BIOSPECTROSCOPY WITH CHEMOMETRICS ANALYSIS OF BLOOD FOR SPECIES IDENTIFICATION AND AGE ESTIMATION

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BIOSPECTROSCOPY WITH CHEMOMETRICS ANALYSIS OF BLOOD FOR SPECIES IDENTIFICATION AND AGE ESTIMATION

by

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LIST OF SYMBOLS

%	Percentage
~	Tilde (approximately equal)
≥	Greater than or equal to
®	Registered sign/brand
™	Trademark sign
ℓ	Litre
µℓ	Microlitre
µm	Micrometre
cm^{-1}	Reciprocal centimetres
mℓ	Millilitre
nm	Nanometre
^{o}C	Degree Celsius
R^2	Coefficient of determination
α	Alpha
β	Beta
d	Day
m	Month
y	Year

LIST OF ABBREVIATIONS

ATR	Attenuated total reflection
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared
BPA	Bloodstain Ageing Analysis
DA	Domesticated-Animals
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
FTIR	Fourier-transform infrared spectroscopy
Hb	Haemoglobin
HbO_2	Oxyhaemoglobin
HC	Hemichrome
IR	Infrared
LDA	Linear Discriminant Analysis
LOD	Limit of detection
LOQ	Limit of quantification
MetHb	Methemoglobin
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
N	Number of samples
NMR	Nuclear Magnetic Resonance
OxyHb	Oxyhaemoglobin
PCA	Principle Component Analysis
PCA-LDA	Principle Component Analysis-Linear Discriminant Analysis
PLS	Partial Least Square

PLSR	Partial Least Square Regression
PLS-DA	Partial Least Square-Discriminant Analysis
PMMA	Polymethyl methacrylate (acrylic)
PMI	Postmortem Intervals
RBC	Red blood cells
R^2	Coefficient Determination
RMSEC	Root Mean Squared Error Calibration
RMSEP	Root Mean Squared Error Prediction
RNA	Ribonucleic acid
RSD	Relative standard deviation
TSD	Time since deposition
UV/Vis	Ultraviolet/Visible
WBC	White blood cells
WHB	Whole human blood
3-D	Three-Dimensional

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BIOSPEKTROSKOPI DENGAN ANALISA KEMOMETRIK DARAH UNTUK PENGENALPASTIAN SPESIS DAN PENGANGGARAN USIA

ABSTRAK

Pengenalpastian spesies dan penanggaran usia kesan darah yang tepat adalah alat penting dalam siasatan forensik, menyediakan maklumat yang tidak ternilai yang boleh memberi impact yang signifikan kepada keputusan kes. Walau bagaimanapun, kaedah sedia ada mencabar kerana prosedur yang merosakkan dan melelahkan. Tesis ini mengkaji keberkesanan teknik biospektroskopik dan integrasi kemometri multivariate untuk penentuan spesies dan usia kesan darah dengan menggunakan sampel daripada lapan jenis spesies: manusia, lembu, ayam, rusa, itik, ikan, kambing dan khinzir. Kajian ini menggunakan analisis tandem daripada Jumlah Pemantulan Terlemah-Spektroskopi Inframerah Transformasi Fourier (ATR-FTIR) dan Ultraungu-Boleh Nampak (UV/Vis), yang dilengkapi dengan Analisis Komponen Utama Dan Analisis Diskriminan Linear (PCA-LDA) untuk menentukan spesies kesan darah. Model PCA-LDA untuk spektroskopi inframerah dan boleh dilihat telah dibina dan menunjukkan perbezaan lengkap antara spektrum manusia dan haiwan. Keputusan menunjukkan bahawa spektroskopi ATR-FTIR mengatasi UV/Vis dengan ketara dalam membezakan darah manusia dan haiwan. Dengan ketepatan pengkelasan 98.3%, model PCA-LDA inframerah berjaya membezakan antara pelbagai spesies, termasuk pengkelasan yang sempurna untuk manusia, ayam, lembu, itik dan ikan. Untuk analisis usia kesan darah in-situ, tompok darah manusia dan haiwan telah disediakan dan disimpan di bawah dua keadaan: di dalam dan di luar. Tompokan kesan darah ditempatkan pada sepuluh substrat berliang dan tidak berliang selama setahun

untuk mensimulasikan tempat kejadian jenayah. Menggunakan spektroskopi ATR-FTIR, dua band protein darah utama (Amides I dan II) boleh dikesan dengan berjaya dalam spektrum darah segar dan lama sehingga satu tahun. Sebanyak 160 model Regresi Kuasa Dua Terkecil Separa (PLSR) telah dibina, dengan prestasi ramalan yang amat unggul terutama bagi tompokan darah luar (RMSE:~0.29-2.42; R^2:~0.56-0.99) berbanding dengan model dalaman (RMSE:~0.51-3.28; R^2:~0.20-0.98). Skor kesilapan purata persegi yang lebih rendah (RMSE) dan skor R^2 yang tinggi untuk tompokan darah pada semua sepuluh substrat, tanpa mengira spesies, meningkatkan praktikaliti penyelidikan ini. Model-model ini digunakan lebih lanjut untuk mewujudkan model Kuasa Dua Terkecil Separa-Analisis Diskriminan (PLS-DA), menunjukkan keupayaan pengkategorikan yang luar biasa sehingga ~99% (dalam) dan ~98% (di luar) bagi kesan darah yang lama pada permukaan porus dan tidak-porus. Kedua-dua hasil pengesahan menunjukkan potensi yang menonjol dan diskriminasi yang boleh dipercayai secara statistik untuk menganggarkan usia tompokan darah pada pelbagai substrat, terutamanya untuk jangka masa penuaan luar dan lebih lama. Secara ringkasnya, kajian ini membuktikan bahawa mengintegrasikan spektroskopi ATR-FTIR dengan kemometrik multivariat menyediakan strategi yang tidak invasif, menyeluruh dan cepat untuk menentukan spesies dan usia kesan darah dalam siasatan forensik dengan aplikasi praktikal yang menjanjikan dalam kes sebenar yang memberi manfaat kepada komuniti forensik dan penguatkuasaan undang-undang.

