DEVELOPMENT OF FTIR SPECTRAL DATABASE FOR THE IDENTIFICATION OF SELECTED WILDLIFE ARTICLES

PROJECT COMPLETION REPORT

Under the scheme

MODERNIZATION OF FOREST FORCE

GOVERNMENT OF TAMIL NADU

Advanced Institute for Wildlife Conservation
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Project Duration: March 2023 – March 2024

ADVANCED INSTITUTE FOR WILDLIFE CONSERVATION

(Research, Training & Education)

TAMIL NADU FOREST DEPARTMENT

VANDALUR, CHENNAI – 600 048

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aiwcrte@tn.gov.in

Declaration
For greater clarity, the title of the approved project has been changed to 'DEVELOPMENT OF FTIR SPECTRAL DATABASE FOR THE IDENTIFICATION OF SELECTED WILDLIFE ARTICLES' Instead of 'DEVELOPMENT OF A SPECTRAL DATA BASE FOR THE IDENTIFICATION OF WILDLIFE ARTICLES BY INFRARED MICROSCOPY'

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About the Back Side Cover
Prema and Shinta are collecting ivory sample for the FTIR analysis.

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Abbreviations & Symbols

ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
cm^{-1}	The number of wavelengths per unit of distance.
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FTIR	Fourier-Transform Infrared Spectroscopy
H_2O	Water
IUCN	International Union Conservation of Nature
KBr	Potassium bromide
kg	Kilogram
mtDNA	Mitochondrial DNA
min	Minutes
μL	Microliter
ng	Nanogram
mM	Millimolar
PCR	Polymerase Chain Reaction
PCA	Principal Component Analysis
PLS-DA	Partial Least Squares Discriminant Analysis
%	Percentage
RPM	Revolutions per minute
Sry	Sex-determining region on the Y chromosome
s	Seconds
UV	Ultraviolet
UNODC	United Nations Office on Drugs and Crime
WCCB	Wildlife Crime Control Bureau

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Abstract

Illegal wildlife trade has attained massive proportions and involves thousands of animal and plant species. Identifying species is essential for combating illicit wildlife trade and protecting species. Generally, morphological methods and DNA analysis are used for species identification. However, each methodology has its limitations, and wildlife forensic laboratories frequently receive samples for analysis that are below standard in terms of quality and quantity. Therefore, it is necessary to develop modern methods/tools for combating wildlife crime. Fourier transform infrared (FTIR) spectroscopy is a powerful analytical technique used to identify chemical components and the structures of compounds in the samples based on vibrational modes of their molecular functional groups. FTIR has been widely applied in various evidence-based investigations, i.e., crime scenes, including analysing questioned documents, banknotes, paints, fibers, hair, gunshot residues, and ivory. However, this technique could not be fully utilized in wildlife forensics due to the lack of a reference IR spectral library/database. In light of this, the current study aimed to (1) develop the FTIR spectral library for Asian elephant ivory and (2) Identification of mongoose hair from domestic cattle, human hair, and synthetic fiber using FTIR spectroscopy analysis. We collected eight samples of Asian elephant ivory ($n = 8$) and hair from four individuals of the Indian grey mongoose (*Herpestes edwardsii*) from the AIWC repository. Additionally, hair from four individuals of domestic cattle (domestic water buffalo and domestic cow) was collected from the farm. Human hair was obtained from volunteers, and synthetic fiber (Polyethylene terephthalate fiber) was collected from the AIWC. All the samples were blended thoroughly using the sterile mortar and pestle; thereafter, 13 mm KBr pellets were prepared, and the FTIR spectrum was recorded. Subsequently, based on the biochemical composition of ivory, hair and synthetic fiber, we identified various characteristics, functional groups, and their respective wavenumbers. The FTIR spectrum recorded for hair samples and synthetic fiber has suggested that visually can be distinguished the synthetic fiber and hair (all the hair used in this study). However, when comparing the FTIR spectra of different hair samples, no significant visual differences were observed, except for a slight variation in the amide-II region, especially observed in the hair of the Indian grey mongoose. The hair FTIR spectra are very similar because all hair is made of keratin proteins. Additionally, relying on visible observation of FTIR spectra can lead to observer bias. To address these issues, we used chemometric (PLS-DA model) analysis to distinguish Indian grey mongoose hair from domestic cattle hair, human hair, and synthetic fiber. The PLS-DA analysis has discriminated all the hair

and synthetic fiber based on the IR absorbance properties and showed an R-square value and an RMSE (root mean square error) value of 0.9 and 0.13 respectively. Our findings have shown that FTIR spectroscopy combined with chemometrics can quickly discriminate Indian grey mongoose hair, domestic cattle, human hair, and synthetic fiber. Next, we used the FTIR spectrum of Asian elephant ivory as a reference to identify the fifteen carvings (n = 15) suspected to be Asian elephant ivory which were received at AIWC. The results revealed that samples 1 -13 are visibly identical to the FTIR spectrum of Asian elephant ivory; in contrast, samples 14 and 15 do not match the FTIR spectrum of Asian elephant ivory. Hence, it is confirmed that out of fifteen samples, thirteen are likely to be of Asian elephant ivory origin. To confirm this finding, we employed the PLS-DA analysis. The result showed an R Square value of 0.9 for calibration and 0.75 for validation, and the model exhibited 100% accuracy in classifying the original and fake ivory samples. To validate the FTIR results, DNA was extracted from all suspected samples (1-15) using the QIAamp DNA Investigator Kit; however, samples 14 and 15 could not lyse during DNA extraction. We also tried all the troubleshooting methods for lysis, but none succeeded. Therefore, we did not process samples 14 and 15 for subsequent experiments. To confirm the FTIR results for suspected samples, we performed PCR amplification of elephant-specific genes, i.e., D-loop (control region) and *Sry* (sex determination region on Y chromosome) from the DNA extracted from suspected samples (samples 1–13). Then, the PCR amplicons were sequenced, and BLAST analysis was performed. The results showed 96.6 to 100% similarity to the elephant. Overall, DNA-based results supported the results from FTIR analysis and confirmed that the suspected samples 1–13 are of elephant origin and 14 and 15 are not of elephant origin. Overall, the current study has demonstrated the identification of ivory (including substitutes) and hair from Indian grey mongoose through FTIR spectroscopy combined with chemometrics, which is rapid, cost-effective, and has excellent potential for forensic analysis.

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Chapter -1

Chapter 1

General Introduction

Wildlife forensic samples can vary from nails to tissues. One of the major aims of wildlife forensic analysis is to identify the taxonomic source (e.g., species, genus, etc.) of non-human biological material, either as items in trade, or direct or indirect evidence in other crimes. Therefore, it is crucial to have reliable and flexible analytical techniques to provide information about the samples collected from crime scenes, including confiscated articles, to prove, beyond a reasonable doubt, whether a suspect is guilty or innocent.

Species identification can be done preliminarily through morphological classification. Morphological examination is a reliable, inexpensive method for identifying articles encountered in forensic casework when sufficient analytical features are present, necessary expertise is available, and suitable comparative reference material is available (e.g., whole animals, intact bones, flight feathers, as in Trail). When these conditions cannot be met, genetic analysis is used to identify species[1].

DNA-based techniques are commonly used to identify species. In wildlife forensics, DNA analysis typically involves sequencing mitochondrial DNA (mt-DNA) for animal identification and using various markers from the nuclear and chloroplast genomes for plant identification **(Figure 1.1).** Specific regions of nucleotide bases in the DNA sequences are used to distinguish between different species. Researchers compare the DNA sequences of unknown specimens to those in databases to identify them based on similarity[1,2]. This process is straightforward when dealing with well-separated species and closely related taxa. However, it becomes more complex when dealing with incomplete taxon sampling and shallow coalescent depths within a group[3].

Furthermore, DNA analysis is the gold standard method, which involves PCR amplification of a partial fragment of the mitochondrial genes from the suspected samples, followed by sequencing analysis, but it often requires several days of turnaround time due to difficulties in extracting DNA from the sample and obtaining PCR-amplifiable DNA, which takes time for species identification and this causes problems in investigation of wildlife cases when quick results are needed. DNA-based species identification is also challenging if samples are preprocessed, contaminated, dry-cured, dried, and decayed[4]. Hence, there is an urgent need to develop rapid, cost-effective, and field-deployable analytic methods to overcome the above-described issues.

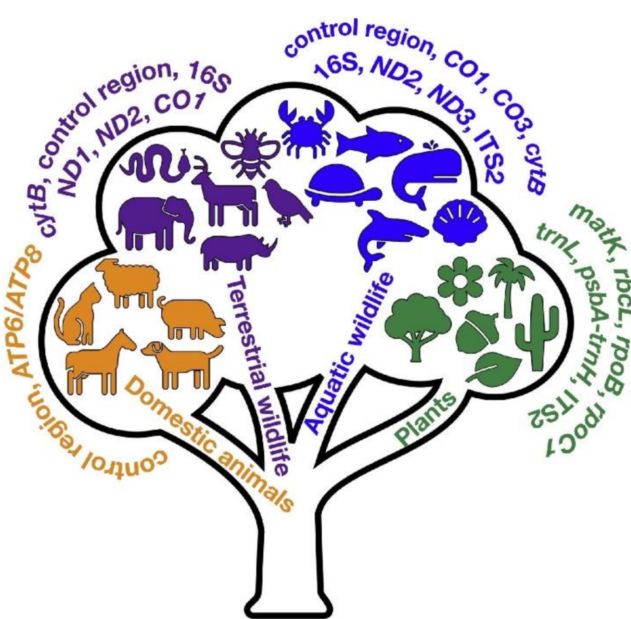

Figure 1.1: Schematic illustration of the Mitochondrial genes used for species identification in wildlife forensic laboratories. 12S: 12S ribosomal RNA; 16S: 16S ribosomal RNA; Cyt-b: Cytochrome b; CO1: Cytochrome c oxidase subunit 1; CO3: Cytochrome c oxidase subunit 3; ND1: NADH dehydrogenase subunit 1; ND2: NADH dehydrogenase subunit 2; ND3: NADH dehydrogenase subunit 3; ATP6: ATP synthase membrane subunit 6; ATP8: ATP synthase membrane subunit 8. 2) Nuclear regions – ITS2: Internal transcribed spacer subunit 2. 3) Chloroplast regions – matK, maturase K; rbcL, ribulose biphosphate carboxylase; rpoB, DNA-directed RNA polymerase subunit beta; rpoC1, plastid-encoded RNA polymerase subunit beta. *The Figure was adapted from Forensic Science International: Animals and Environments, 1, p.100030.*

Fourier Transform Infrared Spectroscopy (FTIR) is a powerful analytical tool for identifying chemical constituents and elucidating compound structures in various real-world samples. It does this by analyzing the vibrational modes of molecular functional groups. FTIR can provide a comprehensive assessment of sample components without requiring sample preparation, which is typically not feasible with other routine analytical methods.

1. IR spectroscopy

The human eye can only see a small part of the broader spectrum of electromagnetic radiation **(Figure 1.2).** The ultraviolet (UV) region is on the high-energy side of the visible spectrum, while the lower-energy side is the infrared (IR). The ideal IR regions for analyzing organic compounds are 2,500 to 16,000 nm in wavelength. There are three type of IR regions: Far (500 and 20 cm^{-1}), mid (4,000 and 500 cm^{-1}), and near-IR (~ 10,000 and 4,000 cm^{-1})[6].

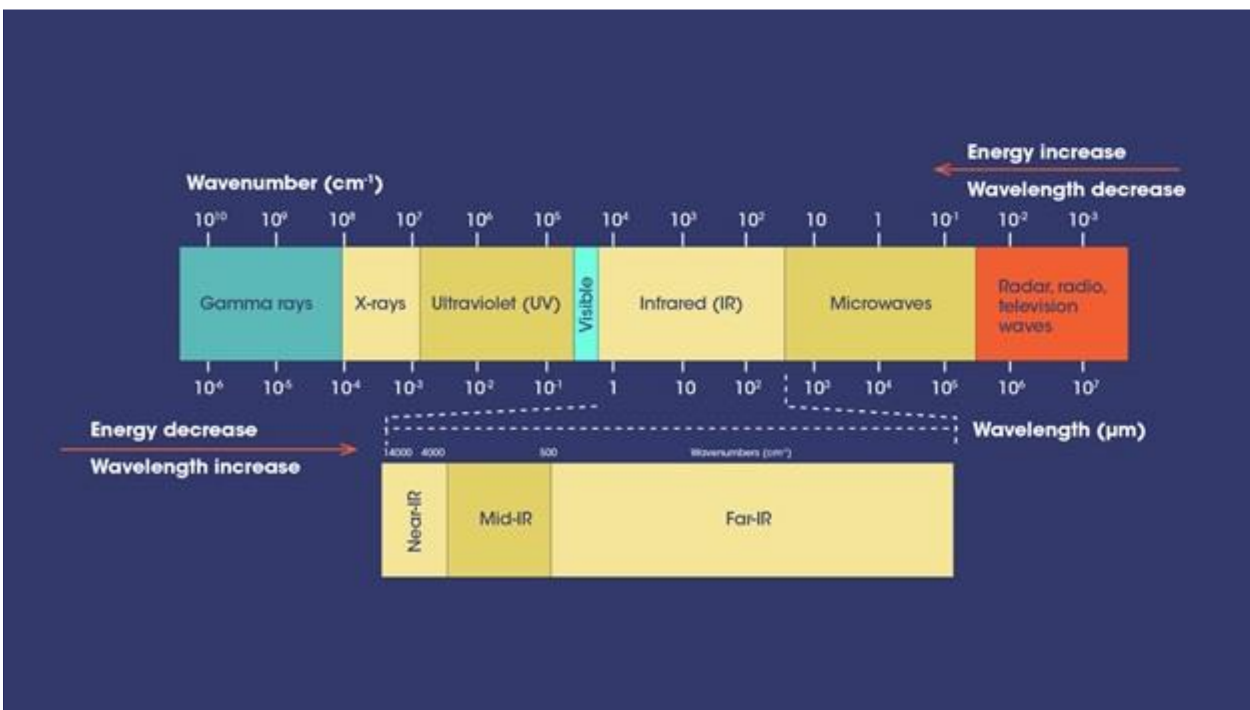

Figure 1.2: Systematic illustration of the electromagnetic spectrum. The IR region indicates the typical sub-region of infrared spectroscopy types. The Figure was adapted from www.technologynetworks.com.

