SPECIES IDENTIFICATION OF GRASS LEAF STAIN IN FORENSIC INVESTIGATION

Paulomi Sharma[1], Ms. Priya Sharma[2]
[1]Student of M.Sc Forensic Science, University Institute of Applied Health Sciences, Chandigarh University, Gharuan, Mohali, Punjab, India, 140413
Email ID: paulomisharma10@gmail.com
[2]Assistant Professor, University Institute of Applied Health Sciences, Chandigarh University, Gharuan, Mohali, Punjab, India, 140413
Email ID: priya.e11161@cumail.in

Abstract:
Forensic science encompasses diverse scientific disciplines applied to legal investigations, and plant materials are increasingly recognized as crucial evidence in many forensic cases. The identification of plant species, specifically in the context of grass leaf stains, has emerged as an area of interest within forensic botany. Grass leaf stains can be encountered in various scenarios, such as crime scenes, environmental investigations, and agricultural disputes. Understanding the species associated with these stains can provide valuable insights into the origin, timeline, and potential involvement of individuals or events. This research paper investigates the application of forensic science in the species identification of grass leaf stains. The analysis and identification of plant materials in forensic investigations play a vital role in criminal cases, environmental forensics, and agricultural investigations. However, limited research focuses specifically on the identification of grass leaf stains. Therefore, this study aims to contribute to the existing knowledge in this area. The research utilizes a multidisciplinary approach that combines morphological analysis, and chemical analysis to identify the species associated with grass leaf stains.

Keywords: forensic science, grass leaf stains, species identification, morphological analysis, chemical analysis, forensic botany.

Introduction

Grass, a common type of plant found in various ecosystems, plays a significant role in forensic investigations due to its wide distribution and frequent interaction with human activities. In forensic science, the analysis of grass stains has proven to be a valuable tool for investigators in understanding crime scenes, identifying suspects, determining movement patterns, and reconstructing events. Grass stains are particularly helpful in forensic investigations because they can provide crucial information about the activities that occurred at a crime scene or the movement of individuals. For example, a grass stain on a suspect's shoe or clothing can indicate their presence at a specific location. By analyzing the grass stain, investigators can potentially determine the type of grass present, which may narrow down the geographic area associated with the stain. This information can be vital in corroborating or disproving an individual's alibi or establishing the sequence of events. One important aspect of grass stains is the composition of grass pigments. Grass contains various pigments responsible for its coloration, such as chlorophylls, carotenoids, and flavonoids. These pigments contribute to the unique coloration of different grass species, providing potential markers for species identification[1]–[4]. The morphological analysis involves the examination of physical characteristics such as shape, size, and color to infer potential species involved in stain formation. Microscopic examination techniques, including light microscopy and scanning electron microscopy (SEM), are employed to study the grass leaf stain's cellular structure and surface features. These techniques provide valuable insights into the specific plant structures and diagnostic features that aid in species identification[5]. Chemical analysis techniques, such as thin-layer chromatography [6]–[8](TLC), high-performance liquid chromatography (HPLC)[7], [9]–[12], and infrared spectroscopy[12], are utilized to analyze the chemical composition of the grass leaf stain. This analysis helps identify unique compounds and pigments associated with particular plant species. By integrating these various analytical methods, this research paper aims to establish a comprehensive framework for the identification of grass leaf stains in forensic investigations.

TLC is a powerful analytical method that allows the separation of complex mixtures into individual components based on their differential migration on a thin layer of adsorbent material. In the case of grass stains, TLC can be utilized to separate the various pigments present in the stain. During TLC analysis, a small portion of the grass stain is dissolved in a suitable solvent[13], [14] and applied to a TLC plate. The plate is then developed in a solvent system, allowing the individual pigments to migrate at different rates. Once the separation is complete, the TLC plate is visualized using specific reagents or under ultraviolet light, revealing distinct pigment bands[15], [16]. By comparing the pigment profile obtained from the stain to reference standards or databases, forensic scientists can potentially identify the species of grass associated with the stain[9], [17], [18]. Therefore, TLC plays a crucial role in identifying grass stains by providing a rapid and efficient method for separating and analyzing the pigments present. This technique enables forensic scientists to determine the specific grass species involved, contributing valuable information to crime scene reconstruction and the overall investigative process. In conclusion, grass stains hold significant importance in forensic investigations[19], [20]. They can provide vital clues about individuals' movements, help establish associations with crime scenes, and assist in reconstructing events. Forensic scientists can separate and identify grass stains by understanding the composition of grass pigments and employing

techniques like TLC, contributing to the successful resolution of criminal cases. The findings of this study will contribute to the advancement of forensic botany and provide valuable insights for forensic scientists, investigators, and researchers involved in plant-related forensic casework [21]– [24].

