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A Novel Approach for Forensic Differentiation of Grass Stains Using ATR FT-IR Spectroscopy and Chemometrics

طريقة جديدة للتمييز الجنائي لبقع العشب باستخدام مطيافية الانعكاس الكلي المخفض المقترن بتحويل فورييه الطيفي بالأشعة تحت الحمراء (ATR FT-IR) والقياسات الكيميائية



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Abstract

Grass evidence often encountered at the crime scene, helps in establishing the primary as well as secondary crime scenes. Due to the limited quantity and intricate nature of samples, there is a need for fast, sensitive and reliable techniques for the effective analysis of this vital evidence. In the current study, an attempt is made to study the feasibility of ATR FT-IR in combination with chemometric tools for chemical characterization and species differentiation of grass stains.

Ten different grass species belonging to three subfamilies, Panicoideae, Chlorodoidae, and Bambusoideae, were collected and analyzed by using the ATR FT-IR spectroscopy combined with the PLSR chemometric tool.

Results showed a clear difference between samples of Panicoideae, Chlorodoidae, and Bambusoideae subfamilies, and between species of each subfamily. To analyse the performance of the classification model, a set of 10 unknown/blind samples (unknown to the analyst) were randomly selected from the training dataset and all unknown/blind samples were accurately assigned in their

المستخلص

يساعد وجود أدلة عشبية في مسرح الجريمة بالإسهام في بناء مسارح الجرائم الرئيسية والثانوية، وبسبب الكمية المحدود وطبيعة حساسية العينات، هناك حاجة إلى أساليب سريعة وحساسة ومعتمدة للتحليل الفعال لهذه الأدلة. ويسهم هذا البحث في دراسة جدوى مطيافية الانعكاس الكلي المخفض المقترن بتحويل فورييه الطيفي بالأشعة تحت الحمراء (ATR FT-IR) والقياسات الكيميائية للتحديد الكيميائي، وتمييز أنواع وفصائل البقع العشبية. وتم جمع وتحليل عشرة أنواع مختلفة من الأعشاب من 3 عوائل فرعية الثماماوات، وكلورساوات، والخيزراناوات باستخدام مطيافية ATR FT-IR مع أداة قياس انحدار المربعات الصغرى الجزئي. ووضحت النتائج تمييزاً واضحاً بين العائلات الفرعية لعينات الثماماوات، وكلورساوات، والخيزراناوات وبين الأنواع من كل عائلة فرعية، ولتحليل أداء نموذج التصنيف، تم اختيار مجموعة من 10 عينات معمة (غير معروفة للمحلل) بشكل عشوائي من المجموعة. وتم تعيين

Keywords: Forensic Science, Forensic Botany, Differentiation, Grass, ATR FT-IR spectroscopy.

الكلمات المفتاحية: علوم الأدلة الجنائية، علم النبات الجنائي، التمييز، الأعشاب، مطيافية ATR FT-IR.



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corresponding group.

ATR FT-IR spectroscopy requires minimal sample preparation and can be successfully used as an eco-friendly, non-destructive, and reliable alternative to other existing methods.

جميع العينات المعمّاة بدقة في مجموعتها المقابلة. ويتطلب التحليل الطيفي لـ ATR FT-IR الحد الأدنى من إعداد العينة، ويمكن استخدامه بنجاح كبديل صديق للبيئة وغير مدمر وموثوق به مقارنة مع الطرق الأخرى الحالية.

1. Introduction

The analysis of botanical evidence is an emerging forensic discipline. Identification of plant species recovered from crime scenes might help in ascertaining the geographic origin, therefore, linking a suspect with a crime scene. Such evidence can also be used to ascertain a primary or secondary crime scene, locating missing bodies and testing alibis. However, the samples often encountered at the crime scene are found in small quantities, degraded, and contaminated. Therefore, the usual techniques such as the study of morphological and anatomical features cannot be used for identification [1,2]

Grasses are frequently encountered botanical evidence at the scene of a crime. Owing to their sticky nature, they can be transferred easily from one place to another in the form of stains, hooks, barbs, soft thin leaves, hairs, or sticky coverings, sticking to the clothes, footwear or the body of a victim or suspect. In the case of outdoor crime scenes, grass associated evidence can be found on weapons used for in crimes and clothing of the victim or suspect. In cases where the criminal has attempted to stage or manipulate the crime scene, the grass samples are found in compromised (degraded, chemically treated, or contaminated conditions) forms. In such cases, species identification becomes crucial for solving the crime [3–5].