BIOSPECTROSCOPY WITH CHEMOMETRICS ANALYSIS OF BLOOD FOR SPECIES IDENTIFICATION AND AGE ESTIMATION

ABSTRACT

Accurate species identification and age estimation of bloodstains are indispensable tools in forensic investigations, providing invaluable information that can significantly impact case outcomes. However, existing methods are challenging due to their destructive and strenuous procedures. This thesis explores the effectiveness of biospectroscopic techniques and multivariate chemometrics integration for species and age determination of bloodstains, using samples from eight species: humans, cattle, chicken, deer, duck, fish, goat, and swine. The present study deployed tandem analysis of attenuated total reflection-Fourier transform infrared (ATR-FTIR) and ultraviolet-visible (UV/Vis) spectroscopy, complemented by principal component analysis and linear discriminant analysis (PCA-LDA) to determine bloodstain species. The PCA-LDA models for infrared and visible spectroscopy were built and showed complete differentiation between human and animal spectra. The results demonstrated that the ATR-FTIR spectroscopy significantly outperformed visible spectroscopy in discriminating human and animal blood. With a classification accuracy of 98.3%, the infrared PCA-LDA model effectively distinguished between various species, including a perfect classification for humans, chickens, cattle, ducks, and fish. For in-situ bloodstain age analysis, human and animal blood spots were prepared and stored under two conditions: indoors and outdoors. The blood spots were deposited on ten porous and non-porous substrates over one year to simulate a crime scene. Utilising ATR-FTIR spectroscopy, two major blood protein bands (Amides I and II) can be successfully detected in fresh and aged

blood spectra for up to one year. A total of 160 partial least squares regression (PLSR) models were developed, with superior predictive performance observed for outdoor bloodstains (RMSE: ~0.29-2.42; R^2: ~0.56-0.99) compared to indoor ones (RMSE: ~0.51-3.28; R^2: ~0.20-0.98). Lower predictive Root Mean Squared Error (RMSE) and high R^2 scores for bloodstains on all ten substrates, irrespective of species, enhanced this research's practicality. These models were further applied to create partial least squares-discriminant analysis (PLS-DA) models, demonstrating outstanding categorisation ability up to ~99% (indoors) and ~98% (outdoors) for aged blood spots on porous and non-porous surfaces. Both validation results showed prominent potential and statistically reliable discrimination for estimating the bloodstain age on various substrates, notably for outdoor and longer ageing terms. In summary, this research proves that integrating ATR-FTIR spectroscopy with multivariate chemometrics provides a non-invasive, conclusive and rapid strategy for determining the species and age of bloodstains in forensic investigations with promising practical applications in real cases benefiting the forensic and law enforcement communities.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Bloodstains are ubiquitous at offence locations and an important information source for forensic investigators. Examining bloodstains provides crucial details about the crime, such as the victim's or perpetrator's identity, the chronological order of events, and the offence's timing. Species identification and age estimation are two main aspects of bloodstain analysis.

Species identification, a pivotal process that discerns human from animal blood, wields significant power in the investigative process. It serves as a compass, guiding the investigation and narrowing the pool of potential suspects. Equally important is age estimation, which establishes a chronological sequence of events in relation to the crime. The forensic analysis of blood, in this context, serves to validate witness statements, establish alibis, and aid in reconstructing crime scenes (Primorac and Schanfield, 2023; Jabor *et al.*, 2024).

Nevertheless, both the process of identifying species and estimating age have distinct difficulties. Species identification can be challenging due to minute blood samples, contamination, or damaged DNA. Age estimation is an intricate procedure affected by various environmental conditions, including temperature, humidity, light exposure, and the surface on which the blood is found (Refn *et al.*, 2023). The variables mentioned substantially influence the rate at which bloodstains deteriorate, which poses a challenging problem in accurately determining their age.

Despite these challenges, advancements in forensic science have led to the development of sophisticated techniques for species identification and age estimation.

By overcoming these hurdles, investigators can extract maximum value from bloodstain evidence, contributing to successfully resolving criminal cases.

1.2 Forensic Blood Evidence

Blood comprises a diverse array of biological and chemical components that can be meticulously analysed to yield crucial insights at various levels of identification. This invaluable information is pivotal in unravelling the sequence of a crime and accurately determining the timeline of events. Blood samples can undergo various sophisticated testing methods in blood analysis. However, in the forensic context, the primary analyses are presumptive screening, which indicates the presence of blood (Cano-Trujillo *et al.*, 2023) and DNA profiling, which is a cornerstone in confirming the identity of the individual associated with the blood sample (Wei *et al.*, 2023). An in-depth comprehension of the composition of these fundamental blood components is imperative for deploying precise and effective analysis techniques. This understanding serves as the foundation for thorough and meticulous forensic investigations, an essential step in the pursuit of justice.

1.2.1 Human Blood Characteristics

Human whole blood has four main constituents, which are red blood cells (RBCs), white blood cells (WBCs), platelets and plasma. It is a fluid connective tissue with a blood cell matrix of 45% and a plasma matrix of 55%. Approximately 7 to 8% of an individual's total body weight is blood, where about 4 to 5ℓ blood volume is for females and 5 to 6ℓ blood volume is for males (Scanes, 2022).

Red blood cells (RBCs) or erthrocyctes are bright-reddish in colour and the most overflowing blood cell for nearly 40 to 45% of their volume. The RBC has a

biconcave disc shape with the centre flattened due to the lack of a nucleus. RBCs comprise a particular protein named haemoglobin (Hb), which is rich in iron (Litwack, 2022). The primary function of haemoglobin in RBCs is to supply oxygen from the lungs to various organs and tissues of the body, then reverts carbon dioxide to the lungs from the body for the exhalation process. Besides, the red pigment from the Hb protein is made up of hemes which gives the blood its red colour (Wiegand and Moreno, 2024).