1.1. FTIR working principle

FTIR works on the absorption principle of an infrared light source on the sample. An intense wavelength of infrared light is passed through the sample. The FTIR instrument measures the absorption wavelengths. An interferometer is used to identify samples by producing an optical signal with the IR frequencies encoded into it. The Fourier transformation technique is used to decode the signal and is a system-based or -generated process, helping in the production of spectral information[6,7] (**Figure 1.3**). In the testing process, the sample is placed in the FTIR spectrometer. Directing a beam of IR at the sample, the absorbance against infrared light at specific frequencies is measured, with thin samples being best for analysis. FTIR spectroscopy is a measurement technique that enables the documentation of infrared spectra, which are extremely helpful in establishing sample structure[8]. There are several types of IR spectroscopy, with the most prominently used being Attenuated Total Reflection (ATR) and Fourier transform infrared (FTIR) spectroscopy. ATR-FTIR spectroscopy is a measurement method where the reflected light travels to the FTIR detector. Within the detector, an interferogram (i.e., the signal format acquired by an FTIR spectrometer) is produced and then mathematically processed into a readable spectrum[9]

(**Figure 1.4**). FTIR spectroscopy results in a high-dimensional data set called a spectrum, comprising hundreds to thousands of data points. Advanced statistical analysis is required to quantify, classify, discriminate, and identify samples. Chemometrics is a powerful statistical tool for uncovering intricate relationships in chemical data[5].

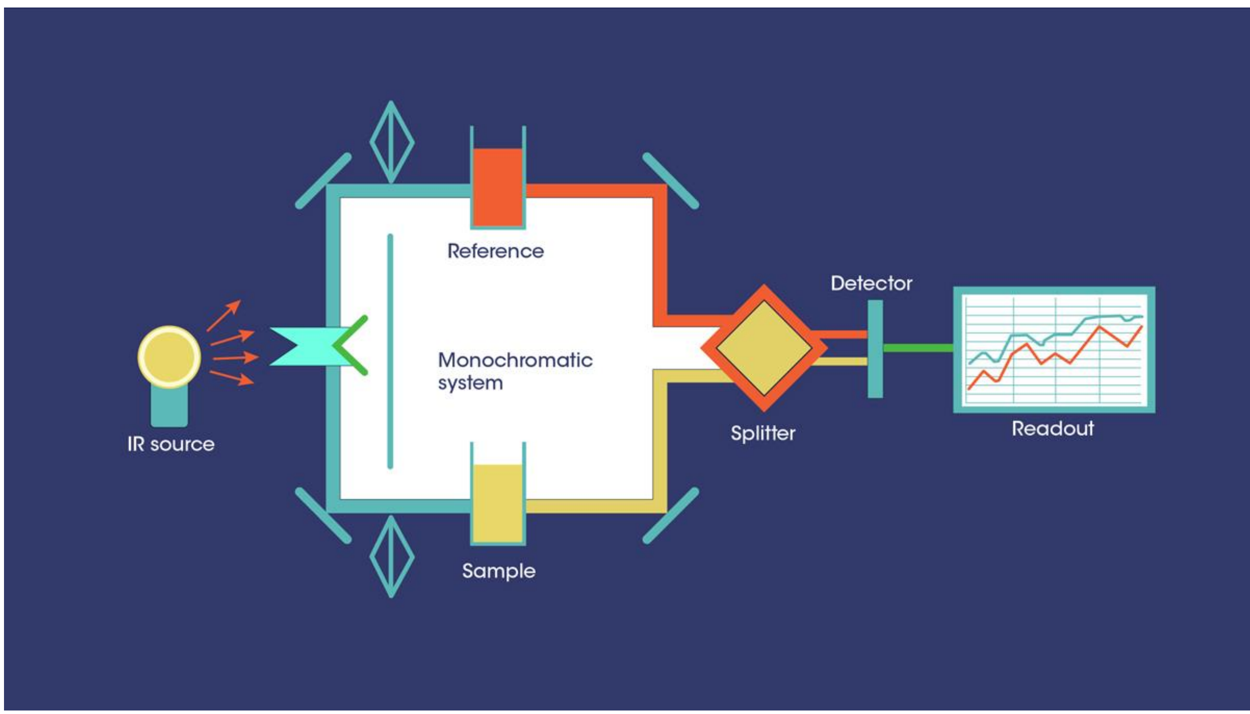

Figure 1.3: Schematic illustration of FTIR working principle. The Figure was adapted from www.technologynetworks.com.

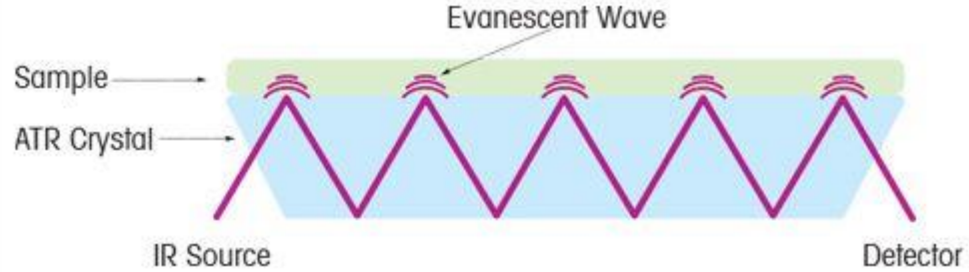

Figure 1.4: Schematic illustration of ATR principle. The Figure was adapted from the 'Mettler Toledo' website.

2. Chemometrics

Chemometrics involves using mathematical and statistical analyses to extract useful information from complex chemical data sets. Spectral output may look like a single line, but it consists of hundreds to thousands of data points forming a spectrum. Analyzing spectral results requires more advanced methods than simple, single-variable data[5]. Therefore, multivariate statistical analyses are necessary to process, quantify, and classify vibrational spectroscopic data. In forensics, standard chemometric analyses fall into three categories: (1) pretreatment techniques, (2) classification methods, and (3) calibration processes.

Pretreatment techniques are employed to prepare the data for classification or calibration methods. This process enhances and organizes the data using mathematical tools to account for chemical variation within a data set. The classification methods of chemometrics can be subdivided into two categories[10,11]: (1) unsupervised and (2) supervised. Unsupervised techniques are a form of exploratory analysis, as no information is provided to the model before sample analysis. These techniques are used to discover underlying trends or patterns within the data set. Principal component analysis (PCA) is one of the main unsupervised techniques. There are four main goals of PCA: (1) clustering the data points, (2) discrimination/differentiation among points, (3) classification/identification, and (4) discriminative power estimation. In preliminary data examinations, cluster analysis, primarily known as hierarchical cluster analysis (HCA), is also used.

In contrast, supervised techniques require a known dataset divided into classes based on different characteristics, allowing the model to 'learn' about the groups. Therefore, when new examples are analyzed, the model can draw inferences about the data and classify the samples based on specific patterns using particular algorithms. Supervised techniques are a form of machine learning as they extract features from raw data to gain knowledge of the sample. They then use this information to solve real-world problems by making decisions, such as identifying illicit substances. There are various forms of supervised techniques, including (1) Partial Least-Squares Discriminant Analysis (PLS-DA), (2) Linear Discriminant Analysis (LDA), (3) k-Nearest Neighbors (KNN), (4) Support Vector Machine (SVM), (5) Random Forest (RF), and (6) Multivariate Regression Methods such as Multiple Linear Regression (MLR), Principal Component Regression, and Partial Least-Squares Regression (PLSR) establish quantitative relationships among multiple predictor variables and a variable response. Supervised techniques

are suitable for classification, discrimination, and the quantitative analysis of samples. Numerous research articles have recently been published highlighting the relationship between chemometrics and forensics analysis[5,11.]

3. FTIR in Forensic Investigations

In forensic analysis, FTIR has been widely used in various criminal cases to examine evidence at a crime scene. This includes analyzing questioned documents, banknotes, paints, fibers, hair, and gunshot residues. Research has also shown that FTIR can effectively analyze biological specimens based on their unique infrared absorption frequencies. It can simultaneously analyze macromolecules such as proteins, lipids, carbohydrates, and nucleic acids. Moreover, recently, FTIR spectroscopy applications in pharmaceutical, biomedical, and clinical fields have gained great attention[12-21].

In light of this, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has recommended various techniques for identifying ivory and ivory substitutes, including Fourier Transform Infrared Spectroscopy (FTIR)[22], and the United Nations Office on Drugs and Crime (UNODC) has recommended physical methods for the identification of narcotics[23]. FTIR has emerged as a potent tool in wildlife forensic analysis; however, this technique could not have been fully utilized due to the lack of a reference IR spectral library/database. Therefore, there is an urgent need to develop a wildlife article-specific FTIR spectral library, which will facilitate quick, reliable, and eco-friendly identification of samples based on their chemical composition. With this background, the current study has been devoted to developing an FTIR spectral library/database for selected wildlife articles.

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Chapter -2

Chapter 2 Development of FTIR spectral library for the identification of Asian Elephant Ivory

Illegal ivory trade remains a prime threat to elephant populations. Prompt identification of ivory is crucial for combating illicit ivory trading. Studies have demonstrated that FTIR spectroscopy is one of the most suitable techniques for identifying ivory and its products. However, this technique could not have been fully utilized due to the lack of a reference IR spectral library/database. In light of this, the present study aimed to develop the FTIR spectral library for Asian elephant ivory. We collected eight Asian elephant ivory ($n = 8$) samples from the repository of Advanced Institute for Wildlife Conservation and recorded the FTIR spectrum. Further, based on the biochemical composition of ivory, we identified various characteristics, functional groups, and their respective wavenumbers. Furthermore, we used the FTIR spectrum of Asian elephant ivory as a reference to identify the fifteen carvings ($n = 15$) suspected to be of Asian elephant ivory. The results revealed that samples 1 -13 are visibly identical to the FTIR spectrum of Asian elephant ivory; in contrast, samples 14 and 15 do not match the FTIR spectrum of Asian elephant ivory. Hence, it is confirmed that out of fifteen samples, thirteen are likely to be of Asian elephant ivory origin. To confirm this finding, we employed the PLS-DA analysis. The result showed an R Square value of 0.9 for calibration and 0.75 for validation, and the model exhibited 100% accuracy in classifying the original and fake ivory samples. The results confirmed that FTIR combined with chemometrics analysis was useful for identifying ivory and fake materials. To validate the FTIR results, DNA was extracted from all suspected samples (1-15) using the QIAamp DNA Investigator Kit; however, samples 14 and 15 could not lyse during DNA extraction. We also tried all the troubleshooting methods for lysis, but none succeeded. Therefore, we did not process samples 14 and 15 for subsequent experiments. Then, amplified elephant-specific D-loop and *Sry* (sex-determining region on the Y chromosome) genes from the DNA extracted from samples 1-13. Subsequently, the PCR amplicons were examined on a 2.5 % agarose gel and observed for samples 1-13, one band at 137 bp (for D-loop) and another at 97 bp (for *Sry*). These results indicated that samples 1-13 are of elephant origin. To confirm this finding, the PCR amplicons (D-loop) from nine samples (1-9) were sequenced, and % similarity was analyzed. The results showed 96.6 % to 100 % similarity to the *Mammuthus primigenius*, *Elephas maximus indicus*, and *Loxodonta africana*. The primers (D-loop and *Sry*) used in this study are elephant-specific and do not distinguish the species. Overall, DNA-based results supported the results from FTIR analysis and confirmed that the suspected samples 1-13 are of elephant origin and 14 and 15 are not of elephant origin. The current study has demonstrated the identification of ivory substitutes through FTIR spectral library for Asian elephant ivory, which is rapid, cost-effective, and has excellent potential for forensic analysis.

Highlights

- The FTIR spectral library was developed for Asian elephant ivory.
- The suspected elephant ivory carvings were identified using the FTIR spectral library of Asian elephant ivory.
- The FTIR results were confirmed through PCR amplification of elephant-specific D-loop and *Sry* genes.
- Overall, the study revealed that ivory and its products can be identified using an FTIR spectral library of Asian elephant ivory.

1. Introduction

The illegal ivory trade remains one of the most pressing threats to the conservation of elephant populations. The Annual Illegal Trade Report (2020) by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has documented that ivory was among the top 10 specimens with the most frequently reported seizures from 2016 to 2020[1]. To understand ivory trafficking, data reported in the World Wildlife Crime Report (2020) by the United Nations Office on Drugs and Crime (UNODC) was reviewed and it was observed that in December 2018, 1,026 tusks (3.2 metric tons) were seized by Cambodian customs. In the year 2019, the Huangpu Anti-Smuggling Bureau seized 2748 tusks (7.48 metric tons) in China, 3,446 kg of ivory were seized in Haiphong, Vietnam, and the Uganda Revenue Authority (URA) seized 762 pieces of ivory (3,299 kg). This report also highlighted that around US$400 in revenue was generated from illegal ivory trafficking from 2016 to 2018. Further, UNODC suggested that illicit supply chain analysis is a powerful tool in our fight against ivory trafficking. It helps us understand the functioning of illegal markets and how organized crime groups interact to organize the often-global illicit trade in goods and services. In addition, it will help us monitor the situation continuously and provide insights on potential weaknesses in the supply chains that allow for disrupting illicit ivory supply chains (the network) and illicit financial flows related to wildlife trafficking[2-4].