Materials and Methods:

Sample Collection

A total of 10 grass leaf stain samples were collected from various locations, including Chandigarh, Patiala, and Ludhiana. The species identification of the collected samples was performed using a morphological method with the aid of taxonomic keys. The morphological characteristics such as shape, size, colour, and other diagnostic features were examined to determine the species associated with each sample. The selected grass samples were collected to create a diverse representation of commonly encountered grass species in forensic investigations. By including a variety of grass types with distinct characteristics, the study aimed to establish a comprehensive reference database for the identification of grass leaf stains in forensic science[23].

Table 1: Sample Collection Details for Grass Species

S. No.	Name of Grass Species	Subfamilies	Location of Collection	Number of Samples
1	Cynodon Dactylon	Chloridoideae	Chandigarh	5
2	Cyperus rotundus	Cyperaceae	Chandigarh	5
3	Paspalum paspaloides	Panicoideae	Ludhiana, Patiala	5
4	Arundinella nepalensis	Panicoideae	Ludhiana, Patiala	5
5	Panicum Repens	Panicoideae	Ludhiana	5
6	Eleusine Indica	Chloridoideae	Ludhiana, Patiala	5
7	Desmostachya aegyptium	Chloridoideae	Ludhiana	5
8	Panicum paludosum	Panicoideae	Patiala	5
9	Paspalidium flavidum	Panicoideae	Ludhiana	5
10	Tragus biflorus	Chloridoideae	Ludhiana, Patiala	5

Sample Preservation

To ensure sample integrity, the collected grass leaf stains were preserved by sandwiching them between paper sheets. This preservation method helped prevent contamination, fading, or degradation of the stains before further analysis[25]–[27].

Sample Preparation

For the staining process, three stains were prepared for each sample, namely the standard stain, stained sample, and blank sample. The staining was conducted on cotton linen cloth, with the stained portion measuring 1.5 cm x 1.5 cm. The cotton cloth was chosen due to its ability to retain stains effectively and provide a suitable substrate for subsequent analysis.

Sample Extraction

The extraction procedure was performed on the stained cloth samples. The stained portions of the cloth were cut and placed in test tubes. Various solvents, including methanol, acetone, and ethanol, were used for extraction[27]. The solubility of the stain in each solvent was noted, and the amount of stain soluble in each solvent was recorded.

Table 2: materials and chemicals required for different processes.

S. No.	Process	Materials and Chemicals required
1	Sample Extraction	Test tubes Measuring cylinder Methanol Acetone Ethanol
2	Analysis	TLC plates Measuring cylinder (200 mL) Capillary tubes Covering lid
3	TLC Solvent System	Toluene Ethyl acetate Formic acid Methanol
4	Visualization	Iodine fuming balls

TLC Analysis

Thin Layer Chromatography (TLC) plates were prepared with a layer thickness of 0.25 mm and a size of 12 cm x 7 cm. The samples were spotted on the TLC plates using a capillary tube. The TLC plates were then placed in a solvent chamber containing a mobile phase consisting of toluene: ethyl acetate: methanol: formic acid (60:15:10:15)[27]. The plates were developed for a specific period (30 minutes) at a temperature of 25°C. After development, the TLC plates were removed from the chamber and subjected to visualization using suitable methods such as iodine fuming. The spots on the TLC plates were observed and recorded using photography.

Methodology (procedure)

Sample Extraction:

1. Collect the stained cloth samples.
2. Place the stained cloth in a test tube.
3. Measure the appropriate volume (1 ml) of methanol, acetone, or ethanol using a measuring cylinder.
4. Add the selected solvent to the test tube containing the stained cloth.
5. Securely seal the test tube and allow the solvent to extract the stain from the cloth.

Analysis:

1. Prepare the TLC plates by cutting them to the desired size.
2. Pour the solvent system consisting of toluene, ethyl acetate, formic acid, and methanol into a suitable ratio (60:15:15:10) into the measuring cylinder (200 mL).
3. Place the TLC plate in a covering lid to ensure a controlled environment during the analysis.
4. spot the extracted stain samples onto the TLC plate using a capillary tube.
5. Carefully place the TLC plate into the lid, ensuring that the plate is immersed in the solvent system but the spots are above the level of the solvent.
6. Allow the TLC plate to develop by capillary action until the solvent front reaches the desired distance.
7. Remove the TLC plate from the solvent and allow it to dry.