White blood cells (WBCs) or leucocytes are vital in the immune system's defence and are located in the bloodstream. Unlike RBCs, they possess a nucleus and hence shield DNA. WBCs present in lower concentrations in blood (4000-11000 per $\mu\ell$) compared to RBCs, and their leading role is to combat disease and illness (McNevin and Padula, 2023). WBCs can be divided into two main groups: granulocytes and agranulocytes. Granulocytes, characterised by the presence of granules in their cytoplasm, include neutrophils, eosinophils, and basophils (Nair *et al*., 2022). Neutrophils, the most prevalent type, play a crucial role in the body's defence against bacteria and fungi. Conversely, eosinophils are primarily involved in allergic reactions and parasite defence, while basophils release histamine and other inflammatory chemicals (Carter, 2024). In contrast, agranulocytes, such as lymphocytes and monocytes, do not have visible granules. Lymphocytes are key players in the immune response, producing antibodies and attacking infected cells, while monocytes, larger in size, can transform into macrophages, which are responsible for removing debris, dead cells, and pathogens (Naeim *et al.*, 2024).

Platelets or thrombocytes, play a vital function in blood clot formations, producing an external seal over an injury and releasing chemical substances that activate the thrombosis in other ways. A healthy platelet level ranges from 150,000 to 450,000 $\mu\ell$ of blood. They vary in size from 2 to 4 µm and are generated by the

megakaryocyte, a giant nucleated bone marrow cell. The megakaryocyte is accountable for developing blood thrombocytes (platelets) essential for natural wound healing (McGuinn and Bussel, 2022).

Plasma is a pale-yellowish aqueous blood component in which proteins, fats, carbohydrates, antibodies, minerals, and platelet compounds are suspended in water, about 95% of the entire blood volume (Du Pasquier, 2024). Plasma makes up approximately 55% of the whole blood's overall amount. Furthermore, plasma is responsible for conserving the human body, maintaining electrolyte stability and protecting the body from infectious diseases (Miura *et al.*, 2024). Blood serum is a component of blood plasma devoid of thrombosis variables; this is frequently the issue when conducting blood ageing or pattern studies in which coagulation will be problematic (Mathew and Bhimji, 2021).

1.2.2 Animal Blood Characteristics

Blood, a vital fluid that courses through the bodies of animals, is a complex mixture of cells, proteins, and fluids. It serves as a dynamic transportation system, carrying oxygen, nutrients, hormones, and waste products to and from the body's cells. Blood is present in animals with a closed cardiovascular system. Their blood comprises RBCs, WBCs, platelets, and plasma (Campbell and Grant, 2022).

The size of RBCs varies significantly across different animal species. Mammalian red blood cells are devoid of nuclei and other organelles. Animal blood may contain four respiratory pigments: haemoglobin, hemerythrin, hemocyanin, and chlorocruorin. Haemoglobin is present in all vertebrates and a few other invertebrates (Anttila and Farrell, 2024).

For many animals, WBCs and platelets are comparable. However, the cell size distributions can sometimes differ between animal species. WBCs come in four forms in certain vertebrate species, such as fish. Platelet binding during thrombosis could also differ between animal species. Platelets of equine blood are the most adherent, while warm-blooded vertebrates are mammal and bird classes (Ortiz and Esteban, 2024).

Different antibodies exist in mammal species such as cattle, horses, cats, and dogs. Monkeys and apes have two varieties of blood groups: human-kind and primate-kind. The blood group types of such animals are comparable. Therefore, DNA or genetic analysis is the most reliable way to differentiate mammal species from human blood species (Tizard, 2023).

1.2.3 Human and Animal Blood Comparisons

Several differences and similarities correlate between human and animal blood. Few studies have found that all mammalian blood, whether animal or human, have identical chemical and physical properties, specifically primate blood (Enos and Moore, 2022). This similarity means that after conducting scientific tests and evaluating the associated bloodstains, it was discovered that the fluids utilised were responding comparably. As a result, many bloodstain experts and crime scene specialists have adopted animal blood for educational purposes and experimental research. This knowledge has significant implications for forensic science, as it allows for more accurate and reliable bloodstain analysis (Dickler *et al*., 2024). However, the differences in diverse species must be comprehended.

Humans have the ABO and Rhesus blood group systems, which are distinct from the varied blood classes found in animals (Carter, 2024). While humans and

certain vertebrates use haemoglobin as the oxygen-carrying pigment, invertebrates exhibit a broader range of pigments, leading to a fascinating array of blood colours. A significant difference is the temperature regulation, with humans being warm-blooded, unlike the variable temperatures of animal blood. These unique characteristics of human and vertebrate blood are not just different, but intriguing and driving exploration of the biological world (Patton *et al.*, 2022).

While human and vertebrate blood exhibit distinct characteristics, several fundamental similarities unite them. They serve as connective tissues, transporting oxygen, nutrients, and hormones throughout the body (Ecker *et al.*, 2021). Additionally, both blood types play a vital role in waste removal and immune response. The circulatory system, which houses and circulates blood, shares a similar basic structure in all vertebrates, including the heart, arteries, veins, and capillaries. Furthermore, all vertebrates have the primary components of blood, including red blood cells, white blood cells, and platelets, albeit with varying specific functions and characteristics (Campbell and Grant, 2022) .

1.3 Forensic Blood-Substrate Evidence

The substrate upon which a bloodstain is deposited significantly impacts its appearance, preservation, and subsequent analysis, particularly in determining its age. Extracting and analysing age-related markers in deeply absorbed blood on porous substrates (fabric, wood, or carpet) is an intricate procedure that necessitates the skills of forensic specialists. Conversely, impermeable substrates (metal or plastic) can better conserve bloodstain details, but environmental factors can influence degradation rates, adding another layer of complexity to the analysis (Hughes, Szkuta, *et al.*, 2024).