Our understanding of plant species, including different species of grasses, has increased exponentially in the last few decades. DNA analysis has proved to be an irreplaceable tool in the identification and differentiation of grass species. However, DNA analysis is expensive and time-consuming. Therefore, there is a substantial need of confirmatory, reliable, rapid, and non-destructive technique for species identification. Infrared spectroscopy has emerged as an effective alternative to many analytical techniques. However, very little research has been conducted on the use of infrared spectroscopy as

an alternative to DNA analysis. Its advantage lies in its non-destructive, high resolution and fast scanning nature [6]. Moreover, barring the one-time instrument expenses, there are very few running expenses, thereby, reducing the per sample cost.

The total internal energy of a molecule can be described as the summation of rotational, vibrational, and electronic energy. Infrared spectroscopy (IR) is the study of the interaction of electromagnetic radiation in an infrared region with the matter. When a molecule is radiated with infrared radiation, there is change in the vibrational state of the molecule. The frequency absorbed by the molecule is specific for a specific molecule, which is represented by the fingerprint region. When the infrared radiation passes through a medium of high refractive index into a medium of low refractive index at a critical angle, total internal reflectance occurs. Even when total internal reflectance occurs, some portion of infrared radiation is refracted and is known as the evanescent wave. The evanescent wave is used for radiating the sample and the spectrum is recorded [7,8]. Attenuated total reflection Fourier transform infrared (ATR FT-IR) spectroscopy is a non-destructive, label-free analytical technique that can be widely utilised to study a range of diverse molecules in a series of distinct conditions [9]. This technique is easy to use with minimal sample preparation required. Since the instrument cost is comparatively less, it is easily available in forensic laboratories [10].

FTIR has been successfully applied in the identification of species of bacillus [11,12] fungi [13,14] yeast [15], mosses [16,17] etc. A review summarizing all work in the field of the identification of microorganisms is also available [18]. However, very little work has been done in the field of identification of grass species using ATR FT-IR spectroscopy. Some initial work has been done for bamboo [19], crops and weed [20]. In this preliminary study, an attempt has been made to apply ATR FT-

IR spectroscopy and chemometrics for the differentiation and identification of grass species belonging to three sub-families, panicoideae, chloridoideae, and bambusoideae.

2. Materials and Methods

2.1. Sample collection and details

Total (n=10) different grass species (five samples per species) were collected from Punjab state in northwestern India. In total, 50 samples belonging to three sub-families of grasses were collected and analyzed. Details of samples collected for the present study is enumerated in Table-1.

2.2 Sample preparation

2.2.1 Stain preparation

Stains were prepared on 4×4 cm white cotton cloth (contamination-free). Grass leaves were rubbed against cotton cloth until the green coloured stain was produced and allowed to dry overnight at room temperature (25±10°C). A total of three stains were prepared from every grass species to check the reproducibility.

2.2.2 Extract preparation

To prepare the stain extract, 2×2 cm piece of stained cloth was cut into small pieces and dipped in 1000 µL (1ml) of ethanol solvent in micro-centrifuge tubes. They were allowed to stay undisturbed overnight. Extracts of

these stains were prepared by using an ethanol solvent as ethanol does not degrade the DNA of the sample [21].

3. Sample analysis

All grass samples were inspected using an ATR FT-IR spectrometer (Bruker eco- Alpha) with ZnSe crystal accessory and Deuterated L-Alanine Doped Triglycene Sulphate (DLATGS) detector. For recording the parameters, such as sample and background scan time, resolution, and scan range, OPUS (version 7.2) software was used. Samples and background were scanned 24 times at a resolution of 4 cm⁻¹ within the range of MIR that is 4000 to 600 cm⁻¹. The surface of the ATR crystal was appropriately cleaned with pre-wetted (isopropyl alcohols and deionized water) cleaning tissues of ATR (part No. 1008033) to avoid the chances of cross-contamination.

Stains were analyzed by placing the stained cloth piece directly on the ATR crystal surface. The background scan of the neat cotton cloth was recorded before analyzing any sample. For the analysis of extract, 10µL of the sample was placed directly on the crystal surface and the first spectrum was recorded immediately after the deposit, the second spectrum was recorded after an interval of five minutes and the third spectrum was recorded after ten minutes. Hence, total of three spectra of one sample were obtained.

The method of chemometrics, partial least square regression (PLSR) analysis, was utilized for the classifica-

Table 1- Details of samples collected for the present study.

Sr. No.	Family	Sub-Family	Species
1			<i>Cenchrus ciliaris</i>
2			<i>Digitaria ciliaris</i>
3		<i>Panicoideae</i>	<i>Echinochloa colona</i>
4			<i>Cenchrus biflorus</i>
5			<i>Panicum paludosum</i>
6	Poaceae		<i>Cynodon dactylon</i>
7		<i>Chloridoideae</i>	<i>Dactyloctenium aegyptium</i>
			<i>Saccharum spontaneum</i>
8			<i>Dicanthium annulatum</i>
9			
10		<i>Bambusoideae</i>	<i>Bambusa vulgaris</i>

tion purposes. PLSR was applied by using the Unscrambler X (CAMO, AS, Oslo, Norway) [22] software on the IR spectroscopic data. Opus format was utilised to import the data into the Unscrambler X software.