All grass leaf stain samples were analyzed in this study, and consistent results were obtained for all samples. The analysis involved three types of samples: pure/standard grass stain, stained grass stain on white cotton cloth (Stained Cotton Cloth), and a blank sample. The stained grass stain on the cotton cloth was subjected overnight and then extracted in methanol. The resulting extract exhibited green colour. However, TLC analysis of the stained extract revealed different colour strands)

- Pure/Standard Grass Stain: The pure grass stain, serving as the control sample, exhibited an immediate green colour upon the addition of methanol. Thin-layer chromatography (TLC) analysis of the pure grass stain revealed distinct colour strands on the TLC plate.
- Stained Cotton Linen Cloth: The stained grass stain on the cotton linen cloth was subjected to overnight extraction in methanol. The resulting extract exhibited a colour. However, TLC analysis of the stained extract revealed significantly diminished strands compared to the pure grass stain. The intensity and clarity of the strands were notably reduced in the stained extract, suggesting potential dilution or extraction limitations associated with the cloth material.
- Stained Cotton Cloth: The grass stain on the cotton cloth was subjected to extraction by keeping it overnight in a different solvent system. The resulting extract exhibited green colour. However, TLC analysis of the stained extract revealed different colour strands.
- Blank Sample (Unstained Simple Cloth): The blank sample, consisting of unstained cloth, served as the negative control. No colour change or observable strands were detected in the blank sample, confirming the absence of any intrinsic grass stain. This control ensured that the cloth used in the experiment did not introduce any interfering pigments or contaminants.

Visualization:

1. Perform visualization of the developed TLC plate using iodine-fuming balls.
2. Gently pass the iodine-fuming ball over the TLC plate to expose it to iodine vapor.
3. Observe the appearance of spots on the TLC plate, which indicate the separation of grass pigments.

Observations:

1. Morphological Analysis: the physical characteristics of the grass leaf stain, such as shape, size, and colour. This analysis can provide clues about the possible species involved.

Table 3: shape and initial colour of grass samples.

S.No.	Grass sample	Shape	Initially Color
1	Cynodon dactylon	Narrow, linear	Dark green
2	Cyperus rotundus	Narrow, linear	Light green
3	Paspalum paspaloides	Broad, lanceolate	Medium green
4	Arundinella nepalensis	Narrow, lanceolate	Light green
5	Panicum Repens	Broad, ovate	Dark green
6	Eleusine indica	Narrow, linear	Light green

S.No.	Grass sample	Shape	Initially Color
7	Desmostachya aegyptium	Narrow, linear	Light green
8	Panicum paludosum	Narrow, lanceolate	Dark green
9	Paspalidium flavidum	Broad, lanceolate	Light green
10	Tragus biflorus	Narrow, linear	Light green

2. Chemical Analysis: Chemical analysis techniques, such as thin-layer chromatography (TLC) was employed to analyze the chemical composition of the grass leaf stain. These techniques can help identify specific compounds or pigments present in the stain, which can be characteristic of certain plant species.

Three types of samples were taken: pure/standard grass stain, stained grass stain on cotton linen cloth, and a blank sample.

i. Pure/Standard Grass Stain: The pure grass stain (control sample) exhibited a green color immediately upon the addition of methanol. It was observed that nutgrass gave a slightly darker green colour compared to scutch grass. When the pure grass stain was analyzed using TLC, green strands were visible on the TLC plate for approximately 25 to 30 minutes. Scutch grass showed both green and yellow light and dark strands, while nutgrass exhibited lighter-coloured strands.

ii. Stained Cotton Linen Cloth: The stained grass stain on the cotton linen cloth was subjected to extraction by keeping it overnight in methanol. The extracted stain showed a light green colour. However, when analyzed using TLC, the strands observed were significantly diminished compared to the stained cotton cloth.

iii. Stained Cotton Cloth: The grass stain on the cotton cloth was subjected to extraction by keeping it overnight in a different solvent system. The resulting extract exhibited green colour. However, TLC analysis of the stained extract revealed different colour strands.

iv. Blank Sample (Unstained Simple Cloth): The blank sample, which consisted of unstained cloth, served as the negative control. No colour change was observed in the blank sample, indicating the absence of a grass stain.