Accurately determining the age of a bloodstain is crucial in reconstructing the timeline of a crime (Wolson, 2023). It can corroborate or refute witness testimonies, narrow the suspect pool, and assist in identifying potential evidence. However, the environmental elements like temperature, humidity, and exposure to light can impact the blood degradation rates adds a layer of complexity to age estimation (Eldridge, 2023).

Furthermore, different bloodstain patterns on various substrates present unique challenges that require the problem-solving skills of forensic investigators. For instance, bloodstain patterns on fabric might be distorted due to the fabric's movement, making analysis more complex (Moza *et al.*, 2023). On the other hand, bloodstains on hard surfaces like glass can retain more clear patterns. Similarly, bloodstains on rough surfaces like wood can create irregular patterns, while bloodstains on smooth surfaces like metal can form more uniform patterns, each presenting its own set of challenges (Meijrink *et al.*, 2023).

In summary, understanding the characteristics of different substrates is essential for forensic investigators when assessing bloodstain age. By considering the substrate type and its potential impact on blood degradation, experts can improve the accuracy of their estimations and contribute valuable information to criminal investigations.

1.3.1 Fabrics

One of the most common scenarios involving fabric substrates and bloodstains occurs in homicide cases. Consider a hypothetical domestic dispute that escalates to violence. The victim, attempting to defend themselves, may struggle with the assailant, resulting in blood transfer onto the attacker's clothing. In a high-profile case, for

instance, the clothing worn by a suspect during a violent crime would be a prime target for forensic examination. Bloodstains on the fabric, particularly those containing DNA, can link the suspect to the crime scene (Faflak and Attinger, 2021). The pattern of bloodstains on the clothing can also provide valuable information about the nature of the assault, such as the position of the victim and assailant during the attack (Powell, 2023). Forensic experts would carefully examine the fabric for bloodstains, document their location and appearance, and collect samples for DNA analysis. Advanced techniques like spectroscopic, RNA degradation, and protein profiling can be used to reconstruct the crime scene by assessing the age of bloodstains on the clothing (Öner *et al.*, 2023). Additionally, the type of fabric can influence the preservation of bloodstains. For example, cotton absorbs blood differently than synthetic fibres, affecting the visibility and recoverability of DNA (Sloan and Robertson, 2023).

Various fibres are available today, including natural, modified plant, and synthetic fibres, each with complex chemical compositions. Modern technology allows for the production of pure, mixed fabrics or polyester-blended fibres. (Harshitha, 2021). Cotton is a natural plant-based fibre derived from the Gossypium genus, commonly found in tropical and subtropical regions worldwide (Nuruzzaman *et al.*, 2024). It has soft, breathable, and permeable natural characteristics and can retain water 24 to 27 times its weight. Cotton fibres are durable, dye-receptive, and resistant to abrasion and high temperature. They become yarn through spinning before weaving into long-lasting products (Radoor *et al.*, 2022).

Denim is a sturdy cotton twill fabric known for its unique diagonal ribbing. The word "denim" is derived from the French expression "serge de Nîmes." It is commonly used in producing jeans and other apparel due to its durability, comfortable feel, and ability to maintain shape over time (Elmogahzy, 2020). Denim is often dyed

with indigo to achieve its classic blue colour and can be blended with other materials for added strength and stretch. The fabric's adaptability and durability lead to a popular choice for diverse clothing and accessories (Talebi and Montazer, 2020).

Polyester is a man-made polymer created from ethylene glycol, which is derived from petroleum, and terephthalic acid (Lutz and Madbouly, 2024). It is versatile and durable, commonly used in clothing and upholstery. Polyester is recognised for its crease-resistant, moisture-wicking qualities, and the power to keep its form and colour. It is often blended with other fibres to create fabrics with different attributes. Additionally, it is used in various household items and industrial materials (Chen *et al.*, 2023).

Satin is a fabric with a history dating back to the Middle Ages. It is known for its luxurious and smooth texture. It is created using a weaving technique that results in a glossy surface and a dull back (History of Clothing, 2021). Typically made from silk, polyester, or nylon, satin is widely used in the fashion and interior design industries. Its lustrous appearance and soft feel make it a popular choice for elegant clothing, bedding, and decorative item (Cicco *et al.*, 2021).

Silk is a natural protein fibre obtained from the cocoons of the mulberry silkworm larvae. It is known for its luxurious feel, lustrous sheen, and strength (Liang *et al.*, 2021). It is comfortable to wear, acts as a good insulator, and is used in various products. Poly-silk is a synthetic fabric made from polyester fibres, designed to mimic the look and feel of natural silk. It is often more affordable but may have different breathability or moisture-wicking properties (Gokarneshan and Rekha, 2021).

Wool is a natural textile fibre known for its softness, warmth, and moisture-wicking abilities. It is obtained from animals like sheep, goats, muskoxen, and rabbits. Due to its excellent insulation properties, wool is used to make warm clothing

(Sabarish *et al.*, 2022). Poly-wool is a blend of wool and polyester fibres, combining the insulating properties of wool with enhanced durability and wrinkle resistance. Poly-wool is commonly used to produce suits, trousers, and formal wear (Triki *et al.*, 2022).

1.3.2 Soil

Soil can serve as invaluable transfer evidence in cases involving blood (Hanslip, 2021). When a crime occurs outdoors or in a location with exposed soil, it's highly probable that soil particles will become embedded in bloodstains, on clothing, or on the bodies of those involved. Forensic experts meticulously collect soil samples containing blood from different locations around the body. These samples are then subjected to a series of analyses (Ogilvie and Lednev, 2023). The soil composition, including its mineral content, particle size, and colour, can be compared to soil samples taken from the suspect's shoes, clothing, or vehicle. If the soil present on a perpetrator's attire conflicts with the soil discovered at the location of the crime, it suggests their presence there and links them to the crime (Karadayı, 2021). The type of soil, its depth, and specific components like pollen, rocks, or organic matter can help recreate the crime scene. Soil on a victim's body can indicate their pre-crime whereabouts, helping to establish a timeline. In some cases, soil can protect DNA within bloodstains from breaking down, improving the chances of obtaining a DNA profile (Fitzpatrick and Donnelly, 2021).