UNODC has reported certain guidelines on methods and procedures for ivory sampling and laboratory analysis[5]. According to these guidelines, morphological examination, Fourier Transform Infrared (FTIR) Spectroscopy/Raman spectroscopy, and mitochondrial DNA-based methods have been recommended for ivory identification. However, the morphological examination can be utilized only if ivory is in its native form, and it cannot be used in the case of processed and furnished ivory products like ornament and carvings. The DNA-based analysis involves pairing and sorting of tusks, sampling of tusks followed by powdering and decalcifying the samples for DNA extraction. However, the time and resources required for a DNA-analysis to identify ivory are often prohibitive, and the quality and amount of DNA extracted from the ivory are critical factors in the molecular identification of ivory, which even becomes more complicated when it is processed ivory[6-9].

Spectroscopy has become an established technique for qualitative and quantitative analysis of various biological and non-biological materials[10-13]. For ivory analysis, several non-destructive

techniques have been developed for the identification and discrimination of ivories including Ultraviolet-visible-near-infrared (UV–vis–NIR) spectroscopy, FT-Raman spectroscopy, fluorescence X-ray spectroscopy, and fluorescence micro spectroscopy. Among the aforementioned methods, FTIR spectroscopy has drawn interest from several researchers due to its advantages over other spectroscopic techniques[14-18]. Firstly, it can be used for chemical and structural characterization of various materials (including amorphous, semicrystalline, and crystalline materials). Secondly, it allows users to use more convenient, rapid, portable, and cheaper equipment for field analysis.

Till today, several studies have been reported for ivory identification using FTIR spectroscopy combined with Attenuated Total Reflectance (ATR) accessory[19,20]. Notably, Wang et al. (2007)[21] and Li et al. (2023)[22] successfully identified ancient ivory tusks from archaeological sites Sanxingdui and Jinsha using FTIR spectroscopy. Banerjee et al in 2008 studied the quality of commercial mammoth ivory using X-ray powder diffraction and FTIR spectroscopy[23]. FTIR spectroscopy can be employed for the identification and differentiation of fossil and modern ivories, as well as ivories from various subspecies of African elephants[24]. On the other hand, FTIR spectroscopy can be employed for the identification of ivory-derived products. Rozalen et al. (2015)[25] used an FTIR to study the origin of material and gilding technique used in Hispano-Philippine ivories sculptures from the 17th century. In 2020 Cartier et al. compared the suspected artefacts with known ivory reference samples using FTIR spectroscopy, to determine whether the artefacts were made of ivory[26].

Nevertheless, no study has focused on establishing a standard reference library for ivory, which would facilitate the rapid identification of suspected articles and decrease the analysis cost and time. Therefore, the current study aimed to establish the FTIR spectral library with chemometrics analysis for Asian elephant ivory identification. Consequently, 15 unknown case samples (carvings) suspected of ivory origin were identified by visually comparing FTIR spectral data of suspected carvings with known reference ivories and chemometrics analysis. Further, FTIR spectral-based prediction of suspected carvings was validated by DNA analysis. Elephant-specific genes, i.e., D-loop and *Sry* (sex-determining region on Y chromosome), were amplified from the DNA extracted from the suspected ivory carvings using a polymerase chain reaction (PCR). Then, the PCR amplicons (sanger chemistry) were sequenced, and a BLAST analysis was performed to identify the % similarity. As per forensic standpoint, the present study is crucial since it will aid

in combating wildlife crimes by allowing for a prompt investigation of illegally imported as well as local ivory and its derived products.

2. Materials and Methods

2.2. Sample collection

2.2.1. Reference ivory samples

To prepare the FTIR spectral library for Asian elephant ivory, eight (n = 8) reference Asian elephant ivories (voucher specimen) were obtained from the repository of Morphometry Laboratory, Advanced Institute for Wildlife Conservation, Chennai, Tamil Nadu, India. No live animals were sampled at any stage of this study.

The samples were cleaned with 70% ethanol (v/v) to remove external contamination and then the inner layer of the ivory was scraped with sterile blades (Glassvan Sterile Surgical Blades, Haryana, India) across three regions (1. Apical, 2. Medial and 3. Basal) and 0.5 mg of powdered sample was collected in 2 ml tubes (Eppendorf Private Limited) independently for each region **(Figure 2.1).**

2.2.2. Unknown case samples (suspected ivory carvings)

Several suspected ivory carvings are received at Advanced Institute for Wildlife Conservation, Centre for Wildlife Forensic Sciences, Chennai, Tamil Nadu, for species identification **(Figure 2.2).** 15 unknown case samples (suspected ivory carvings) were used in this study to determine whether the ivory was used in their preparation. The samples were processed to powder form in the same manner as mentioned above for the reference ivory samples (section 2.2.1).

Figure 2. 1. (a) Eight Asian elephant ivory (*n* = 8) samples were collected from the Morphometry Laboratory, Advanced Institute for Wildlife Conservation, Chennai, Tamil Nadu, India, for the development of an FTIR spectral library. Ivory-1 and 2: 52 cm long and 15 cm mid-girth; Ivory-3 and 4: 51 cm long and 17 cm mid-girth; and Ivory-5 and 6: 80 cm long and 18 cm mid-girth; Ivory-7: 28 cm long and 15 cm mid-girth; Ivory-8: long 29 cm and mid-girth 18 cm. (b) The Sampled area is highlighted with numbers (I, II, and III), for each ivory, three samples were taken, and the spectrum was recorded.

Figure 2.2. The Advanced Institute for Wildlife Conservation, Centre for Wildlife Forensic Sciences, Chennai, Tamil Nadu, received fifteen (*n* = 15) suspected ivory carvings for the identification of elephant origin. (1) Elephant statute (2) Saraswathi statue (3) Female big statue (4) Female doll (5) Bullock cart (6) Sindoor Box (7) Pot (8) Cup (9-12) Small elephant statute (13) Big elephant statute with human rider (14-15) suspected ivory.

2.3. Sample preparation for FTIR spectroscopic analysis

A pinch of scraped sample and 1.0 g of KBr powder (FTIR grade, Sigma Aldrich) were taken into a sterile mortar and blended thoroughly using the pestle. Subsequently, a 13 mm KBr pellet was prepared. All the samples (reference ivory, suspected ivory carvings and antlers) were prepared similarly. FTIR spectrum was recorded using the specified instrumental parameters described below.

2.4. Instrumentation

FTIR spectroscopy measurements were performed using a Bruker Alpha-II FTIR spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a Deuterated triglycine sulfate (DTGS) detector. Data were recorded in the spectral range from 4000–500cm^{-1} in transmittance mode with a spectral resolution of 4 cm^{-1}. Averaged 24 scans were acquired for both background and sample spectra. All samples (08 reference ivory, 15 suspected ivory carvings and 06 spotted deer antlers) were prepared and analyzed in triplicates, totally 87 spectra (Reference ivory 8 nos × 3 = 24; 15 nos suspected ivory carvings × 3 = 45; 06 spotted deer antlers × 3 = 18) were recorded. All experiments were performed at room temperature (25˚C ± 0.5 ˚C). After each experiment, all the instrument accessories for preparing the pellet and the sample holder were cleaned with acetone (analytical grade) to avoid any cross-contamination.

2.5. Data analysis and spectra preparation

The recorded FTIR spectral data was pre-processed using OPUS software (Version 8.8) (Bruker Optics, Ettlingen, Germany). Atmospheric compensation for reducing the influence of CO_2 and H_2O absorption bands and baseline correction were employed for every recorded spectrum before the analysis and graphing. The FTIR spectra were prepared (Origin Lab, Premium version-2023) individually for each sample and after that, the functional groups were annotated for the primary chemical compounds present in the sample.

2.6. Multivariate data analysis

Chemometric tools were used to analyze the complex spectral data, providing objectivity to the findings. The current study utilized the PLS-DA (Partial Least Square-Discriminant Analysis) chemometrics tool to determine the origin of ivory based on its IR absorbance properties[27]. Ivory consists of dentin, a hard calcareous material composed of calcium hydroxyapatite and protein (primary chemical composition)[28]. Studies reported that similar chemical composition is present in other wild articles such as antlers, bones, teeth, etc[29, 30]. We

selected the antler to construct the PLS-DA model because deer antlers have been used as a substitute for ivory, and it is a bit challenging to identify after pre-processing. Therefore, we collected six (n = 6) spotted deer antlers from our institute, and the samples were prepared and recorded the FTIR spectrum as described in *sections 2.1 and 2.2*, respectively.

To construct the PLS-DA model, FTIR spectral data from 12 samples (six each from ivory and antler) was initially used. All the data analysis was performed using MATLAB R2020b equipped with PLS Toolbox 8.2 (Eigenvector Research, Inc. WA, USA) on a personal computer with a Windows 11 operating system (Microsoft Corporation, Redmond, WA, USA).

Partial Least Squares Discriminant Analysis (PLS-DA) was performed for classifying and differentiating the ivory and antler samples. For constructing the PLS-DA model for classification/validation, the FTIR spectra between 3500 cm^{-1} to 500 cm^{-1} were taken. All the spectral data were pre-processed before model construction. The data was converted from transmittance to absorbance, then smoothened using a second-order Savgol Filter (Savitzky-Golay algorithm), normalized and mean-centred. The analysis was performed in three ways: (i) the six ivory and antler data were initially used to construct the PLS-DA model to distinguish ivory and antler. (ii) Validation analysis was performed using two ivory samples and finally (iii) External blind validation was performed using fifteen (n = 15) carvings (suspected for ivory). The performance of the PLS-DA model was analyzed using internal cross-validation and external prediction sensitivity and specificity.

2.6.1. Validation test

To evaluate the effectiveness and classification ability of the developed PLS-DA model, validation was performed. Eight FTIR spectral data for ivory samples were split into two subsets (6 for model construction and 2 for validation), and the validation of the constructed PLS-DA model was performed with two ivory samples (other than the training dataset). Subsequently, specificity and sensitivity were determined for validation set, to validate the discriminating power of the developed PLS-DA model.

2.6.2. Identification of suspected carvings using an established PLS-DA model.

We used the PLS-DA model to identify if the suspected carvings are made from elephant tusks. First, we preprocessed the FTIR spectral data for the 15 suspected samples, as described in *section 2.5*. Then, we applied the data to the developed PLS-DA model. After that, we calculated the sensitivity and specificity values.

2.7. Molecular species identification of suspected ivory carvings

2.7.1. Sampling

All suspected ivory carvings were first cleaned with 70% ethanol (v/v) and air-dried for 10-15 minutes. Then, we scrapped the samples and collected approximately 200 mg of sample in a 2 ml sterile tube from each carving. To prevent contamination between samples, we wiped the drill bit with acetone (analytical grade) before and after the sampling.

Step 1: Decalcification:

1. Firstly, 1.5 ml of 0.5 mM EDTA (pH 8.0) was added to the samples and incubated at 56°C for 1 hour in a ThermoMixer (Eppendorf India Private Limited). Every 10 minutes, the samples were mixed thoroughly to ensure proper lysis.
2. After incubation, the samples were centrifuged at 5000 rpm for 5 minutes, and the supernatant was discarded. Subsequently, the pellet was washed thrice with phosphate-buffered saline (PBS) (pH 7.0) and added 1.5 ml of 0.5 mM EDTA (pH 8.0) and the solution was incubated at 37°C for 1 hour in a ThermoMixer. Following the incubation, the samples were washed again with PBS, and the resulting pellet was used for DNA extraction.
3. During decalcification, we noticed that samples 1-13 were lysed properly; however, samples 14 and 15 were unable to lyse. We also tried all the troubleshooting methods for lysis, but none succeeded. Therefore, we did not process samples 14 and 15 for subsequent experiments.

Step 2: DNA extraction

The pellet from Step 1 was taken, and the QIAamp DNA Investigator Kit (Qiagen, Hilden, Germany) was used for DNA extraction as per the manufacturer's instruction. Extracted DNA was quantified using NanoDrop (Thermo Fisher, Massachusetts, United States) and stored at - 20°C until further analysis.

2.7.2. Multiplex PCR amplification for elephant-specific genes

To confirm the FTIR results for suspected ivory carvings, we performed PCR amplification of elephant-specific genes, i.e., D-loop (control region) and *Sry* (sex determination region on Y chromosome) from the DNA extracted from suspected carvings (samples 1-13) using the primers reported by Gupta et al., (2006)[31] i.e. (D-loop: F: 5’-GAGGCCCTAACACAGTCAAGCAAC-3’; R: 5’-CGTGTACGCTGGGAATTTAGGTT-3’; *Sry*: F: 5’ GGGATACCAGTGGAAAATGCTTA-3’; R: 5’ - GTTCGGGTATTTCTCTCGGTGCA3’). Briefly, a 25 µl total volume PCR reaction was carried

out with 12.5µl 1X red dye master mix (Ampliqon, USA), 0.5 µL D-loop forward and reverse primers (5 pM each), 1 µl *Sry* forward and reverse primers (5 pM each), 50 to 100 ng of extracted DNA as template and the volume was adjusted to 25 µL with nuclease free water. The PCR reaction conditions were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 40s, annealing at 60°C for 1 min, extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min. The PCR amplicons were analysed on 2.5% agarose gel electrophoresis and photographed in a Gel Doc + system (Bio-Rad, Laboratories, USA).

2.7.3. Sanger sequencing and validation

To ensure the primers correctly amplified the specific regions of D-loop (elephant species specific gene), the PCR amplicons from nine carvings samples (1-9) were selected for Sanger sequencing. About 75 µl of each PCR amplicon was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified PCR amplicon was sequenced bidirectionally following the instructions of the BigDye™ Terminator v3.1 Kit (Thermo Fisher Scientific Inc, Waltham, Massachusetts, United States) using an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, United States). The sequences from samples (1-9) were analyzed for quality and compared with sequences in the NCBI database using the Basic Local Alignment Search Tool (BLAST) to identify the % similarity.