4. Partial least square regression analysis (PLSR)

PLSR is a supervised clustering dimension reduction tool, used to work with high dimensional data set to recognize the significant similarities and differences within the score plots. The labelled training data set is utilised to locate the patterns in the data and this stored data is used for the purpose of validation study. The next phase is the 'prediction phase' also known as the testing phase, in which the data, which were not included in the training data set, are validating using the parameters of the first phase. PLSR establishes the linear relation of the potential independent variants to the potential dependent variant. PLSR score plot can be used to establish the similarities and differences between the samples [23–26].

Pre-treatment methods used for generating the PLSR model were linear baseline correction and baseline offset, de-resolve transform (18 channels were used for convolution) and orthogonal signal correction (OSC). To make the PLSR model, the non-linear iterative partial least squares (NIPALS) algorithm was used. This algorithm handles missing values and is suitable for computing only the first few factors of a dataset. A total of 25 calibration samples were used with four factors suggested by PLSR model.

5. Results and Discussion

The generated spectra were acquired from 4000 to 600 cm^{-1} range with 4 cm^{-1} resolution. Spectra obtained from stained samples created noise due to the interference caused by peaks due to substrates. The subtraction approach used in FTIR did not provide a pure spectrum of grass components. Significant peaks were masked due to the presence of cotton substrates therefore, it has been concluded that it is not possible to differentiate grass species in stained form. To overcome this limitation, stained samples were extracted with ethanol solvent and analyzed. A total of three spectra of each sample was recorded. The first spectrum was recorded immediately after

deposition of sample on ATR crystal surface, the second spectrum was recorded after a five minutes interval and the third spectrum was recorded after a ten minute interval. Significant peaks of grass were observed in spectra analyzed after a ten minute time interval due to the complete evaporation of ethanol solvent. Based on the above observation, spectra obtained after 10 minutes were considered as significant for the purpose of differentiation.

5.1 Visual discrimination of ATR FT-IR spectrum of grass extract

Grass leaves are composed of many organic components, so they produce complex ATR-FTIR spectra with so many absorption bands. Figure-1 shows the representative ATR FT-IR spectra of grass leaf extracts. First a broad and intense peak placed at about 3354 cm^{-1} attributed to the O-H stretching vibrations. Sharp peaks were observed at approximately 2914 and 2846 cm^{-1} which correspond to long chain aliphatic compounds such as methylene (CH_2) stretching attributed to polysaccharide, lipids and carbohydrates. As grass leaves are rich in proteins, a peak positioned at approximately 1630 cm^{-1} attributed to the presence of amines (proteins), and amide I vibrations ($\text{C}=\text{O}$ bending). Next, peaks positioned at approximately 1421 and 1415 cm^{-1} are due to the amide II ($\text{C}-\text{H}$ stretch) with a vibrational group of $\text{C}-\text{O}/\text{O}-\text{H}$ bending. Another strong and sharp peak positioned at approximately 1029 cm^{-1} corresponds to oligosaccharides, glycoproteins, and cellulose ($\text{C}-\text{O}$ stretch). Peaks positioned in the range of 1300-600 cm^{-1} (fingerprint region) corresponds to low molecular weight monosaccharides, polyols, and carbohydrates [17,27–30].

The FTIR spectra of grass species of different subfamilies exhibit similar values of absorbance at nearly identical wavenumbers. Figure-2 shows the overlaid ATR FT-IR spectra of representative species of panicoidae, chloridoideae and bambusoideae subfamilies. It is quite difficult to identify the species based on visual variations within the obtained spectra. Therefore, a chemometric method, PLSR, was used to analyze the absorption bands in the selected MIR range. In this study, the PLS is trained by acquiring spectra of all the collected samples of grass species. The aim of the current study is to discriminate among samples of three subfamilies of