Table 4: Solubility of Grass Species in Different Solvents

S. No.	Grass Species	Solubility in Ethanol	Solubility in Methanol	Solubility in Acetone
1	Cynodon dactylon	++	+++	+++
2	Cyperus rotundus	++	+++	+++
3	Paspalum paspaloides	++	+++	++
4	Arundinella nepalensis	++	+++	++
5	Panicum repens	++	+++	++
6	Eleusine indica	++	+++	+++
7	Desmostachya aegyptium	++	+++	+++
8	Panicum paludosum	++	+++	++
9	Paspalidium flavidum	++	+++	++
10	Tragus biflorus	++	+++	+

Note: -: Not soluble (stain not dissolved); + : Sparingly soluble (some part of stain dissolved); ++: Soluble (stain dissolved but with difficulty); +++: Highly soluble (stain dissolved easily)

Table 5: Rf Values and Colours at Rf Values for Grass Samples

S.No.	Grass Sample	Component	Rf value	Color
1	Cynodon dactylon	A	0.92	Green
		B	0.80	Green
		C	0.73	Yellow
2	Cyperus rotundus	A	0.85	Dark green
		B	0.73	Basil green
		C	0.70	Yellowish green
3	Paspalum paspaloides	A	0.88	Light green
		B	0.78	Pale yellow
		C	0.67	Yellowish brown
4	Arundinella nepalensis	A	0.91	Pale green
		B	0.82	Light yellow
		C	0.7	Brownish green

5	Panicum Repens	A B C D E	0.89 0.81 0.75 0.49 0.15	Light green Pale yellow Brownish green Dark green Dark green
6	Eleusine indica	A B C D	0.93 0.79 0.72 0.63	Dark green Light green Pale yellow Dark green
7	Desmostachya aegyptium	A B C	0.88 0.77 0.69	Light green Pale yellow Yellowish green
8	Panicum paludosum	A B C	0.86 0.76 0.68	Dark green Light green Pale yellow
9	Paspalidium flavidum	A B C D E F	0.87 0.79 0.73 0.71 0.63 0.49	Light green Pale yellow Brownish green Basil green Dark green Dark green
10	Tragus biflorus	A B	0.84 0.75	Dark green Light green

Results and Discussion:

The present study focused on developing a Thin Layer Chromatographic (TLC) method for the species identification of grass leaf stains. The results obtained from the TLC analysis were compared with the previous study conducted by Rajinder Singh Chandela and Priyanka Jindal [27]. The TLC method utilized a specific solvent system composed of toluene, ethyl acetate, methanol, and formic acid in a ratio of 60:15:10:15. This optimized solvent system was chosen to ensure efficient separation and resolution of the components present in the grass leaf stains. The development time was set at 25 minutes, and the analysis was conducted at a temperature of 25°C. The TLC results (Table 5) provided valuable information on each grass sample's Rf values and colour observations. These parameters served as characteristic markers for identifying and differentiating the grass species based on their stains. The Rf values ranged from 0.71 to 0.94, indicating distinct migration patterns of the compounds present in the stains. Comparing the findings of our study with the previous research by Rajinder Singh Chandela and Priyanka Jindal, it is evident that both studies share a common objective of identifying and distinguishing grass stains. Despite the methodological differences, the results obtained from our TLC analysis demonstrate promising outcomes for the identification of different grass species based on their stains. The TLC method offers advantages such as simplicity, cost-effectiveness, and accessibility, making it a suitable choice for forensic applications where rapid and reliable identification of grass stains is crucial. It is important to note that further validation and comparison of the TLC method with other analytical techniques, including the one used in the previous study, would enhance the reliability and applicability of the method in forensic casework. Expanding the sample size and including a wider range of grass species would provide more comprehensive and conclusive results.

Conclusion:

The developed Thin Layer Chromatographic method shows potential for the species identification of grass leaf stains. The obtained TLC patterns, Rf values, and colour observations demonstrate the potential of this method in differentiating between grass species based on their stains. The conclusion also emphasizes the relevance of the research findings in forensic investigations, particularly in cases involving outdoor crimes where grass stains are often encountered as evidence. The ability to accurately identify the grass species from their stains can provide valuable information in linking suspects and victims to crime scenes or verifying alibis. With its advantages of being quick, reliable, and cost-effective, TLC can serve as a valuable tool in forensic science for analyzing grass leaf stains. Additionally, it may suggest future directions for research, such as exploring the use of advanced techniques like high-performance liquid chromatography (HPLC) or DNA-based analysis for enhanced species identification.

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