3. Results and Discussion

3.1. Development of FTIR spectral library for Asian elephant ivory

The goal of this work is to establish the FTIR spectral library for Asian elephant ivory and to accomplish this, the primary biochemical composition of the ivory was determined by analyzing the FTIR spectra of reference samples. The results are tabulated in **Table 2.1**; briefly, spectral peaks were observed at 1658 cm^{-1} and 1547 cm^{-1} associated with organic materials amide I, and amide II respectively. The peak at 1242 cm^{-1} corresponds to Amide III, and the peaks at 1453 cm^{-1} and 871 cm^{-1} represent $\nu_3(CO_3^{2-})$ and $\nu_2(CO_3^{2-})$ respectively both of which depict the CO_3 on the OH site of hydroxyapatite. In addition, the peak at 1411 cm^{-1} represents $\nu_3(CO_3^{2-})$ along with the CH_2 wag modes and peaks within the range of 1117 cm^{-1} and 1027 cm^{-1} associated with $\nu_3(PO_4^{3-})$. Additionally, the peaks at 600 cm^{-1} and 559 cm^{-1} corresponds to the inorganic orthophosphate group of $\nu_4(PO_4^{3-})$, and a narrow peak at 965 cm^{-1} represents $\nu_1(PO_4^{3-})$. The PO_4^{3-} bands were identified as less intense in the modern Asian ivories[32-36] **(Figure 2.3).**

Table 2.1: Assignment of IR absorption bands in the spectra of the Asian elephant ivory.

S. No.	Peak (cm^{-1})	Assignment	Source	References
1.	1658	Amide I	Collagen Type I	32-36
2.	1547	Amide II	Collagen	
3.	1453	$\nu_3(CO_3^{2-})$	Hydroxyapatite - $Ca_5(PO_4)_6(OH)$	
4.	1411	$\nu_3(CO_3^{2-})$	Hydroxyapatite - $Ca_5(PO_4)_6(OH)$	
5.	1242	Amide III	Collagen	
6.	1117	$\nu_3(PO_4^{3-})$	Hydroxyapatite - $Ca_5(PO_4)_6(OH)$	
7.	1027	$\nu_3(PO_4^{3-})$	Hydroxyapatite - $Ca_5(PO_4)_6(OH)$	
8.	965	$\nu_1(PO_4^{3-})$	Hydroxyapatite - $Ca_5(PO_4)_6(OH)$	
9.	871	$\nu_2(CO_3^{2-})$	Hydroxyapatite - $Ca_5(PO_4)_6(OH)$	
10.	600	$\nu_4(PO_4^{3-})$	Hydroxyapatite - $Ca_5(PO_4)_6(OH)$	
11.	559	$\nu_4(PO_4^{3-})$	Hydroxyapatite - $Ca_5(PO_4)_6(OH)$	

Elephant ivory comprises 70% carboned hydroxyapatite crystals (inorganic), 20% collagen (constituted with type 1) and 10% water[23]. Thus, researchers use carboned hydroxyapatite and collagen as preliminary chemical compounds for identifying elephant ivory [32-36]. In this study, FTIR spectral peaks observed at 1453 cm^{-1}, 1411 cm^{-1}, 1117 cm^{-1}, 1027 cm^{-1}, 965 cm^{-1}, 871 cm^{-1}, 600 cm^{-1}, and 559 cm^{-1} are attributed to carboned hydroxyapatite. FTIR spectral peaks observed at 1658 cm^{-1}, 1547 cm^{-1}, and 1242 cm^{-1} are attributed to amide I, amide II, and amide III (Protein source is collagen) respectively.

To gain a better understanding, the findings of this study were compared to FTIR spectral data from African elephant ivory. Regardless of elephant species, the ivories are constituted of hydroxyapatite and collagen. Yannick Le Guennec documented the FTIR spectral bands of African elephant ivory at 1674 cm^{-1}, 1554 cm^{-1}, and 1250 cm^{-1} that are attributed to amide-I and amide-II from collagen protein, and the bands at 1445 cm^{-1}, 1410 cm^{-1}, 1123 cm^{-1}, 960 cm^{-1}, and 557 cm^{-1} are attributed to hydroxyapatite[24]. However, based on the comparison it can be observed that the

IR spectral data of the two ivories (Asian and African elephant ivory) do not differ much because they comprise the same chemical constituents.

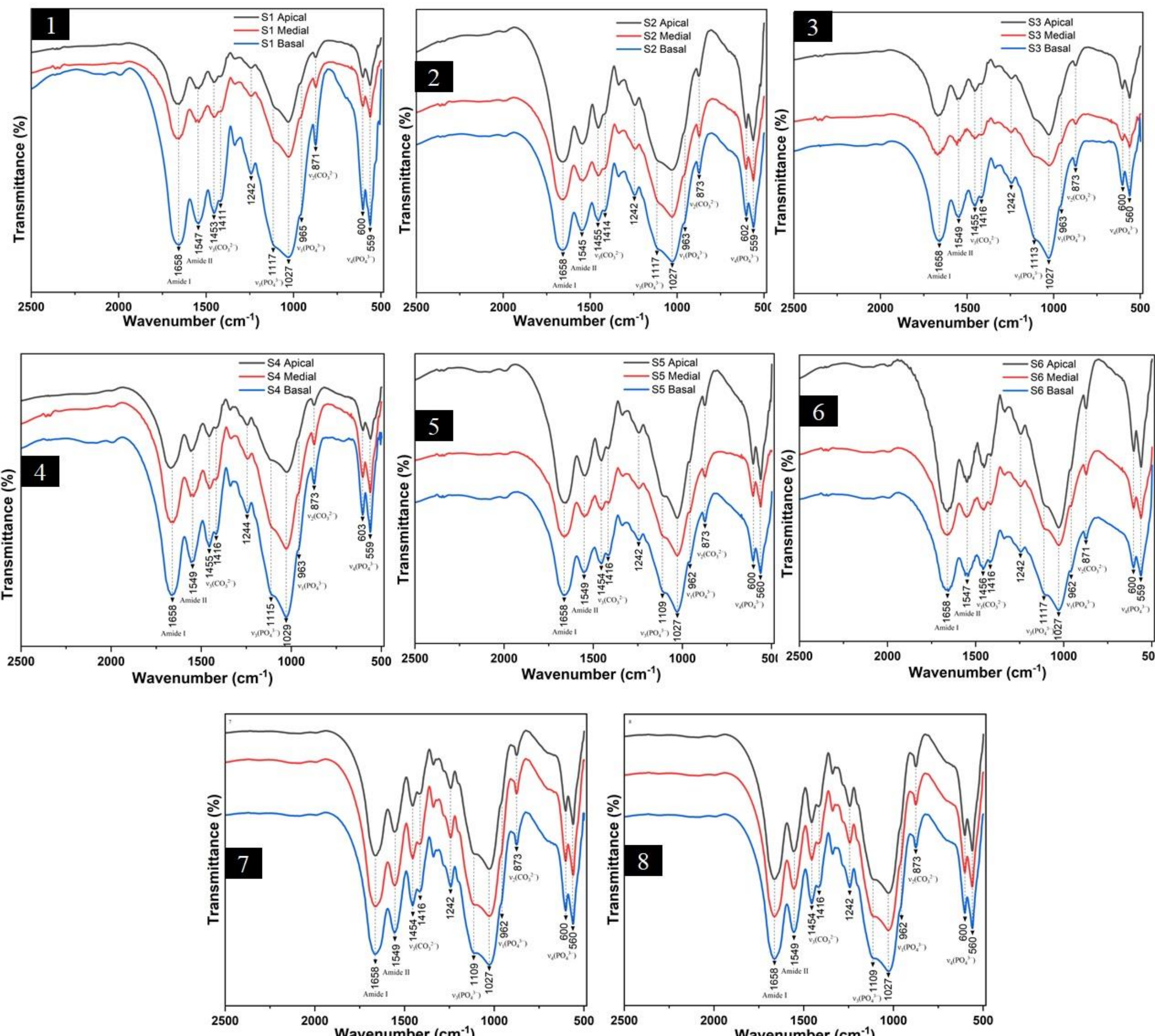

Figure 2.3. FTIR spectrum recorded for eight Asian elephant ivories (1-8). Annotated the spectral peaks associated with the ivory chemical compounds, i.e., carboned hydroxyapatite and collagen. The experiments were performed in triplicate.

3.2. PLS-DA model construction

The UNODC World Wildlife Crime Report-2024 documented that ivory was a high-value exclusive good and accounted for 3% of seizure records during 2015–2021[37]. National and international laws limit commercial ivory trading, and to meet market demands, traders used various materials (articles from other wild and domestic animals, other chemical materials, etc.) to prepare fake ivory products[38]. This is a major concern to identify ivory origin from counterfeit materials. Therefore, the current study attempted to construct the PLS-DA model to identify ivory/products from ivory. In light of this, the study used Asian elephant ivory and spotted deer antlers, which have a similar chemical composition to ivory. The results showed two clusters in the PLS-DA model: ivory (class-I) and antlers (class-II), with an R Square value of 0.9. Additionally, we did not observe any overlap between ivory and antlers; however, we noticed a scattered distribution for ivory, which reflected the biochemical concentration-dependent and spatial variations of the spectra recorded for ivory samples **(Figure 2.4).** Previously published reports were reviewed to understand the biochemical concentration variations in the ivory. Notably, Raubenheimer et al. (1998)[39] studied the impact of geographic variations in the chemical composition of ivory of the African elephant. They observed that ivory's inorganic fraction and hydroxyproline content vary based on the geographic location. Singh et al. (2006)[40] studied the elemental and isotopic composition of Asian elephant ivory and found that the nitrogen isotopic ratio (^{14}N and ^{15}N) varies based on location. They also observed that ivory from different states of India has different concentrations of nitrogen isotopic ratio. For example, Karnataka has a medium to high nitrogen isotopic ratio, and Uttarakhand has a low nitrogen isotopic ratio. Meanwhile, Assam, Kerala, and Tamil Nadu ivory had a medium nitrogen isotopic ratio. The researchers concluded that nitrogen isotopic ratio variation in elephant ivory is due to different plant types and food species in various states. Besides this, no study has been conducted to understand what parameters play important roles in the biochemical concentration variations in ivory. However, it is well documented that ivory is composed of collagen and hydroxyapatite molecules, and these biochemical compounds can be used to identify ivory and distinguish it from original ivory and

fake ones. The current study also observed the presence of collagen and hydroxyapatite molecules in all the Asian elephant ivory samples taken to develop the FTIR spectral library **(Figure 2.3).**

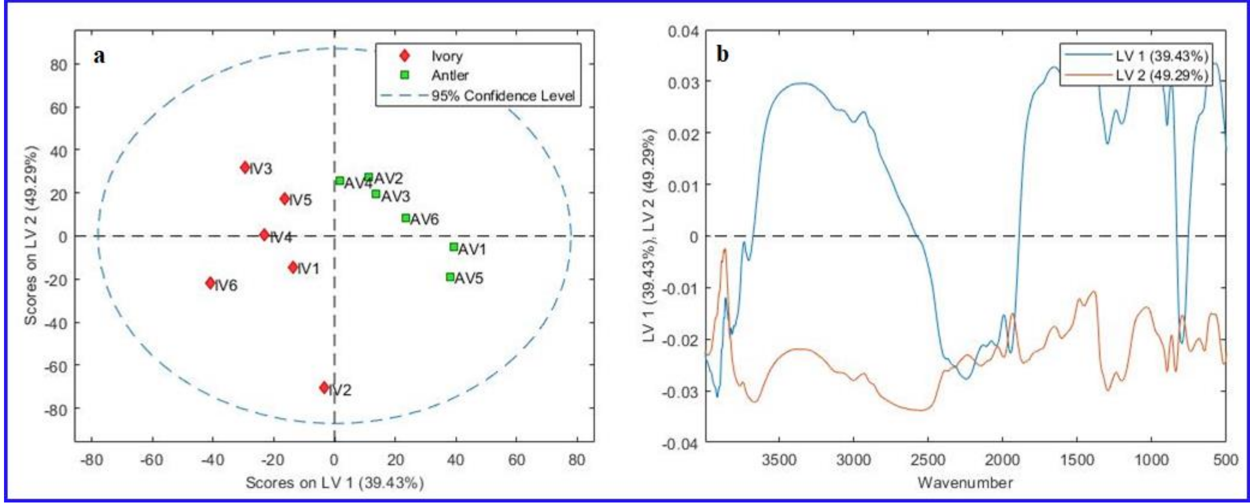

Figure 2.4. (a) PLS-DA score plot to discriminate the ivory (n = 6) and antlers (n = 6) (contain similar chemical composition to ivory) (b) Loading plot for PLS-DA analysis.

3.2.1. PLS-DA model validation

To evaluate the working performance of the constructed PLS-DA model, a validation analysis was performed with two Asian elephant ivory samples (the dataset was not used for training). The results indicated that two ivory samples were accurately categorized (grouped) into ivory class with an R2 value of 0.75 and a Root Mean Square Error of cross-validation (RMSECV) of 0.31 **(Figure 2.5).** Based on these results, we understand that the developed PLS-DA model can discriminate between ivory and articles containing similar chemical compositions. To test this hypothesis, we employed the PLS-DA model to investigate whether the fifteen suspected ivory carvings are of elephant origin.