However, analysing soil mixed with blood has challenges, such as interference with soil analysis and soil variations even within short distances. Proper collection and storage of soil-blood mixtures are crucial to prevent contamination and degradation.

Despite these challenges, advances in forensic science have improved the ability to extract valuable information from soil-blood mixtures (Hirashima *et al.*, 2022).

The soil composition includes geological, chemical, biological, and physical characteristics. Oxisol and ultisol are identified under soil taxonomy as lacking essential minerals for plant life (Owens and Libohova, 2023). These components, including gibbsite, goethite, hematite, and kaolinite, comprise about 72% of the terrain in elevated areas like mountains and hills. The tropical climate of a country leads to oxidation and nutrient loss, resulting in an accumulation of sesquioxide in the soil, as seen in red soil (Norazwani *et al.*, 2022).

Red soil covers about 13% of the Earth's land and is rich in potassium, varying from sandy to clayey. Productivity depends on its management, making it either productive or infertile (Dondeyne *et al.*, 2023). Red soil, including alfisol, oxisol, and ultisol, is rich in iron, giving it a distinctive reddish colour. Through effective agricultural practices, red soils are predominantly used for cocoa, oil palm, and rubber cultivation (Haruna, 2019).

1.3.3 Metal

Metal substrates have been crucial in many high-profile cases, providing valuable evidence in bloodshed crimes. In homicide cases, metal objects such as knives, axes, or blunt instruments are often used as murder weapons. Blood spatter patterns on these metal surfaces can reveal important details about the crime, such as the number of blows inflicted, the victim's position, and the attacker's movements (Singh *et al*., 2021). In cases of burglary and assault, a metal object like a crowbar used to force entry into a home may come into contact with blood if the homeowner resists, potentially linking a suspect to the crime scene (Larkin, 2015). Additionally,

in hit-and-run accidents involving pedestrians, the vehicle's metal components, such as grilles and bumpers, can retain bloodstains, providing crucial evidence for identifying the vehicle involved. Moreover, in industrial accidents, metal machinery can become contaminated with blood, which, when analysed, can help reconstruct the accident and identify potential safety hazards (Vigato *et al.*, 2022).

However, challenges associated with using metal substrates as evidence include preservation difficulties, microscopic traces of blood, and the potential interference of rust with bloodstain analysis and DNA extraction (van Oorschot *et al.*, 2021).

Metals are essential in various applications due to their abundance and versatility. Different metals display distinctive characteristics and are used for specific functions. Among these is aluminium, which is widely utilised metal, represented by the chemical symbol Al and has an atomic number of 13 (Vijaya *et al.*, 2021). Aluminium is known for its silvery-white appearance, softness, non-magnetic nature, and ductility within the boron group. Moreover, it is highly esteemed for its lightweight, durable, and corrosion-resistant characteristics (Hughes, Szkuta, *et al.*, 2024). This metal is used extensively in industries to produce various goods, such as aircraft, automobiles, packaging materials, cookware, and foil. Additionally, its reflective nature makes it suitable for electrical transmission lines and as a mirror coating (Hughes *et al.*, 2023).

1.3.4 Plastic

Plastic, a ubiquitous material in modern life, often becomes an unintended part of crime scenes and plays a significant role in forensic investigations, particularly concerning blood evidence. Common scenarios involving plastic include weaponry,

bags and containers holding blood-stained items, and plastic items with blood transfer. While plastic can preserve bloodstain patterns, its smooth surface may present challenges in analysis. DNA recovery from plastic can be difficult, but advancements in forensic techniques have improved success rates. Plastic can also retain other types of evidence, such as fibres, hair, or skin cells, which can contribute to investigations. However, evidence may be compromised if the plastic item has been cleaned or tampered with (Schalike and Illes, 2020).

Plastic, a multifaceted artificial polymer originating from petroleum, natural gas, and other chemicals. It comprises long chains of molecules called polymers, giving plastic unique properties (Bakar *et al*., 2022). There are various types of plastic, each with its distinct characteristics and uses. Such widely recognised is polymethyl methacrylate (PMMA), a diaphanous thermo-softening plastic that is lightweight, shatter-resistant and has optical clarity similar to glass. It is commonly known by the brand names Plexiglas or Lucite (Wypych, 2016). PMMA is highly regarded for toughness, weatherability, and effortless fabrication. It finds applications in diverse products, including car headlights, signage, aquariums, medical devices, and display screens. Its versatility and clarity make it popular in industries where transparency and impact resistance are required (Bryant, 2022).

1.3.5 Rubber

Rubber can be both porous and non-porous, commonly found in many everyday items. Its porosity properties significantly influence forensic bloodshed investigations (Gent, 2020). Porous rubber substrates (car mats, gym mats, or certain types of footwear) can absorb blood, making traditional bloodstain analysis challenging but still allowing techniques like luminol to reveal it. DNA recovery from

porous rubber can be more complex and time-consuming, but trace evidence (fibres, hair, or soil particles) can be valuable in linking a suspect to the crime scene. On the other hand, non-porous rubber (car tyres or certain types of footwear soles) allows for a more straightforward bloodstain analysis (Rodriguez *et al.*, 2020). However, it may limit the amount of blood collected for DNA recovery. Both porous and non-porous rubber substrates can trap trace evidence and understanding the properties of rubber substrates is essential for effective crime scene investigation (Chandrasekaran, 2017).

Rubber is a natural or synthetic elastic material with a wide range of applications. Natural rubber, a product of the rubber plant species Hevea brasiliensis, is harvested from the tree's latex. In contrast, artificial rubber is created by petrochemical products (Guerra *et al.*, 2021). There are various types of rubber and neoprene is another non-porous synthetic rubber known for its resistance to oil, water, weather, and flame. Due to its durability and flexibility, it is commonly used in the production of automotive fan belts, wetsuits, and gaskets (Rubber-Cal, 2023).