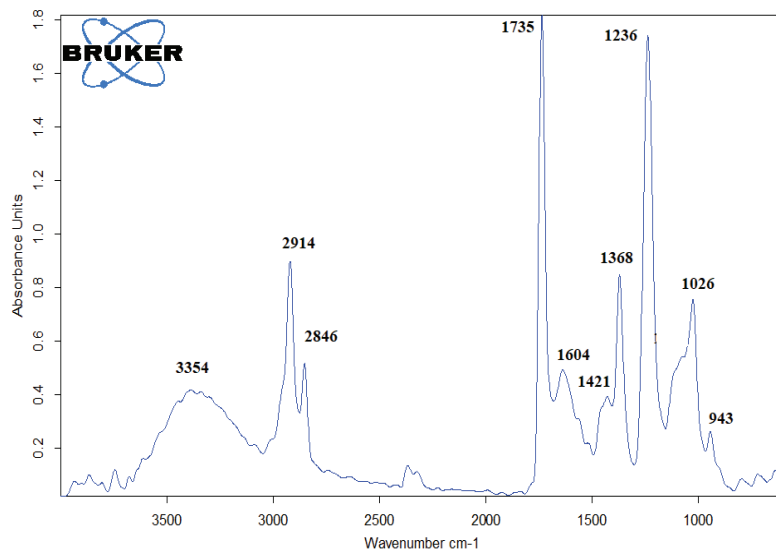


Figure 1- Representative ATR FT-IR spectra of grass leaf extract [1].

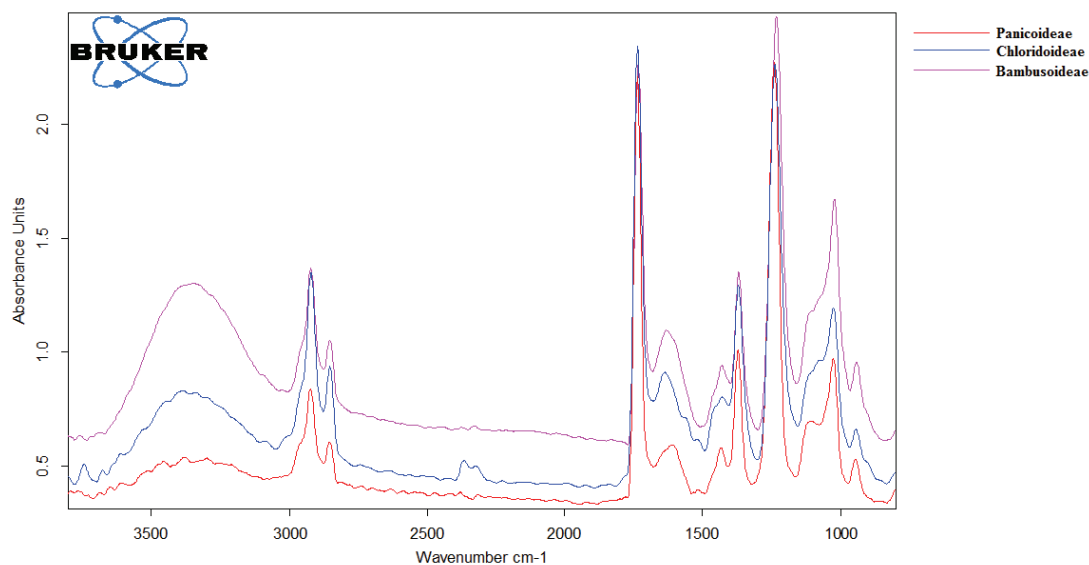


Figure 2- Overlaid ATR FT-IR spectra of representative species of Panicoideae, Chloridoideae and Bambusoideae subfamily.

panicoideae, chlorodoidae, and bambusoideae. Further differentiation is attempted between samples of the individual subfamilies.

5.2 Precision and Repeatability test

The precision test was carried out by using the spectra of three replicates of the same sample of grass species. The repeatability test was conducted by preparing the in-

dependent samples of the same species under the same set of experimental conditions. No visual difference between obtained spectra was observed as can be seen in Figure-3 and Figure- 4.