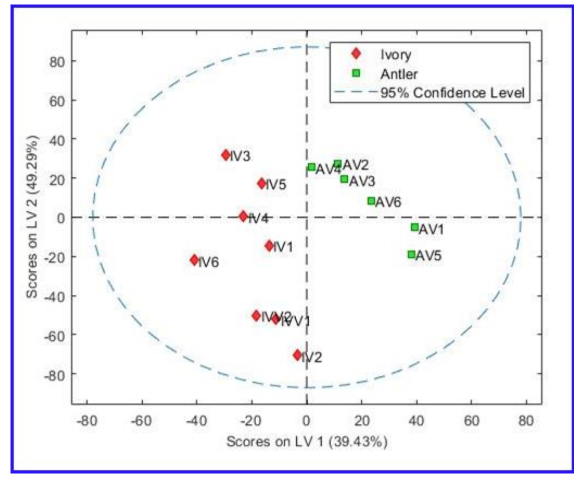

Figure 2.5. PLS-DA score plot for validation. IVV = Ivory validation

3.3. Identification of suspected ivory carvings through established FTIR spectral library for Asian elephant ivory

The primary objectives of developing an FTIR spectral library for Asian elephant ivory are to identify ivory and its modified products and distinguish between original and fake ivory. In this study, FTIR spectra of 15 suspected ivory carvings were visually compared with the FTIR spectra of 6 reference Asian elephant ivory samples. It was observed that spectra of suspected ivory carvings (samples 1-13) contain peaks at 1658 cm-1, 1547 cm-1, and 1242 cm-1 which are attributed to the collagen protein, and peaks attributed to hydroxyapatite are present at 1453 cm-1, 1411 cm-1, 871 cm-1, 1117 cm-1, 1027 cm-1, 600 cm-1, and 559 cm-1 **(Figure 2.6)**. In the case of samples 14 and 15, no spectral peaks associated with hydroxyapatite and collagen protein were observed (Figure 6). In addition, slight variations were observed in the intensity of spectral peaks (samples 1-13) when compared to reference ivory samples. This is due to ivory being subjected to pre- and post-processing to reach the form of carvings. However, visual comparison of FTIR spectra can be biased and error-prone therefore, PLS-DA were employed to identify whether the carvings are of ivory origin.

In the PLS-DA analysis for suspected ivory carvings, the model showed an R2 value of 0.9 and a Root Mean Squared Error (RMSE) of 0.19 with 100% classification accuracy. Out of fifteen suspected carvings (SS), the results showed that samples 14 and 15 are not of ivory origin, and these materials do not contain any hydroxyapatite and proteins. Further, except samples (SS3, SS6, and SS8); all other samples are grouped with ivory **(Figure 2.7)**. The results suggested that (1) during ivory modification into different articles, they are subjected to various chemical treatments and pre-processing procedures. During this process, the chemical composition of ivory is likely to denature, and it impacts the IR absorbance properties. (2) The current PLS-DA model was constructed with six ivory samples and validated with two ivory samples. Therefore, it is necessary to use a high volume of ivory samples to develop comprehensive PLS-DA for ivory; additionally, it is also essential to validate the PLS-DA model with articles containing similar chemical composition to ivory (e.g., bone and antlers).

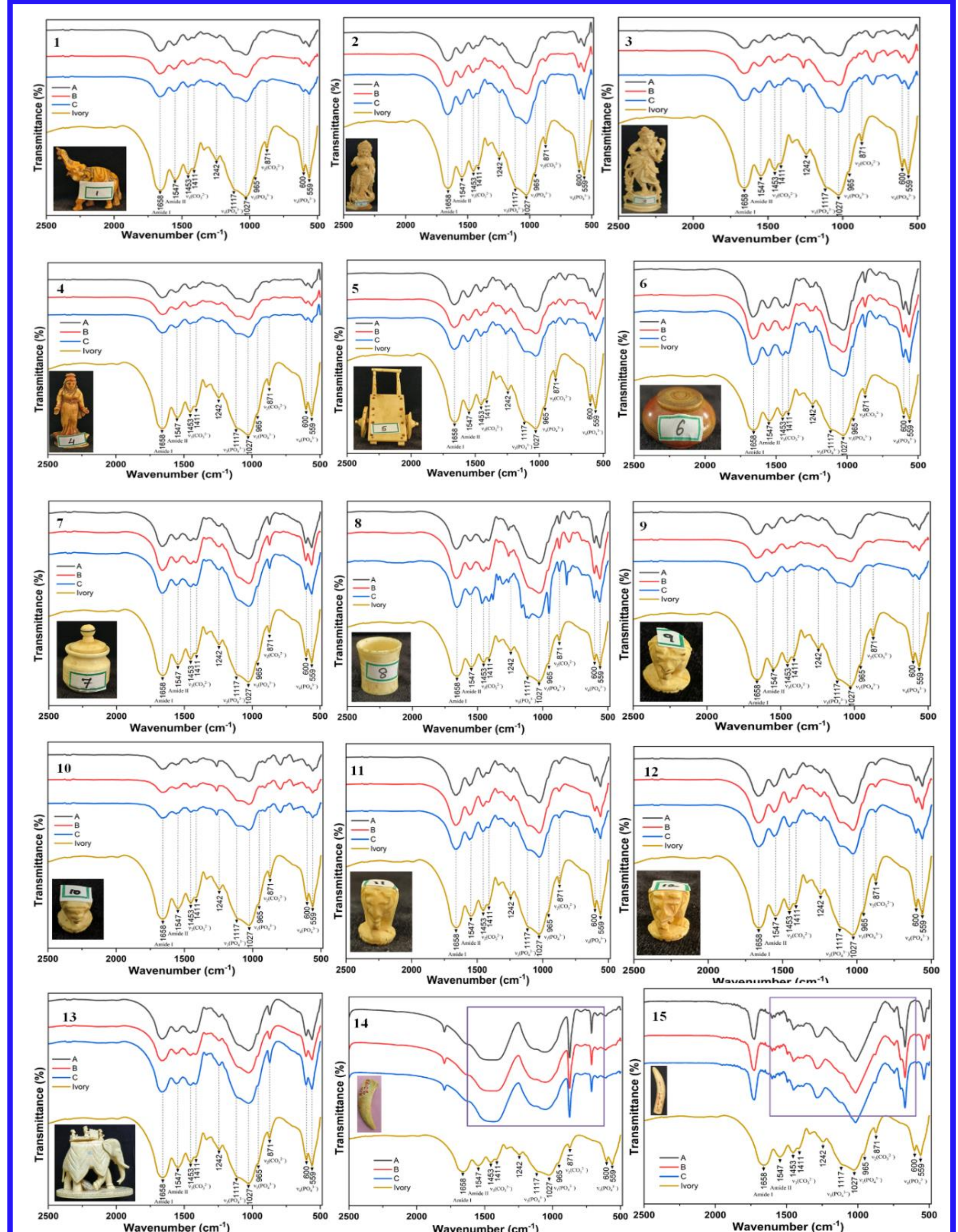

Figure 2.6. FTIR spectra were recorded for fifteen (n = 15) suspected ivory carvings, then compared with the spectra recorded for Asian elephant ivory (which served as a reference). The FTIR spectra of the reference and suspected ivory carvings demonstrate a spectrum pattern and chemical composition similar to ivory (1-13 samples). However, the FTIR spectra for samples 14 & 15 did not match with the reference ivory spectra (14–15). Spectra were recorded in triplicate for each suspected artefact and ivory. *Ivory* = Reference (FTIR spectrum for Asian elephant ivory; one spectrum was randomly selected from this study).

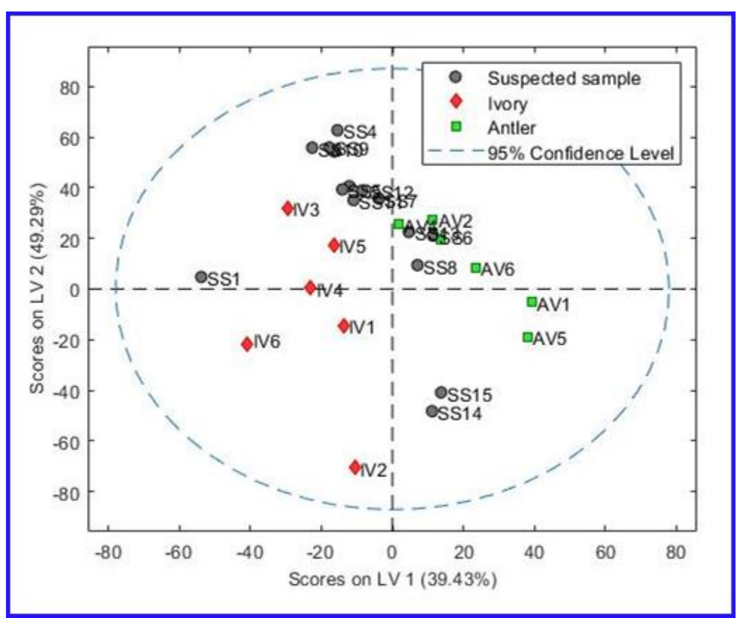

Figure 2.7. PLS-DA score plot for the identification of suspected ivory carvings. *SS = suspected samples.*

3.4. Validation of FTIR results by amplification of elephant-specific gene from suspected carvings

Elephant-specific genes amplification was carried out to validate the overall results of FTIR spectroscopy and chemometrics analysis for identifying suspected carvings. Out of 15 samples, DNA was only extracted from 13 samples (1-13) while no DNA was extracted from samples 14 and 15. Although all possible DNA extraction methodologies were employed, none of them were successful. Hence, it was presumed that samples 14 and 15 had no DNA, and PCR amplification was not carried out for samples 14 and 15. The PCR was carried out to amplify elephant-specific genes (*Sry* and D-loop genes) from the DNA extracted from samples 1 to 13. All of the samples showed PCR bands at 137 bp (D-loop) and 97 bp (*Sry*), which indicated that elephant specific *Sry* and D-loop genes were successfully amplified in suspected carvings (1-13) **Figure 2.8**. These results confirmed that the DNA extracted from 13 suspected carvings is of elephant origin. On the other hand, the results of this study were found to be compatible with the sex identification of elephants reported by Gupta et al. (2006)[31]. DNA analysis strongly confirmed that the suspected carvings (1-13) are of elephant origin. To validate these results, we sequenced the PCR amplicons of D-loop gene (137 bp) from nine suspected carving samples (1-9), and

BLAST analysis was performed. The results show similarity to the *Mammuthus primigenius, Elephas maximus,* and *Loxodonta Africana* (**Table 2.2**). The primers (D-loop and *Sry*) used in this study are elephant-specific and do not distinguish the species. Therefore, it is confirmed that suspected ivory carvings (1-13) received for species identification are of elephant origin. In contrast, in PLS-DA analysis, suspected ivory carvings (SS3, SS6, and SS8) are not grouped with ivory, but the DNA analysis confirmed that these samples are of elephant origin. These results clearly indicate that it is necessary to strengthen the PLS-DA model with a high number of ivory samples to overcome the issue stated above. Overall, the current study demonstrated the development of the FTIR spectral library for Asian elephant ivory and enabled the identification of elephant origin from the suspected ivory carvings using the FTIR spectral library for Asian elephant ivory developed from this study.

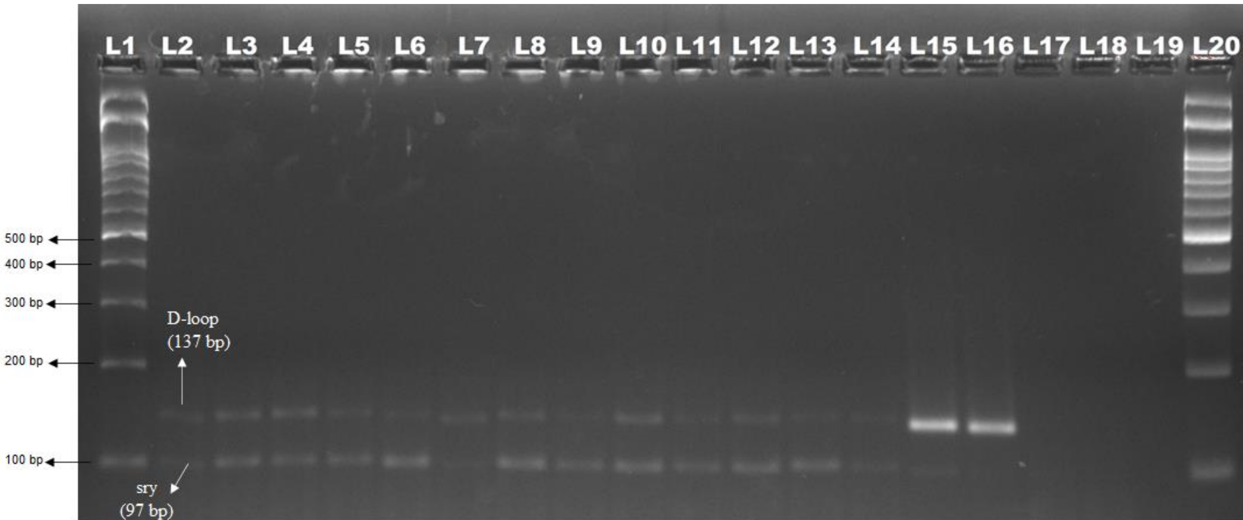

Figure 2.8. Multiplex PCR amplification of the elephant-specific gene from the DNA extracted from the suspected carvings. L1 – 100 bp DNA ladder; L2 to L14, suspected carvings multiplex PCR products, and Lane L15 –Male elephant positive control, L16 –Female elephant positive control; L17 - L19 - Negative control (No template DNA); L20-100 bp DNA ladder.

Table 2.2: Basic Local Alignment Search Tool (BLAST) analysis is used to identify species from the suspected carvings and validate the FTIR results.