1.4 Protein and Its Structural Properties

Protein is naturally made up of about 20 standard types of amino acids. Amino acids (polypeptides) are carbon, oxygen, nitrogen, and hydrogen atoms arranged on a planar backbone chain (Gromiha *et al.,* 2024). The primary protein structure is the amino acids' linear sequence in a peptide chain. The secondary structure or protein conformation is the three-dimensional folding shape of a peptide chain attributed to hydrogen linkages between adjacent backbone amide and carbonyl. Alpha-helices and beta-pleated sheets are two essential elements of secondary structures in polypeptide strands (Austin, 2024). Approximately 80% of haemoglobin proteins are comprised of

the α-helix type. Each helical turn of an α-helix protein contains 3.6 residues bonded by hydrogen and Van der Waals forces. The α-helix formed within two neighbouring amino acids is spatially arranged three to four amino acids away in the strand pattern (Yadav, 2020).

Another typical protein shape is the β-sheet. Unlike α-helix proteins, β-sheet proteins produce two residues every rotation, creating strong inter and intramolecular hydrogen bonding at two amino acids apart. Certain regions of a polypeptide backbone that lose their typical secondary structure are called random coils and loops (Prasad *et al.*, 2023).

Hydrophobic interactions and disulphide linkages form the protein tertiary structure, stabilising the secondary structure fold in a close overall protein conformation (Rehman *et al.*, 2020). Quaternary shape describes the polypeptide sequence arrangement in the multimeric proteins (Godbey, 2022). **Figure 1.1** illustrates the primary, secondary, tertiary, and quaternary protein arrangements (Designua, 2023).

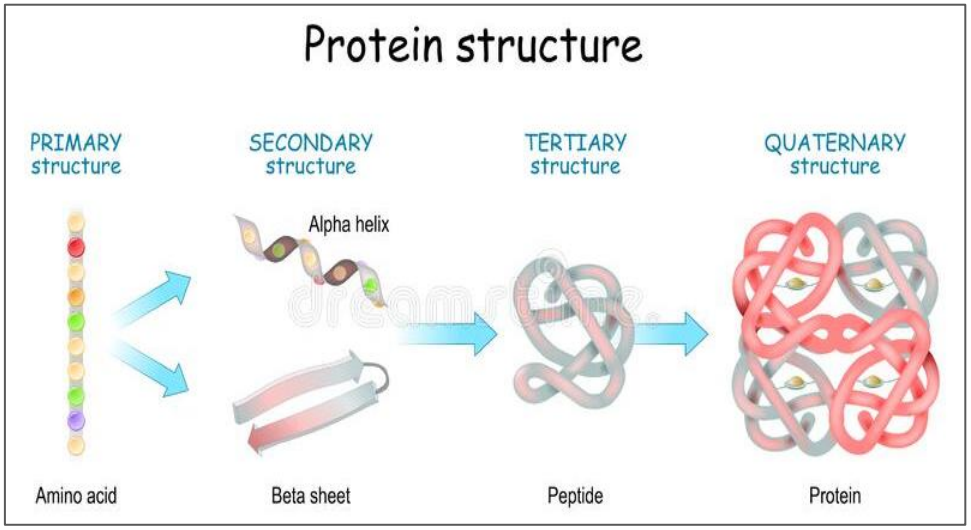

Figure 1. 1: Hierarchy of protein structure.

1.5 Hemoglobin Decomposition

Haemoglobin, an iron-rich protein, transfers oxygen between the lungs and the body's cells (Ohmori *et al.*, 2019). Comprising nearly all of the blood's dry weight, it has various forms or derivatives. These forms can transform differently within and outside the body.

Inside the body, haemoglobin primarily exists as deoxyhaemoglobin (without oxygen) and oxyhaemoglobin (with oxygen). While arterial blood is typically rich in oxygen, venous blood contains less (Ha and Bhagavan, 2023). A small portion of haemoglobin naturally converts to methaemoglobin, which reverts to its original form by the enzyme cytochrome b5 (Tiessen *et al.*, 2022). However, when haemoglobin is exposed to oxygen outside the body, it fully saturates with oxygen. Without cytochrome b5 to reverse the process, haemoglobin irreversibly transforms into methaemoglobin, altering its colour from crimson red to dark brown. Later, the methaemoglobin is denatured to become hemichrome (Bremmer *et al.*, 2011). This transformation results from the different light-absorbing properties of heme molecules' oxyhaemoglobin, methaemoglobin, and hemichrome structure (Zhang *et al.*, 2023).

Furthermore, other external factors such as temperature, humidity, and storage settings may also influence the blood drying process result (Kaur *et al.*, 2020). Thus, to validate the drying performance, different researchers created diverse approaches to obtain the best outcomes. However, most research experiments mimicked bloodstain samples in controlled laboratory settings (Lee *et al.*, 2023). In an actual crime scenario, fluctuating environmental variables may influence the bloodstain's degradation and coagulation rate, contributing to the intricacy and challenge of bloodstain age determination.

1.6 Problem Statement

Accurate and timely species identification and age estimation of bloodstains are critical in forensic investigations for establishing evidence timelines, linking suspects to crime scenes, and ultimately achieving justice. While advancements in forensic science have improved techniques for bloodstain analysis, challenges persist in obtaining reliable and precise results, particularly in cases involving degraded or small amounts of blood.

The current issues include using time-intensive and laborious methodologies with varying degrees of accuracy in species identification, especially when dealing with non-human blood or mixed samples, and the lack of standardised protocols for accurately estimating bloodstain age. Furthermore, environmental factors affecting blood degradation and its impact on DNA recovery and analysis require further investigation. Preserving blood samples is crucial, as current procedures can be invasive and jeopardise the integrity of DNA's compositional structure. Additionally, many existing methods for species identification and age estimation of bloodstains lack comprehensive validation and reliability testing across diverse sample sets.

