kernel PCA as a chemometric technique. Their model showed excellent blood species categorisation among human and non-human blood specimens. Despite that, it was not easy to precisely interpret the outline from each principal component, and this chemometric tool requires complex preparation of datasets which is time-consuming (26,41). In this present study, a quicker and easier datasets preparation namely factor analysis, covariance matrix, eigenvalues, and eigenvectors are applied in this chemometric method of PCA-LDA for blood traces identification while Kernel PCA applies complicated algorithms in dimensionality reduction for classification purposes.

Identification of blood species was also presented by Zhang et al. (42) by employing near-infrared diffuse transmitted spectra with the advanced chemometric approach of PLS-DA model to distinguish three species of blood such as human, macaque, and mouse. Their PLS-DA model presented 100% accuracy for the three blood species samples. However, this technique does not support detailed information of chemical components of each blood class but focused solely on the differences in spectroscopic mathematical of whole components.

There also have been many research studies conducted in the previous years for blood species distinction with other spectroscopic approaches combined multivariate analysis. Such to that were, Espinoza and team members have proposed an electrospray-ionising mass spectrometry technique for the rapid analysis of blood samples to identify and differentiate species (43). Another research on determination and discrimination of bloodstains among human and non-human blood was distinguished by portable Raman spectroscopy coupled with chemometric analysis. Eleven blood species (cat, chicken, cow, dog, horse, mouse, pig, rabbit, rat, sheep and human) were discriminated and identified using PCA chemometric tool (44). A study carried out by Mistek et al. (25) had distinguished between human and animal blood samples combining ATR-FTIR with PLS-DA models. The study utilised three blood species (cat, dog, and human) and PLS-DA models were developed to separate and characterise the blood species. According to the author, the theory of blood species differentiation is unique between every spectroscopic method, and

the sample quantity also varies for each method. Our study has further demonstrated that the application of ATR-FTIR spectrocopy coupled with chemometrics resulted in a higher rate of successful classification of animal blood species (≥ 90%) as compared to visible spectroscopic technique.

CONCLUSION

The main goal in the current research was to examine the potential application of analytical spectroscopic analysis for species differentiation of animal blood samples in forensic blood traces identification. The results demonstrated that ATR-FTIR spectroscopy had been an ideal technique and suitable tool for determination and discrimination of blood traces analysis as compared to visible spectroscopy with the former showed a higher number of a successful classification of animal blood species. PCA-LDA classification using ATR-FTIR and visible spectral dataset demonstrated an accuracy of 94% and 88%, respectively. The combined application of chemometrics and spectroscopic analysis has been a prominent way in extracting and gathering the key information of spectral dataset. The PCA-LDA method in chemometrics analysis has been proven to produce a higher correct classification of blood from various animal species, although the spectral features are similar via visual spectral observation.

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