Sample ID	Sample name	D-loop sequences	% Similarity	Quarry cover (%)	Species#
S1	Elephant statute	GACTGATTGAGTATTAGATCTGGCACGGTATATATGGGGTATTATTCAGTCAATGCTCGGAGGACA	98.39	92	*Mammuthus primigenius*
			98.39	92	*Elephas maximus indicus*
			98.39	92	*Loxodonta africana*
S2	Saraswathi statue	TACTGATTGAGTATTAGATCTGGCACGGTATATATGGGGTATTATTCAGTCAATGCTCGGAGGAA	98.33	90	*Mammuthus primigenius*
			98.33	90	*Elephas maximus indicus*
			98.33	90	*Loxodonta africana*
S3	Female big statue	GACTGATTGAGTATTAGATCTGGCACGGTATATATGGGGTATTATTCAGTCAATGCTCGGAGGACA	98.39	92	*Mammuthus primigenius*
			98.39	92	*Elephas maximus indicus*
			98.39	92	*Loxodonta africana*
S4	Female doll	TACTGATTGAGTATTAGATCTGGCACGGTATATATGGGGTATTATTCAGTCAATGCTCGGAGGACA	98.39	92	*Mammuthus primigenius*
			98.39	92	*Elephas maximus indicus*
			98.39	92	*Loxodonta africana*
S5	Bullock cart	TACTTGATTGAGTATTAGATCTGGCACGGTATATATGGGGTATTATTCAGTCAATGCTCGAGAA	96.67	90	*Mammuthus primigenius*
			96.67	90	*Elephas maximus indicus*
			96.67	90	*Loxodonta africana*
S6	Sindoor Box	TATAATTGATTAAGATCTGGCACGGTATATATGGGGTATTATTCAGTCAATGCTCGGAGGAA	100	85	*Mammuthus primigenius*
			100	85	*Elephas maximus indicus*
			100	85	*Loxodonta africana*
S7	Pot	GGACTTGATTGAGTATTAAGATCTGGCACGGTATATATGGGGTATTATTCAGTCAATGCTCGGAGGAC	98.48	95	*Mammuthus primigenius*
			98.48	95	*Elephas maximus indicus*
			98.48	95	*Loxodonta africana*
S8	Cup	TAACTGATTGAGTATTAGATCTGGCACGGTATATATGGGGTATTATTCAGTCAATGCTCGGAGGAA	98.33	89	*Mammuthus primigenius*
			98.33	89	*Elephas maximus indicus*
			98.33	89	*Loxodonta africana*
S9	Small elephant statute	GACTATTGAGTATTAGATCTGGCACGGTATATATGGGGTATTATTCAGTCAATGCTCGGAGAA	98.28	90	*Mammuthus primigenius*
			98.28	90	*Elephas maximus indicus*
			98.28	90	*Loxodonta africana*

#The primer used in this study did not differentiate the elephant species and will confirm whether the suspected samples were of elephant origin or not. Based on BLAST analysis, it is confirmed that the suspected carvings received for analysis are of elephant origin.

4. Conclusion

The development of innovative analytical methods for biological traces is in demand in the forensic sciences for both human and wildlife. Wildlife forensic laboratories receive various samples for species identification, i.e., antlers, feathers, quills, tusks, cheek teeth, tail hair, scales, claws, and canines including modified articles. DNA extraction from these samples is time-consuming, and getting quality DNA for PCR amplification is a major challenge. Currently, elephant ivory is one of the major trafficked articles around the world, and traders use various methods to modify the ivory to convert it into various products. During this process, the DNA gets degraded or fragmented. As a result, the molecular identification of ivory becomes even more challenging. In light of this, the current study demonstrated the development of an FTIR spectral library for Asian elephant ivory and documented the spectral peaks associated with chemical compounds present in the ivory (i.e., hydroxyapatite and collagen). The spectral data was also subjected to advanced chemometric methods i.e., PLS-DA to eliminate biases and provide objectivity to the results. Interestingly, 13 of the 15 suspected ivory carvings were found to be of ivory origin when compared to reference samples using visual and chemometric examinations. To support and validate the findings of FTIR spectroscopy analysis, PCR amplification of elephant-specific D-loop and *Sry* (sex-determining region Y gene) genes was performed on suspected ivory carvings and confirmed the FTIR findings. This study proposed that the FTIR spectral library created for Asian elephant ivory can be used to solve cases where suspected elephant ivory or its products are encountered in a quick, reliable, environmentally friendly, and cost-effective manner. However, further investigation is needed to account for potential variations (i.e., visible observation in the FTIR spectra and functional groups characteristics) in similar type of samples/materials (e.g., bone) and need to be assessed prior to this method being applied to forensic applications (especially, while examining modified products from ivory origin). The current study used only six ivory samples to develop the FTIR spectral library for Asian elephant ivory**.** We believe that the sample size used in this study to create an FTIR spectral library for the Asian elephant, which was less in number, and future expansion of the spectral library may be created as and whenever we receive authentic Asian elephant ivory. A large number of samples is necessary to strengthen the FTIR spectral library, increasing its confidence level for forensic applications.

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Chapter -3

Chapter 3 Discrimination of mongoose hair from domestic cattle, human hair, and synthetic fiber using FTIR spectroscopy and chemometric analysis

Mongoose hair is used to prepare fine brushes, which increases the demand for the mongoose to be poached from the wild and brutally bludgeoned to death. Mongooses are listed as Schedule I species under the Indian Wildlife (Protection) Act 1972. Species identification of wildlife case-related samples is necessary to convict a person under this legislation. Microscopy and DNA-based techniques are commonly used to identify mongoose hair in seized brushes. However, in the painting brushes, the roots, and the lower part of the hair are mostly trimmed, and only the upper part is used to make the brushes. In addition, brushes are often prepared with mixed hair from mongoose, domestic cattle, human hair, and synthetic fibre. Therefore, the identification of mongoose hair by microscopy and DNA-based techniques is restricted due to the lack of complete strands of hair and the absence of hair roots. Therefore, there is an urgent need to develop an alternative methodology for the identification of mongoose hair from seized articles. FTIR spectroscopy for forensic analysis has gained significant attention over the years because of its sensitivity, specificity, and non-destructive nature. The present study aimed to discriminate Indian grey mongoose (*Herpestes edwardsii*) hair from domestic cattle (domestic water buffalo and domestic cow), human hair, and synthetic fiber based on their chemical composition using FTIR spectroscopy and chemometric analysis. We have taken hair from four individuals for each species, namely Indian grey mongoose, domestic cattle, human hair, and synthetic fibre. The FTIR spectrum was recorded, and Partial Least-Squares Discriminant Analysis (PLS-DA) was used to discriminate hair and synthetic fiber. The established PLS-DA model showed an R-square value and an RMSE (Root Mean Square Error) value of 0.9 and 0.13 respectively. Our preliminary findings have shown that FTIR spectroscopy combined with chemometrics can quickly discriminate Indian grey mongoose hair, domestic cattle, human hair, and synthetic fiber, providing crucial evidence for judicial proceedings.

Highlights

1. FTIR spectroscopy combined with chemometrics analysis has been established to identify hair from mongoose.
2. FTIR spectroscopy analysis is a rapid, cost-effective, and field-deployable tool for wildlife forensics.

1. Introduction

India is a megadiverse country, home to 7-8% of the world's flora and fauna. It accommodates more than 45,000 species of plants and 91,000 species of animals[1]. Wildlife in India is protected by the Wildlife (Protection) Act 1972 (WPA), which prohibits the trade of more than 1800 species of wild animals, plants, and their derivatives[2-4]. Despite strong legal protection, poaching and illegal trade have been increasing over the years, posing a significant threat to several iconic species and pushing them to the brink of extinction. Mammals are frequently poached for their skin, meat, hair, and wool, which are widely traded for different purposes, such as fur coats, leather purses, belts, shoes, and carvings, etc[5,6].

In recent years, mongooses (*Herpestes* sp.) have been poached illegally and their hair is used to produce painting and shaving brushes[7,8.] The various seizures made by law enforcement agencies indicate that poaching of these animals is still regular and concerns the conservation of these animals[7]. For example, in 2023, the Andhra Pradesh Forest Department seized more than 18000 paint brushes from several premises allegedly used by dealers in Hyderabad city, and forensic analysis confirmed that the paint brushes were prepared with Indian grey mongoose (*Herpesten edwardsi*)[9]; The Tamil Nadu Wildlife Crime Control Bureau seized 14,000 mongoose hair paint brushes from various districts of Tamil Nadu, such as Chennai, Coimbatore, and Madurai[10]. Wildlife Crime Control Bureau (WCCB), India, has conducted enforcement operations in seven Indian states from 2002 - 2021, leading to the recovery of more than 1.6 lakh mongoose hair paint brushes and 292.7 kg of raw mongoose hair[11]. Additionally, the Centre for Wildlife Forensic Sciences, Advanced Institute for Wildlife Conservation, Tamil Nadu Forest Department, Vandalur, Chennai, has received several cases involving the use of mongoose hair. A study documented that there are seven mongoose species in India[7] namely (1) *Herpestes edwardsii*, (2) *Herpestes smithii,* (3) *Herpestes urva*, (4) *Herpestes palustris*, (5) *Herpestes javanicus*, (6) *Herpestes vitticolis* and (7) *Herpestes brachyurus*. All mongoose species are protected under the Indian Wildlife (Protection) Act 1972 and classified as Schedule I species[12], and the International Union Conservation of Nature (IUCN)[13] categorized mongoose species as ‘Least Concern’.

In general, microscopy was used to identify mongoose hair in the seized brushes. The Wildlife Institute of India (WII)[14] has demonstrated the identification of Indian grey mongoose from guard hair. The Zoological Survey of India (ZSI)[15] and the Wildlife Crime Control Bureau (WCCB)[16] have reported microscopic structures of dorsal guard hairs for identification of

mongoose species. However, the ZSI report lacks detailed descriptions of microscopic hair characters, and the WCCB report has no microscopic images. As a result, the practical application of these findings for forensic case analysis is limited. To address this issue, Sahajpal *et al.* (2009)[7] demonstrated the identification of four mongoose species (*H. edwardsii, H. smithii, H. palustris, and H. urva*) by microscopic examination of the pattern of the dorsal guard hair band and discriminate functional analysis (DFA). However, all studies have exhibited the identification of mongoose species by microscopy examination of the dorsal guard hair band pattern. Still, hairs from other regions of the mongoose body (e.g., tail) are also used in brush manufacturing. Therefore, further studies must be employed and compared with the reference data to overcome this limitation. Moreover, the identification of species from hair is subject to several factors, such as the availability of complete strands of hair, the expertise of the forensic examiner, the quality and quantity of the hair, the availability of known samples (reference hair samples) and the instrumentation used for analysis[17-21]. The results of microscopy analysis may sometimes not be conclusive; therefore, further study is needed to identify the species[21].

Next, DNA analysis is the most common method used to detect hair at the molecular level[22]. Before analysis, a forensic analyst classifies the hair based on the morphology of the hair roots to apply the appropriate analytical methods[23,24]. In forensic analysis, hair samples with anagen follicular tissue are ideal for DNA analysis[25]. However, up to 95% of hair collected as forensic evidence has been documented to be telogen, which is completely keratinized and contains a small amount of cellular material[26,27]. To overcome this issue, Bourguignon et al. (2008)[28] classified telogen hairs found at the crime scene such as type 1, without visible soft tissue; type 2, with a small amount of soft tissue attached; and type 3, with a large amount of soft tissue attached.

In most cases, the seized brushes received for forensic analysis were type 1 and DNA extraction; subsequent analysis is very challenging[7,22]. However, a few studies have reported the identification of species based on hair shafts, though the success rate is subject to various parameters, including the content of keratin[29]. In addition to this, during our forensic examination of the brushes received for analysis, we observed that the brushes prepared with mongoose hair were often mixed with hair from other domestic cattle, such as domestic water buffalo (*Bubalus bubalis*), domestic cow (*Bos taurus*), human hair, and synthetic fibers. Therefore, the identification of mongoose hair from seized brushes is a very tough task using microscopy and DNA-based techniques due to three primary reasons: (1) lack of complete hair structure (hair strands), (2) the

hair samples received for analysis are telogen, and (3) the seized brushes often mixed with hair from domestic cattle, human hair, and synthetic fibres. Therefore, it is a fact that identifying mongoose hair from seized brushes and discriminating it from counterfeit (i.e., hair from domestic cattle, humans, and synthetic fibres) is a major concern, and there is an urgent need to develop robust techniques/methods to distinguish mongoose hair from counterfeit materials.

The application of vibrational spectroscopy for forensic purposes has gained significant attention over the years because of its sensitivity, specificity, and non-destructive nature[30]. The infrared spectrum displays the vibrational characteristics of a sample based on the different absorption frequencies of the individual functional groups present in the sample (e.g., based on the chemical composition of the sample)[30]. In light of this, FTIR is used to analyse various materials in forensic science, including biomedical samples[31], paint[32], fingerprints[33,34], and ink[35]. Furthermore, studies have documented that the identification of species from hair using Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)[36-40]. Notably, Manheim et al. (2015)[41] described the identification of human, animal, and synthetic fiber by ATR-FTIR spectroscopy. Boll and co-workers (2017)[42] researched the use of ATR-FTIR spectroscopy for the classification of dyed and non-dyed hairs; Bhatia et al. (2024)[43] showed the identification of hairs from Royal Bengal Tiger (*Panthera tigris tigris),* Indian Leopard (*Panthera pardus fusca)* and snow Leopard (*Panthera uncia*) using the ATR-FTIR spectroscopic technique in combination with chemometric analysis. Hence, previous studies have well documented that species, biological, and non-biological articles can be identified by ATR-FTIR spectroscopy and chemometric analysis. Therefore, we have initiated a study to employ FTIR spectroscopy combined with chemometric tools for the identification of mongoose hair from other hair (i.e., hair from domestic cattle and human).

In light of this, the current study demonstrates FTIR spectroscopy combined with chemometric tools for the identification of Indian grey mongoose hair (*Herpestes edwardsii*) and discriminating it from domestic cattle (domestic water buffalo (*Bubalus bubalis*), domestic cow (*Bos taurus*)), human hair, and synthetic fiber. We selected the Indian grey mongoose for this study because it is the most common mongoose found in southern India and its proximity to human habitation[9]. As a result, Indian grey mongooses are often poached and their hair is used for brush preparation. The results of this study will play an important role in the wildlife forensics in identifying Indian grey mongoose hair from seized articles.

2. Materials and Methods

2.1.1 Collection of Indian grey mongoose hair (voucher sample)

We collected 100 hair samples (25 hair strands from each individual) from four Indian grey mongooses in our Morphometry Laboratory at Advanced Institute for Wildlife Conservation (AIWC), Chennai, Tamil Nadu, India. No sample was collected from live animals.