It is necessary to tackle these concerns to improve the probative value of bloodstain evidence and enhance the general efficiency of forensic investigations. Thus, adopting a non-invasive, rapid, robust, and validated strategy is essential and holds great promise for advancing the forensic field.

1.7 Research Aims and Objectives

The **primary aims** of this research are:

- To investigate the robustness of the modern biospectroscopic approach as a powerful, systematic, validatory, and inexpensive tool in blood species identification.
- To develop an ideal method for the age estimation of bloodstains since deposition in forensic investigation.

The highlighted **specific objectives** of this study were:

i. To develop a proper methodical sampling procedure and operational workflow for non-destructive spectroscopic analysis for forensic blood species identification.
ii. To explore the efficiency of bio-spectroscopic techniques and chemometric analysis in the definitive species identification of blood samples.
iii. To assess the practicability of ATR-FTIR spectroscopy and chemometric strategy for estimating the age of bloodstains deposited on ten different substrates at varying time points from 0 to 365 days.
iv. To study the time since deposition (TSD) of the bloodstain exposed to two conditions; indoor and outdoor environments.

1.8 Significance of Study

Accurately determining a bloodstain's species and age is pivotal in forensic investigations. It lays the foundation for subsequent analyses, such as DNA profiling and bloodstain analysis, which can significantly impact case outcomes.

Identifying bloodstain origin is significant in differentiating between human and animal species in crime scene investigations, which can help exclude irrelevant evidence, focusing investigative efforts on human involvement in cases such as

homicide and burglary (Lee *et al.*, 2023). It can also establish links between suspects and crime scenes, providing valuable investigative leads (Boman, 2023). In wildlife forensics, identifying endangered or protected species bloodstains can help prosecute wildlife crimes, disrupt illegal trade networks, and provide evidence of habitat destruction or human-wildlife conflict (Sharma *et al.*, 2023). Additionally, in mass disaster identification, determining the species of bloodstains can help prioritise recovery efforts and identify human victims (Singh *et al.*, 2022). Similarly, identifying animal bloodstains in affected areas can facilitate assessing the impact of disasters on wildlife populations (Soniya and Kumar, 2022).

The correct identification of the species of a bloodstain not only saves time and resources by preventing unnecessary tests and analyses and ensures evidence integrity by preventing contamination of human DNA samples with animal DNA (Dickler *et al*., 2024). It can strengthen the prosecution's case by providing compelling evidence linking suspects to crime scenes or exonerating innocent individuals. Besides, identifying zoonotic diseases through bloodstain analysis can help prevent the spread of infectious diseases between animals and humans, while understanding the impact of human activities on wildlife populations can contribute to conservation efforts (Shaheen, 2022).

Ultimately, accurately identifying the species origin of a bloodstain is a cornerstone of modern forensic science. It provides invaluable information for investigators and enables them to build more substantial cases, protect public health, and contribute to environmental conservation. This present study also tackles a few of the bloodstain identification contexts, including:

- "Can we establish non-destructive analytical techniques?"
- "Can we optimise bloodstain detection specificity?"

Additionally, to improve bloodstain detection analysis, there is a clear need to accurately estimate the age of bloodstains since their deposition. Presently, no standard protocol describes the steps to measure the age of bloodstains found at the scene. In a real-life scenario, the surroundings may promote denaturation and coagulation of the bloodstain, making it difficult to measure its age (Alkhuder, 2022).

One of the significant applications is determining the timeline of events that led to a crime. This can aid in crime scene reconstruction, suspect identification, and DNA evidence interpretation (Jabor *et al.*, 2024).

Advanced spectroscopic methods such as Raman spectroscopy and Fourier-transform infrared spectroscopy have been explored to estimate the age of bloodstains. These techniques offer advantages over traditional chemical tests and visual inspection, including non-destructive analysis and the ability to detect small quantities of bloodstains (Zhang *et al.*, 2023).

Bloodstain age estimation can also provide crucial information in cases where the exact time of the crime is unknown or disputed. By analysing the age of bloodstains found at the scene, investigators can estimate when the crime was committed, which can help narrow the list of suspects and identify potential witnesses (Sijen and Harbison, 2021).

Furthermore, bloodstain age estimation can be used to analyse historical forensic or cold cases. In such cases, bloodstains may have been preserved for years or even decades, and the age of these stains can provide important information about when the crime was committed. This information can be used to re-examine the evidence and identify potential suspects or witnesses that may have been overlooked in the original investigation (Hetchler, 2023),

In summary, the study of bloodstain age estimation holds excellent significance in forensic science due to its outstanding crime solution abilities, such as crime scene reconstruction, suspect identification, DNA analysis, time since death determination, crime timing estimation and analysis of historical forensic cases.

In the past, non-invasive spectroscopic techniques coupled with chemometrics have been successfully employed to confirm the identification of various blood species and to predicate bloodstain age. Therefore, this research project combined the ATR-FTIR technique with multivariate chemometric analysis to identify and discriminate different blood species and evaluate fresh and aged human and animal bloodstains on various substrates. The outcomes of this research could aid in improving forensic and criminal investigations.

1.9 Scopes of Study

Establishing the origin and age of bloodstains has numerous advantages in forensic biological analysis. However, it can be a challenging initiative due to the need for more effective, invasive, and well-received procedures. The feasibility of combining ATR-FTIR spectroscopy with multivariate chemometric for species identification and bloodstain age estimation was explored in the present work.

Identifying and individualising bloodstains is vital since humans and animals can engage in a particular crime. In the current work, species identification and discrimination study have been examined. The principal component analysis with linear discriminant analysis (PCA-LDA) chemometric approach was applied to distinguish and classify human blood from non-human blood samples. For this

differentiation and classification project, seven animal blood samples were used: chicken, cattle, deer, duck, fish, goat, and swine.