5.3 Classification between Panicoideae, Chlorodoidae, Bambusoideae subfamilies

A two-dimensional (2-D) PLSR model was made by

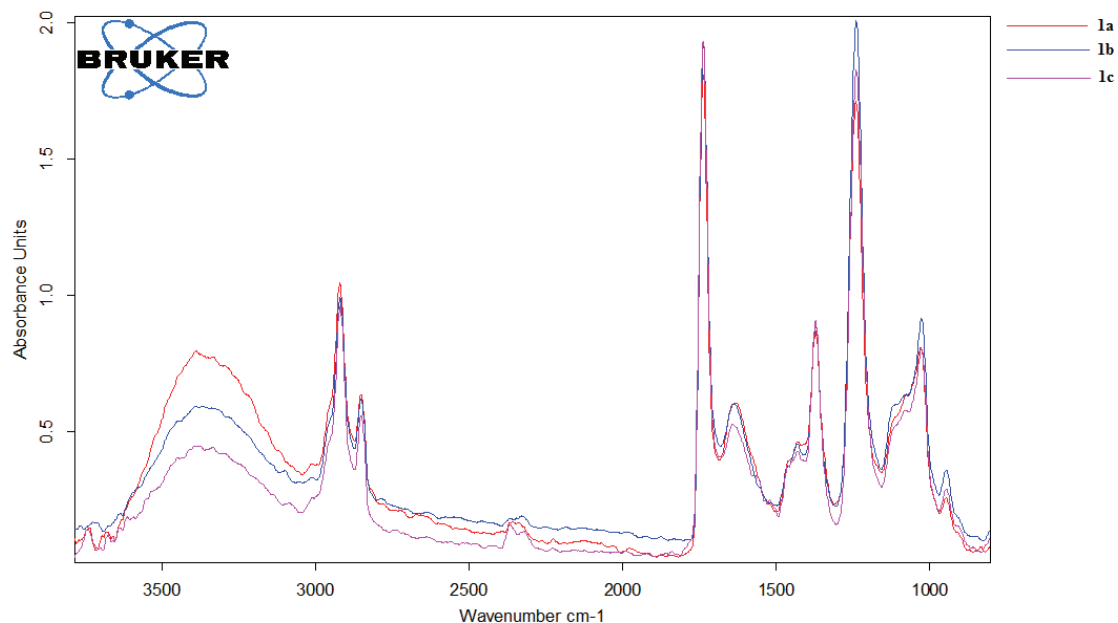


Figure 3- Representative ATR FT-IR spectra to check repeatability [Sample number 1].

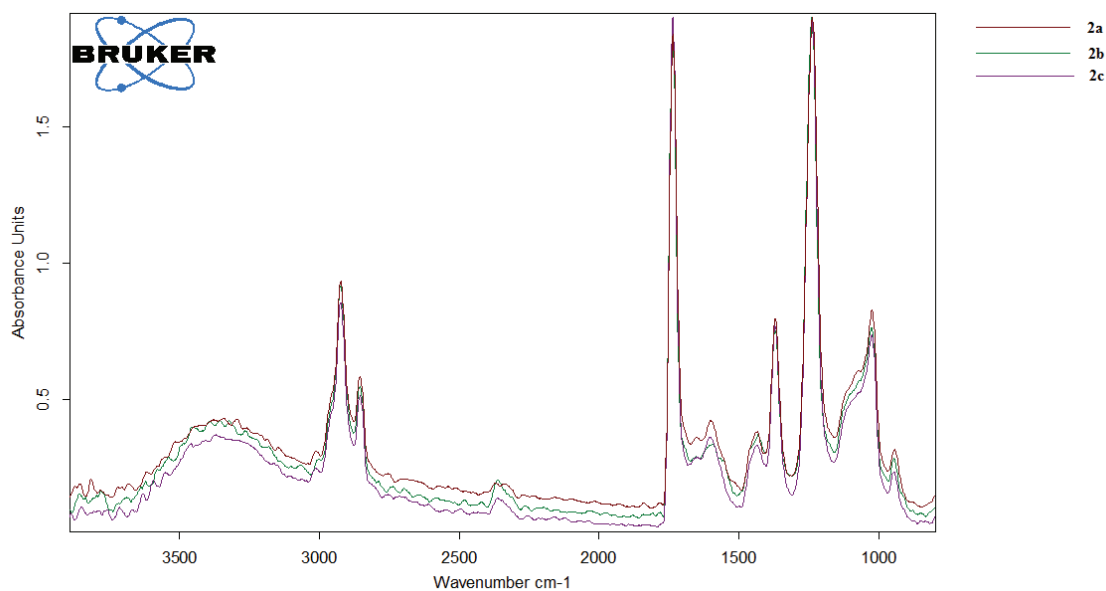


Figure 4- Representative ATR FT-IR spectra to check the precision [Sample number 2].

using the training dataset containing 50 IR spectra belonging to three subfamilies [panicoideae ($n=25$), chloroideae ($n=20$), and bambusoideae ($n=5$)] with two specified latent factors or variables from PLS regression. Based on the PLSR score plot, samples of panicoideae, chloroideae and bambusoideae subfamilies were entirely separated. Figure-5 shows the PLSR model to differentiate between panicoideae, chloroideae, and bambu-

soidae. As shown in figure-5 panicoideae designated with blue squares lie in class 1, chloroideae designated with red circles lie in class 2 and bambusoideae designated with green triangles lie in class 3. Twenty-five samples of panicoideae, 20 samples of chloroideae and 5 samples of bambusoideae subfamilies were differentiated using the PLSR model. A distinct differentiation between the samples of three subfamilies can be observed however,