2.1.2 Collection of domestic cattle hair samples

From the Kolapakkam Farm house, Chennai, India, approximately 25-30 tail hairs were collected from each of the four individuals of domestic water buffalo (*Bubalus bubalis*) and domestic cow (*Bos taurus*). Before FTIR spectroscopy analysis, the species was confirmed by molecular analysis.

2.1.3. Human hair collection

Human hair samples were obtained for this study from four volunteers willing to donate a small amount of scalp hair. For all hair donors, only their age and biological sex were recorded; donor names and all other personally identifiable information were not collected. The donated hairs were visibly found to be natural (non-dyed) and black.

2.1.4. Synthetic fiber

In this study, we used Polyethylene terephthalate fiber. For FTIR analysis, we chopped small pieces of fiber and prepared the samples as described below.

2.2. Sample Preparation for FTIR spectroscopy Analysis

About 25 hair strands were taken from each individual, chopped into small fragments and finely powdered using a sterile mortar and pestle. The powdered samples were then thoroughly mixed with 1.0 g of KBr powder (Merck Millipore, IR spectroscopy grade) and a 13 mm KBr pellet was prepared. The mortar and pestle were cleaned with acetone (Analytical grade) and then dried before and after the preparation of the sample to avoid cross-contamination.

2.2.1. Instrumentation and Spectral Collection

FTIR spectra were recorded using Bruker FTIR model: Alpha II (Bruker Optics, Ettlingen, Germany) equipped with a ZnSe crystal accessory and a DTGS detector. A blank KBr pellet without the sample was run for the background scan before switching to the subsequent sample. The sample holder and instrument accessories to prepare the pellet were cleaned with analytical grade acetone after each experiment to avoid cross-contamination. The spectra were measured in

transmittance mode within the range 4000-500 cm^{-1} (mid-infrared region) with a spectral resolution of 4 cm^{-1}. All experiments were averaged from 24 scans and performed at room temperature. Each sample was analyzed in triplicate and a total of 48 reference spectra were recorded (16 samples $\times$ 3 = 48). Furthermore, we also recorded the FTIR spectrum for four synthetic fibers (4 samples $\times$ 3 = 12). In total, we recorded 60 spectra for this study.

2.3. Data Analysis

2.3.1. Spectra Preparation, Analysis, and Comparison of Spectra

The FTIR spectral data obtained from the OPUS software (Version 8.8, Bruker Optics, Ettlingen, Germany) were pre-processed within the software. Atmospheric compensation was applied to reduce the influence of the CO_2 and H_2O absorption bands, and a baseline correction was performed on every spectrum before any analysis. Graphical plots for the reference and suspected samples were created using Origin Pro 9. Then, the functional groups present in the primary compounds in the hair were annotated.

2.4.2. Chemometric Analysis

Chemometrics analysis is an interdisciplinary field of applied mathematics and statistics that helps to design, gather, analyse, and validate the information from an intrinsic set of data obtained from various analytical techniques[44]. In our study, the Partial Least Squares Discriminant Analysis (PLS-DA) is used to analyse the spectral data. PLS-DA is a linear classification method that combines the properties of partial least squares regression and the discriminative ability of classification technology[45]. Hence, the current study used the PLS-DA model to discriminate Indian grey mongoose hair from human hair, synthetic hair, and domestic cattle. Before applying the PLS-DA model, the data was pre-processed using the Savitzky-Golay algorithm for smoothing, the Mean centering method, and normalization. A classification model was constructed using PLS-DA to classify Indian grey mongoose hair, domestic cattle, human hair, and synthetic fiber. Four samples were taken from each species to construct the calibration set of the PLS-DA model. All data were analyzed using MATLAB R2020b (The MathWorks, MA, USA) equipped with PLS Toolbox 9.3 (Eigenvector Research, WA, USA).

3. Results and Discussion

3.1. FTIR spectroscopy analysis for Indian grey mongoose hair, domestic cattle hair, human hair, and synthetic fiber

Identifying seized wild animal parts is a great challenge due to a multitude of species involved in the illegal wildlife trade and the various forms of seized items. These articles are often made from materials derived from domestic animals and other sources (materials); therefore, species identification is more complex using conventional methods[46]. For example, CITES has documented that wild mammal skins (leather, fur) and products (hair, wool) are widely traded, often mixed with counterfeit furs, making the identification of wildlife products challenging[47]. Therefore, there is an urgent need to develop alternative methods to identify intra- and interspecies variations in wild and domestic species. This will enable a better implementation of national and international laws to effectively control wildlife trade.

In this study, FTIR spectroscopy was used to discriminate Indian grey mongoose hair, domestic cattle hair, human hair, and synthetic fiber. Hence, we recorded the FTIR spectrum for the hair of the Indian grey mongoose, domestic cattle hairs, human hair and synthetic fibres. The findings revealed that we could visually distinguish synthetic fiber from hair (all the hair used in this study). The results suggested that through FTIR analysis, it can be visually confirmed if seized brushes contain hair or any other materials (including other than hair origin). On another hand, a slight spectral difference, especially in the amide-II region, was observed in the hair of Indian grey mongoose compared to domestic animals **(Figure 3.1)**. Similar results were observed in the FTIR spectrum recorded for intra-species (different individuals from the same species) (Supplementary figures I). The primary biochemical component of animal fibres is keratin, a fibrous structural protein widely found in human and animal organs, including the epidermis, hoof, horn, hairs, and feathers[48]. Gallagher (2009)[49] detailed the protein composition of some animal fibers, while Espinoza et al. (2009)[50], Manheim et al. (2016)[41], Agarwal et al. (2019)[51], Wang et al. (2021)[48], and Xu et al. (2022)[52] characterized hair proteins using spectroscopy techniques and described their chemical composition and corresponding wavenumbers **(Table 3.1)**.

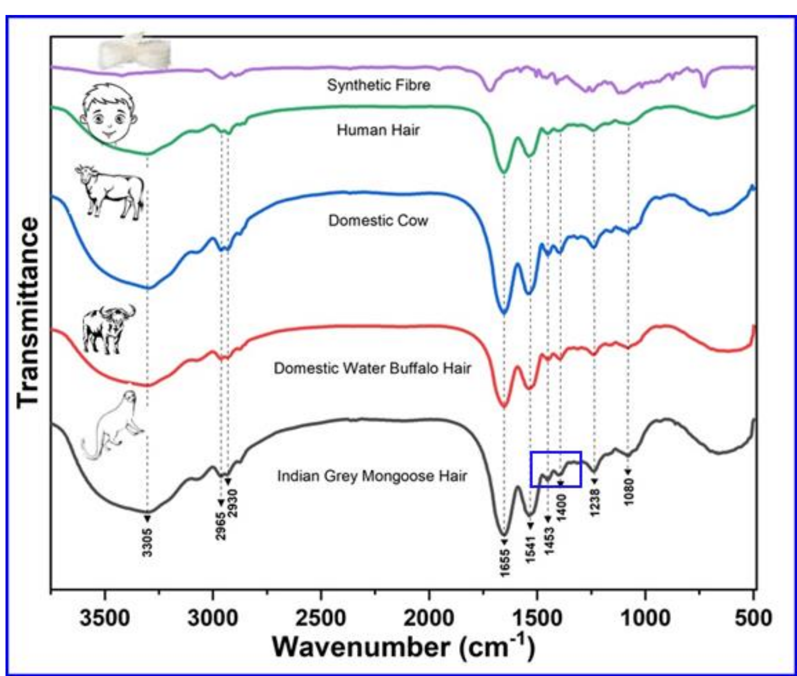

Figure 3.1. FTIR spectra of hair from Indian grey mongoose, domestic water buffalo, domestic cow, human hair and synthetic fiber. The inserted clipart was collected from online sources (for better visualization purposes), and Microsoft PowerPoint was used to prepare the figure. The blue box indicates the spectral differences in mongoose hair compared to other animal hair used in this study (including human hair).

Table 3.1: Assignment of IR absorption bands in the spectra of hair.

S. No.	Wavelength (cm^{-1})	Assignment	Source	Ref
1.	3100–3500	Amide A **(**Overlap of amide hydrogen N–H region and O–H region)	N-H stretching vibrations	48-53
2.	2850–3000	ν (CH3) asymmetric; υ(CH3) symmetric	Saturated and unsaturated C=H, C–H region	
4.	1600–1700	Amide I (Carbonyl oxygen C=O region)	C=O stretching vibrations	
5.	1500–1572	Amide II (Amide hydrogen N–H bond)	N-H formation vibrations and C-N stretching vibrations	
6.	1361–1470	Amide III δ(CH2) (CH3) deformation	C–N region	
7.	1210–1290	Amide III (Carbonyl oxygen C–O region)	C-N and C-O stretching vibrations, N-H and O=C-N bending vibrations	
8.	998–1100	Sulphur oxygen (S–O vibration, C–H bond)	Cystine oxides	

In our study, the peak observed at 3305 cm^{-1} is related to the organic material Amide A (primary amide) from the overlap of the N-H and O-H hydrogen amide regions (NH stretching vibrations). Based on previous studies on keratin, the peaks at 2965 cm^{-1} and 2930 cm^{-1} represent asymmetric and symmetric ν (CH3), respectively, and Amide I is identified at 1655 cm^{-1} which corresponds to the stretching vibrations of the C=O bond (amide group). The detected peak at 1541 cm^{-1} is the Amide II band that arises from vibrations of NH bending and C-N stretching, and the narrow peaks at 1453 cm^{-1} and 1400 cm^{-1} are due to the deformation in δ(CH2) (CH3) and δ(CH3) respectively. Similarly, a weak peak at 1238 cm^{-1} illustrates Amide III, a combination of stretching vibrations of the C-N and C-O bonds and bending vibrations of the N-H and O=C-N bonds. As reviewed and documented by Espinoza et al., 2009, the region between 1200 to 1000 cm^{-1} is associated with vibrations of the sulfur-oxygen groups of keratins, and hence the peak at 1080 cm^{-1} is of νs (SO) from cysteine oxide[50]. Based on the FTIR spectra recorded for hair samples, we found no visual difference in the spectra (except Indian grey mongoose hair), as all hair is made of keratin proteins. Additionally, relying on visible observation of FTIR spectra can lead to observer bias. To address these issues, we used chemometric analysis to distinguish Indian grey mongoose hair from domestic cattle hair, human hair, and synthetic fiber.

3.2. Chemometric modelling for discrimination of Indian grey mongoose hair, domestic cattle hair, human hair, and synthetic fiber

The PLS-discriminant analysis (PLS-DA) method is commonly used for predictive, descriptive modelling, and discriminative variable selection[54]. PLS-DA has various applications in various fields, such as forensic science, banking, medical diagnosis, food analysis, metabolomics, and soil science[54-58]. Keeping in view, this study used PLS-DA analysis to differentiate between Indian grey mongoose hair, domestic cattle hair, human hair, and synthetic fiber based on their intensity of IR radiation absorption by biochemical composition of each hair sample.

The PLS-DA model was calculated as three latent variables (LV), namely LV1 (87.09%), LV2 (7.22%), and LV3 (2.82%); consequently, it contributed to the separation of the five groups. Score plots between LVs are shown in **Figures 3.2** and **Figure 3.3.** The plot clearly showed species wise cluster and differentiation; the latent variables effectively highlighted the differences in the FTIR spectra recorded for each hair sample. The developed model has shown an R-square value of 0.9 and an RMSE value (root mean square error) of 0.13. Hence, the PLS-DA analysis has successfully discriminated the Indian grey mongoose hair, domestic cattle hair, human hair, and

synthetic fiber based on the IR transmittance variations of the chemical compositions present in the hair samples (especially keratin protein).

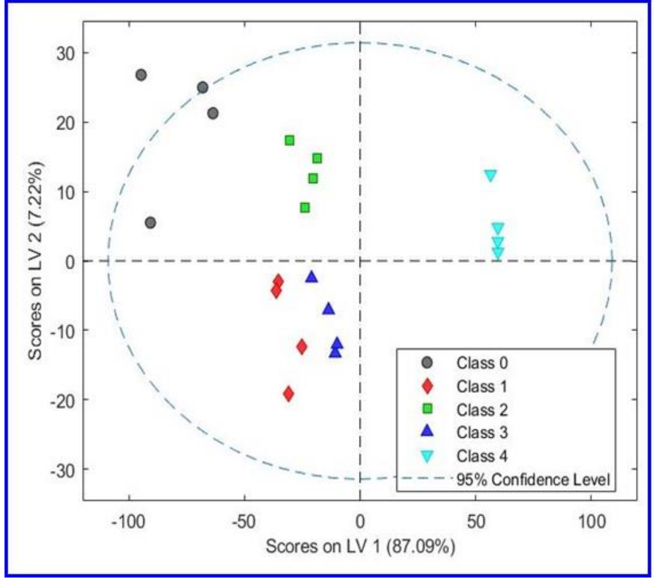

Figure 3.2. Score plot for the PLS-DA classification model with LV1 *vs* LV2. Class 0- Indian grey mongoose, class1- domestic water buffalo, class 2- domestic cow, class 3 -Human hair and class 4 – Synthetic fiber.

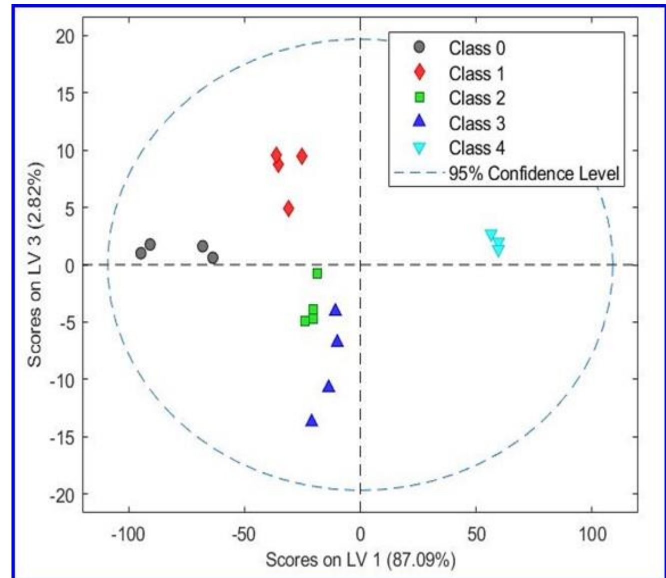

Figure 3.3. PLS-DA classification model: Score plot LV1 *vs* LV3. Class 0- Indian grey mongoose, class1- domestic water buffalo, class 2- domestic cow, class 3 -Human hair and class 4 – Synthetic fiber.