Bloodstain age prediction is essential in forensic investigations as it could reveal when an actual crime occurred. Past studies in this research area have featured numerous instrumentations. Nevertheless, these technologies have yet to be brought into forensic work because most require extensive prior sample preparation, which might cause contamination or constant damage to the samples. The ATR-FTIR spectroscopy was applied in conjunction with partial least squares regression (PLSR) and partial least squares-discriminant analysis (PLS-DA) to estimate and characterise the age of indoor and outdoor bloodstains up to one year old on diverse substrates.

Furthermore, this study incorporated all ageing external elements (climate, humidity, and temperature), contamination (soil, dirt, and sand), and substrate effects analyses, whereas previous studies only evaluated these elements independently. These findings are a breakthrough toward the actual utilisations of bloodstain identification and age estimation in forensic casework, where a wide range of situations may be addressed. **Figure 1.2** specifies the parameters and elements that are covered in this research.

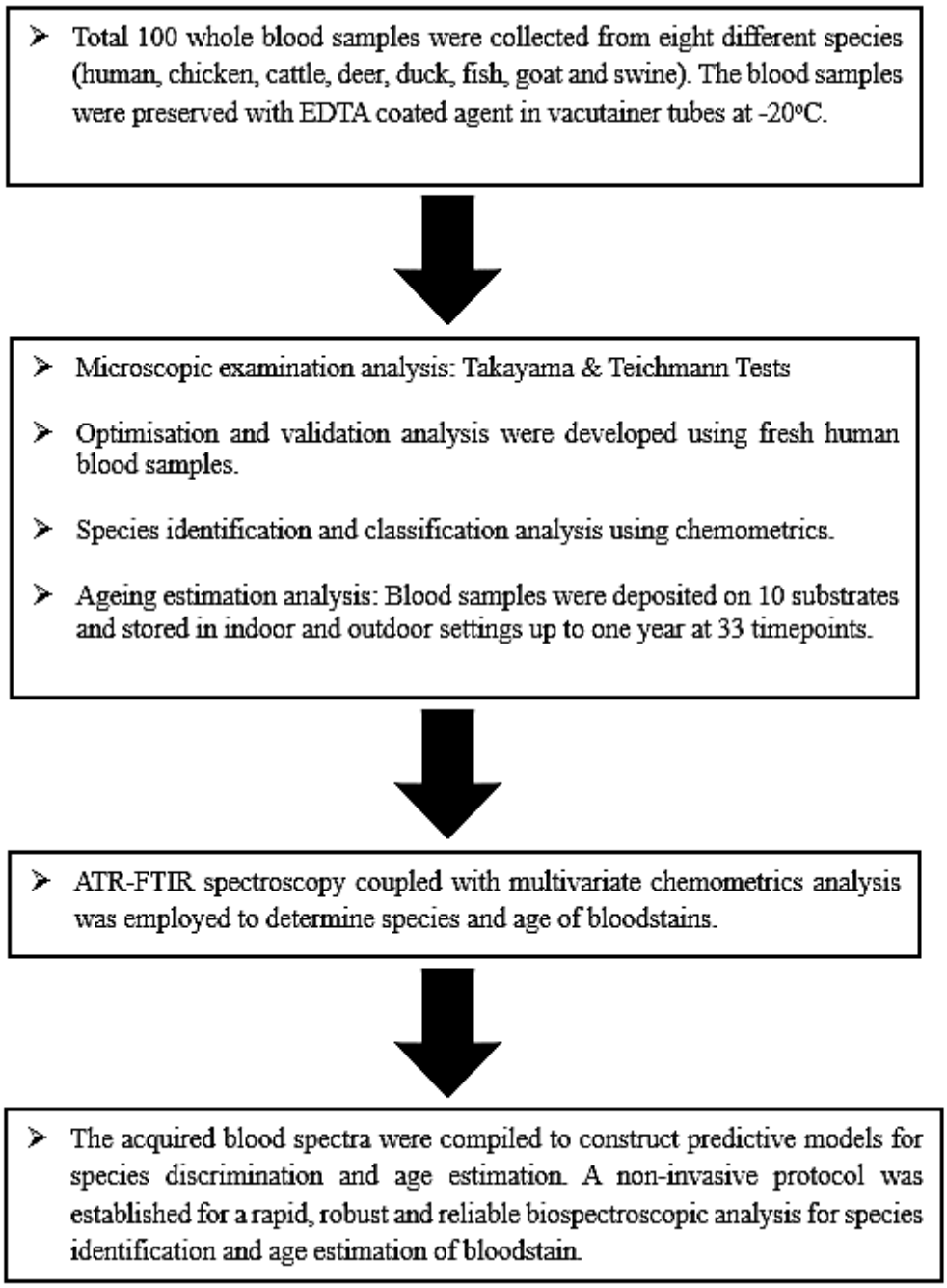

Figure 1. 2: A detailed operational workflow representation of the research scope.

1.10 Thesis Outline

The first chapter of this study addresses the research background, the research problem, and the research objectives. The chapter also covers a comprehensive

description of the contexts of this study. The second chapter reviews and cites the relevant literature works for the core elements of this thesis.

Chapter three elaborates on sampling, procedures, instrumentation, optimisation, validation, collection, and blood samples storage. The analysis of blood samples in this chapter was divided into three main sections. The first segment demonstrates the confirmatory blood tests, including detecting the blood cells by Takayama and Teichmann crystal tests under microscopic observation. In the second part, blood samples are identified and distinguished by combining multi-spectroscopic and chemometric strategies based on their spectral characteristics. The third section investigates the effects of bloodstain age estimation under two storage settings deposited on diverse porous and non-porous substrates.

The fourth chapter describes and interprets the outcomes of this research. This section compares and discusses the significant or insignificant results of past literature studies with the latest empirical findings from this experiment and achieves the research objectives and solutions for the research problem of this thesis.

Chapter five is the conclusion that summarises and addresses limitations and suggestions for the future work of this study. It explains the implications of this novel research and highlights the feasibility of a modern bio-spectroscopic method in blood species identification and age prediction models.