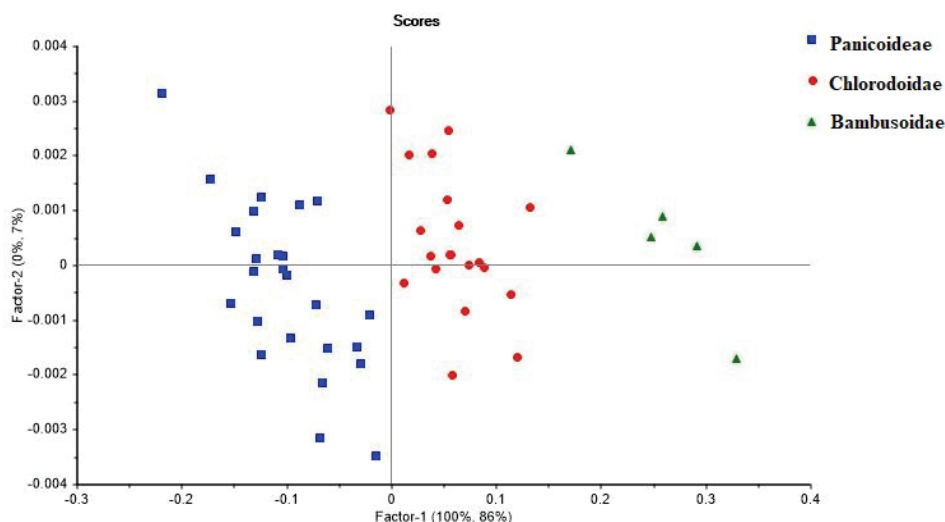


Table 2- Predicted Y (unknown samples of grass species of Panicoideae subfamilies) with predicted, deviation and reference values.

Predicted Y (Unknown Grass Species)	Predicted	Deviation	Actual Identity
11	0.9013	0.1679	1
12	0.9827	0.1157	1
13	1.9654	0.1321	2
14	3.0036	0.1262	3
15	4.9638	0.1224	4

Table 3- Predicted Y (unknown samples of grass species of Chlorodoideae subfamilies) with predicted, deviation and reference values.

Predicted Y (Unknown/ Blind Sam- ples)	Predicted	Deviation	Actual Identity
16	6.0032	0.1297	6
17	7.0092	0.1459	7
18	7.9662	0.1437	8
19	8.9850	0.1505	9
20	6.0282	0.1048	6

**Figure 5-** PLSR model to differentiate between Panicoideae, Chlorodoideae, and Bambusoideae.

few samples from each subfamily can be seen as outliers. A 100% accuracy rate in terms of positive classification was observed.

The R- square value in the PLSR model ascertains anticipated compatibility for future predictions with a pre-defined number of factors. The R- square values acquired from the calibration and validation set are 0.998 and 0.967 respectively. The obtained RMSE values for

the calibration and validation error are 0.02 and 0.11 respectively. The value of the slope is closer to 1 that is 0.99, indicates that the data is well modelled. The high R- square value signifies the high predictive accuracy and low error rate. Hence, the generated PLSR model is a good fit for the collected samples of the current study.

Figure-6 displays the loading plot of factor 1. The loadings have the homogenous profile as the original

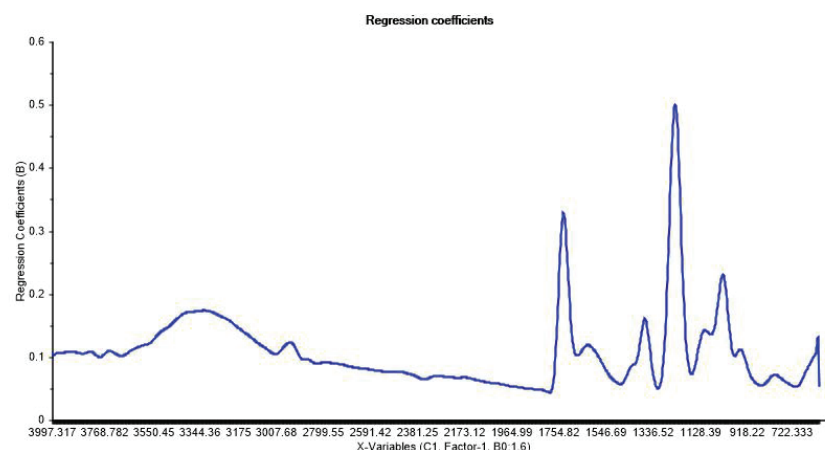


Figure 6- Loading plot of factor 1.

data and thus it emphasizes the regions of high importance and the factors provide noteworthy information. In loading plot, the region of amines (proteins), and amide I vibrations (C=O bending), amide II (C-H stretch) with a vibrational group of C-O/O-H bending, 1029 cm⁻¹ (C-O stretch) and peaks in fingerprint region due to monosaccharides, polyols, and carbohydrates are the dominant variables for the discrimination of selected grass species. Factor 1 shows the positive correlation in the whole region of the loading plot.