Proteins are made of hundreds of amino acids folded into a well-defined structure that is stabilized by thousands of interactions. Infrared (IR) spectroscopy can reveal the structure of

proteins via vibrational resonances from the polypeptide backbone and/or side chains. Such resonances depend on the protein structure and local interactions (such as hydrogen bonding between amino acids)[59]. In light of this, studies documented that FTIR spectroscopy analysis of polypeptides and proteins reveals nine characteristic IR absorption bands: amide A, B, and I−VII. The amide I and II bands are the most prominent vibrational bands of the protein backbone. The amide I band (1700−1600 cm−1) is the most sensitive spectral region to the protein's secondary structural components and is primarily due to the C = O stretch vibrations of the peptide linkages. On the other hand, the amide II band is mainly derived from in-plane NH bending and the CN stretching vibration[48-53].

In this regard, the current study aimed to discriminate the Indian grey mongoose hair, domestic cattle hairs, human hair, and synthetic fibres based on their biochemical composition using FTIR analysis. The FTIR analysis of hair showed the characteristic peaks at 1655 cm^{-1} (amide I), 1541 cm^{-1} (amide II), and peaks at 1453 cm^{-1} and 1400 cm^{-1} are due to the deformation in δ(CH2) (CH3) and δ(CH3) respectively[60]. These spectral peaks were attributed to hair keratin protein (abundant in the outer layer of skin, hair, and nails) and similar peaks were observed in all the hair samples, excluding slight differences in Indian grey mongoose hair. Studies reported that in the protein analysis through IR spectroscopy, the amide I band is sensitive to the secondary structure and is not strongly influenced by side chains of proteins. In contrast, the IR absorption properties vary in the α-helices, β-sheets, random coils, and loops (i.e., protein secondary structure elements) due to hydrogen bonding of different strength from one peptide –C=O group to H–N– group of a neighboring amino acid. The strength of the hydrogen bond plays a vital role in the absorption frequency of the C=O vibration in the amide I, corresponding to different secondary structure segments within the protein[61-63].

Considering that keratin protein is present in all the hair used in this study, the amino acid compositions of hairs vary from species to species. Subsequently, the hydrogen bonding patterns change within keratin proteins (keratin in different hair types). As a result, it impacted in IR absorption properties of keratin proteins present in each hair type. The small variations in amide I and amide II regions of hair cannot be identified by the naked eye. However, PLS-DA analysis identified the difference in IR absorption properties in the amide I, amide II, and amide III regions of hair and discriminated[63]. At this movement, the current study demonstrated discrimination of

mongoose hair, domestic cattle, human hair, and synthetic fiber using FTIR spectroscopy combined with chemometric analysis.

However, the number of samples was limited to only four in the current study; therefore, we were unable to validate the results. The study showed that FTIR spectroscopy combined with chemometric analysis could discriminate Indian grey mongoose hair, domestic cattle (domestic water buffalo and domestic cow), synthetic fiber, and human hair. The results indicate that this method can be used to identify Indian grey mongoose hair from seized articles and can discriminate it from domestic cattle hair, human hair, and synthetic fiber.

Furthermore, it is believed that FTIR spectroscopy combined with chemometric analysis will facilitate the rapid identification of Indian grey mongoose from seized articles and will be cost-effective compared to molecular techniques. Furthermore, creating an FTIR spectral library for Indian grey mongoose hair could be beneficial, as it could be utilized in the field, and border force and law enforcement officials can use it to identify mongoose hair in seized articles. This study showed positive signs for the identification of mongoose hair in seized articles, including mixed mongoose hair from domestic cattle, human hair, and synthetic fiber. However, to enhance the forensic applications of this methodology, additional studies should be performed to validate it with a large number of hair samples, which will facilitate the construction of a comprehensive classification and discriminant model.

Our study recorded the FTIR spectra of samples made with potassium bromide (KBr) pellets. As a result, we used a fine powder of 25 hair samples to record the spectra of one sample (in triplicate). Due to the non-availability of hair samples, we are unable to use a high volume of samples for this study. Consequently, the data generated are inadequate to validate this methodology. Furthermore, India is home to seven species of mongoose; our study specifically focused on identifying Indian grey mongoose hair. Therefore, future studies should aim to identify and differentiate the other mongoose species. Despite these limitations, our proof-of-concept study marks a significant advance in species discrimination based on hair analysis. The results obtained from this study will have practical applications in wildlife forensic investigations.

Conclusion

In wildlife forensics, discriminating or identifying individual species from an unknown sample is necessary to convict a person under the law. The current study attempted to identify Indian grey mongoose hair and distinguish it from domestic cattle hair, such as domestic water

buffalo, domestic cow, human hair, and synthetic fiber, using FTIR spectroscopy combined with chemometric analysis. The preliminary results of this study documented that FTIR spectroscopy, combined with chemometric analysis, is a powerful tool for discriminating Indian grey mongoose hair from other animal hair and has great potential for forensic applications. However, the current study is carried out with a minimum sample size; as a result, we were unable to validate the results. Therefore, to apply these methods to forensic practice, we will improve the experimental setup with a high volume of samples in the future research to resolve the problem stated above.

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Summary

Summary

The illegal wildlife trade is growing rapidly every year. UNODC World Wildlife Crime Report 2024: Trafficking in Protected Species has documented almost all species recorded in seizures during 2015–2021. For example, corals are 16%; crocodilians are 9%; elephants are 6%; bivalve molluscs are 6%, etc. The report urged an urgent need to develop rapid, cost-effective, and field-deployable techniques/tools to control illegal wildlife trade, which will help conserve species. FTIR spectroscopy for forensic analysis has gained significant attention over the years because of its sensitivity, specificity, and non-destructive nature. So far, several studies have reported FTIR based forensic science, involving biomedical samples, paint, fingerprints, and ink. However, this technique could not have been fully utilized in wildlife forensic analysis due to the lack of a reference IR spectral library/database. In light of this, the current study has demonstrated the development of the FTIR spectral library for Asian elephant ivory. The developed library has been used to identify whether the suspected ivory carving is of elephant origin. Further, the FTIR results for suspected ivory carving analysis have been validated with elephant-specific gene amplification, and the results were similar to those of the FTIR results. The results confirmed that FTIR analysis could be employed to identify elephant origin and could be discriminated from counterfeit articles. Additionally, the present study has shown FTIR-based identification of hair from Indian grey mongoose and discriminating it from domestic animal hair, human hair and synthetic fibre. The results are promising and have great potential for wildlife forensic applications. In summary, this study has explored FTIR-based identification of Asian elephant ivory and hair from mongoose, and the results are hopeful and have forensic applications.

Future Perspectives

Future perspectives

We hope that the results here demonstrate that methods of FTIR spectroscopic analysis have great potential as viable analysis tools for forensic science applications. FTIR spectroscopic imaging is a powerful approach for identifying and characterizing unknown wildlife forensic samples with rapid, cost-effective, non-destructive methods. Currently, much research has reported FTIR-based analysis in wildlife forensic science applications; however, to use this technique in wildlife forensics with high potential, it is necessary to develop a universal FTIR library. In light of this, the current study attempted to develop an FTIR library for Asian elephant ivory and hair from mongoose. The preliminary results are promising and open the door for a new direction in wildlife forensic analysis and can be employed for highly traded wildlife articles, for example, pangolin scales and animal hair, etc., and to discriminate wildlife articles from counterfeit materials. FTIR spectroscopic analysis will help the forensic personnel in two ways: (1) whether the suspected article is from a biological source or not, and (2) Based on the FTIR library, it can make decisions or understand the major chemical compositions present in the sample. Therefore, FTIR analysis has excellent potential in wildlife forensic analysis.

Publications

Discover Applied Sciences

Brief Communication

Development of FTIR spectral library for the identification of Asian elephant ivory: an innovative approach in wildlife forensics

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Abstract

The illegal ivory trade remains a prime threat to elephant populations. Identification of ivory is crucial for combating illicit ivory trading. Studies have demonstrated that FTIR spectroscopy is one of the most suitable techniques for identifying ivory and its products. However, this technique could not have been fully utilized due to the lack of a reference IR spectral library/database. In light of this, the present study aimed to develop the FTIR spectral library for Asian elephant ivory. We collected eight Asian elephant ivory ($n = 8$) samples from our institute repository and recorded the FTIR spectrum. Further, based on the biochemical composition of ivory, we identified various characteristics, functional groups, and their respective wavenumbers. Furthermore, we used the FTIR spectrum of Asian elephant ivory as a reference to identify the fifteen carvings ($n = 15$) suspected to be of Asian elephant ivory. The results revealed that samples 1 -13 are visibly identical to the FTIR spectrum of Asian elephant ivory; in contrast, samples 14 and 15 do not match the FTIR spectrum of Asian elephant ivory. Hence, it is confirmed that out of fifteen samples, thirteen are likely to be of Asian elephant ivory origin. To confirm this finding, we employed the PLS-DA analysis. The result showed an R Square value of 0.9 for calibration and 0.75 for validation, and the model exhibited 100% accuracy in classifying the original and fake ivory samples. The results confirmed that FTIR combined with chemometrics analysis was useful for identifying ivory and fake materials. To validate the FTIR results, we extracted the DNA from suspected samples 1–15 and amplified elephant-specific D-loop and *Sry* (sex-determining region on the Y chromosome) genes. Subsequently, the PCR amplicons were examined on a 2.5% agarose gel and observed for samples 1–13, one band at 137 bp (for D-loop) and another at 97 bp (for *Sry*). These results indicated that samples 1–13 are of elephant origin, whereas samples 14 and 15 are not of elephant origin. To confirm this finding, the PCR amplicons (D-loop) from nine samples (1–9) were sequenced, and % similarity was analyzed. The results showed 96.6 to 100% similarity to the *Mammuthus primigenius*, *Elephas maximus indicus*, and *Loxodonta africana*. The primers (D-loop and *Sry*) used in this study are elephant-specific and do not distinguish the elephant species. Overall, DNA-based results supported the results from FTIR analysis and confirmed that the suspected samples 1–13 are of elephant origin and 14 and 15 are not of elephant origin. The current study has demonstrated the identification of ivory substitutes through FTIR spectral library for Asian elephant ivory, which is rapid, cost-effective, and has excellent potential for forensic analysis.

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Discrimination of mongoose hair from domestic cattle hair, human hair, and synthetic fiber using FTIR spectroscopy and chemometric analysis: a rapid, cost-effective, and field-deployable tool for wildlife forensics†

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Mongoose hair is used to prepare fine brushes, which increases the demand for mongooses to be poached from the wild and brutally bludgeoned to death. Mongooses were listed as Schedule I species under the Indian Wildlife (Protection) Act 1972. Species identification of wildlife case-related samples is necessary to convict a person under this legislation. Microscopy and DNA-based techniques are commonly used to identify mongoose hair in seized brushes. However, in painting brushes, the roots, and the lower part of the hair are mostly trimmed, and only the upper part is used to make the brushes. In addition, brushes are often prepared with mixed hair from mongoose, domestic cattle, human hair, and synthetic fibre. Therefore, the identification of mongoose hair by microscopy and DNA-based techniques is restricted due to the lack of complete strands of hair and the absence of hair roots. Therefore, there is an urgent need to develop an alternative methodology for the identification of mongoose hair from seized articles. FTIR spectroscopy for forensic analysis has gained significant attention over the years because of its sensitivity, specificity, and non-destructive nature. The present study aimed to discriminate Indian grey mongoose (*Herpestes edwardsii*) hair from domestic cattle hair (domestic water buffalo and domestic cow), human hair, and synthetic fiber based on their chemical composition using FTIR spectroscopy and chemometric analysis. We have taken hair from four individuals for each species, namely Indian grey mongoose, domestic cattle, human hair, and synthetic fibre. The FTIR spectrum was recorded, and partial least-squares discriminant analysis (PLS-DA) was used to discriminate hair and synthetic fiber. The established PLS-DA model showed an R-square value and an RMSE (root mean square error) value of 0.9 and 0.13 respectively. Our preliminary findings have shown that FTIR spectroscopy combined with chemometrics can quickly discriminate Indian grey mongoose hair, domestic cattle hair, human hair, and synthetic fiber, providing crucial evidence for judicial proceedings.

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1. Introduction

India is a megadiverse country, home to 7–8% of the world's flora and fauna. It accommodates more than 45 000 species of plants and 91 000 species of animals.[1] Wildlife in India is protected by the Wildlife (Protection) Act 1972 (WPA), which prohibits the trade of more than 1800 species of wild animals, plants, and their derivatives.[2–4] Despite strong legal protection, poaching and illegal trade have been increasing over the years, posing a significant threat to several iconic species and pushing them to the brink of extinction. Mammals are frequently poached for their skin, meat, hair, and wool, which are widely traded for different purposes, such as fur coats, leather purses, belts, shoes, carvings, *etc.*[5,6]

In recent years, mongooses (*Herpestes* sp.) have been poached illegally and their hair is used to produce painting and shaving brushes.[7,8] The various seizures made by law enforcement agencies indicate that poaching of these animals is still regular and affects the conservation of these animals.[7] For example, in 2023, the Andhra Pradesh Forest Department seized more than 18 000 paint brushes from several premises allegedly used by dealers in Hyderabad city, and forensic analysis confirmed that the paint brushes were prepared with Indian

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