5.3.1 Differentiation of grass species of Panicoideae subfamily

Five species of the panicoideae subfamily such as the *Cenchrus ciliaris*, *Digitaria ciliaris*, *Echinochloa colona*, *Cenchrus biflorus* and *Panicum paludosum* were analyzed and differentiated using the PLSR multivariate data analysis. Based on the PLSR score plot, these species were entirely separated from each other. The results showed complete discrimination between these species. Figure-7 shows the PLSR model to differentiate between five species of panicoideae subfamilies [class I- *Cenchrus ciliaris*, class II- *Digitaria ciliaris*, class III- *Echinochloa colona*, class IV- *Cenchrus biflorus*, class V- *Panicum paludosum*]. The green triangle represents the species of *Cenchrus ciliaris* (class I), red circles represents *Digitaria ciliaris* (class II), green pentagon represents *Echinochloa colona* (class III), blue diamond represents *Cenchrus biflorus* (class IV), brown inverted triangle represents *Panicum paludosum* (class V).

The interspecies differentiation can be clearly observed however, there are certain intra species differences. In case of *Cenchrus ciliaris*, three distinct groups can be observed. Similarly, in case of *Echinochloa colona* two groups and *Cenchrus biflorus* three groups can be observed. This intra group variation may be due to the limited number of the sample size or different environmental conditions from which the samples were collected.

Digitaria ciliaris, *Cenchrus biflorus*, *Echinochloa colona* and *Panicum paludosum* showed 100 % accurate classification. *Cenchrus ciliaris* showed an 80% accurate classification as one sample in this class was not classified in any group. The R-square value obtained for this model is 0.996, which is highly significant.

5.3.2 Discrimination of species of Chlorodoidae subfamily

As shown in figure-8 the species in the chlorodoidae subfamilies that is *Cynodon dactylon*, *Dactyloctenium aegyptium*, *Saccharum spontaneum*, and *Dicanthium annulatum*. are also differentiated on the basis of their PLSR score plot. Blue squares represent the species of *Cynodon dactylon* (class 1), red color circles represent *Dactyloctenium aegyptium* (class 2), green triangles represents *Saccharum spontaneum* (class 3), and light blue diamonds represents *Dicanthium annulatum* (class 4).

Using the PLSR model, the samples are classified into four distinct groups; however, few closely placed samples of different species are observed. One sample



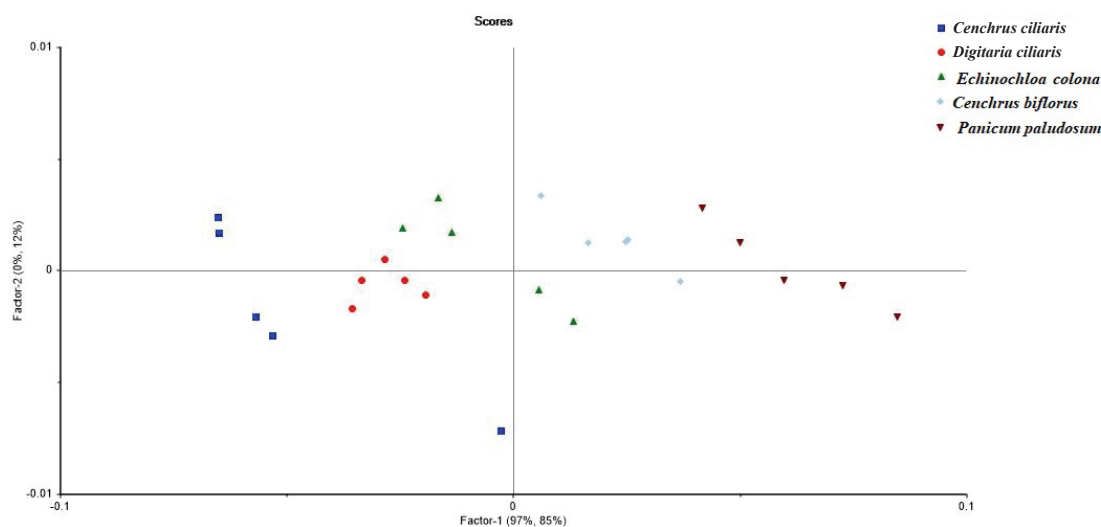


Figure 7- PLSR model to differentiate between 5 species of Panicoideae subfamilies [class I- *Cenchrus ciliaris*, class II- *Digitaria ciliaris*, class III- *Echinochloa colona*, class IV- *Cenchrus biflorus*, class V- *Panicum paludosum*].

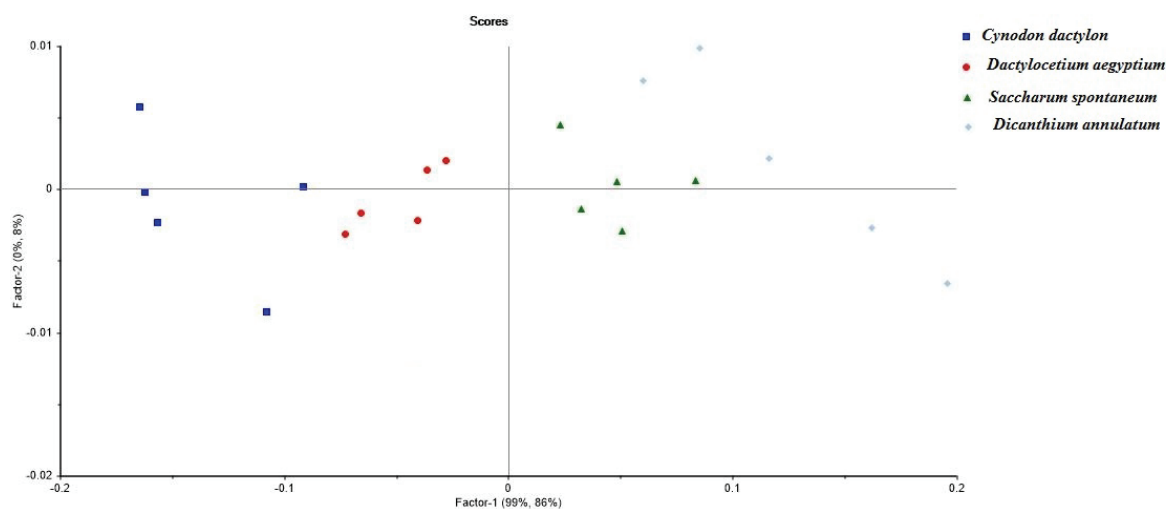


Figure 8- PLSR model to differentiate between 4 species of Chlorodoidae subfamilies [class I- *Cynodon dactylon*, class II- *Dactyloctenium aegyptium*, class III- *Saccharum spontaneum*, and class IV- *Dicanthium annulatum*].

of *Cynodon dactylon* is very close to the *Dactyloctenium aegyptium*. This might be because of the high chemical composition similarity between these two grass samples. They have been found to be of the same ancestor and sister group in the phylogenetic tree [30]. Overall, an accurate classification of 95% was obtained. *Cynodon dactylon* showed an 80% accurate classification (1 out of 5 samples was closely placed with class II). *Dactyloctenium aegyptium*, *saccharum spontaneum*, *dicanthium annulatum* each showed 100% accurate classification as shown in figure 8. The obtained R-Square value for the current model is 0.996.

The non-destructive approach of ATR FT-IR spectroscopy and the performance of classification ability of the PLSR model make this technique well-suited for the discrimination of grass species. Results suggested that the complete differentiation between selected grass species was possible even in cases of the same subfamilies.

6. Blind test

For the evaluation of the performance of the classification model, PLS (mean-centered) was used for the prediction of unknown samples with four components.

Unknown/blind grass samples can easily be identified by its nearby position of the known group of the training data set. A “dummy” value is assigned to each class/group for model building purposes. The value of 1, 2, 3, 4, 5, 6, 7, 8, and 9 is used to construct the PLSR model corresponding to the *Cenchrus ciliaris*, *Digitaria ciliaris*, *Echinochloa colona*, *Cenchrus biflorus*, *Panicum paludosum*, *Vynodon dactylon*, *Factylocetium aegyptium*, *Daccharum spontaneum*, and *Ficanthium annulatum* respectively.

In Figure-9 & 10 and Table-2 & 3 known groups (Actual identity) are given, which were constructed based on the training data set. Table-2 shows the predicted Y (unknown samples of grass species of the panicoideae subfamilies) with predicted, deviation and reference values. Table-3 shows the predicted Y (unknown samples of grass species of the chlorodoideae subfamilies) with predicted, deviation and reference values.

In the prediction columns, it is observed that the unknown/blind grass samples are positioned at their individual class. Five unknown samples of panicoideae (11-15) and five unknown samples of chlorodoideae (16-20) subfamilies were taken. Based on these predictions, unknown/blind samples were correctly classified in their respective species groups.

Figure-9 shows the predicted outcome with the deviation plot of unknown grass species of the panicoideae subfamily. Unknown/blind sample number 11 and 12 lie in group 1 that is *Cenchrus ciliaris*; sample number 13 belongs to *Digitaria ciliaris*; sample number 14 belongs to *Echinochloa colona*; and sample number 15 belongs to *Panicum paludosum*. Figure-10 indicates the predicted outcome with the deviation plot of unknown species of Chlorodoideae subfamilies. Unknown sample number 16 and 20 belong to the *cynodon dactylon*; sample number 17 belongs to *Dactylocetium aegyptium*; sample number

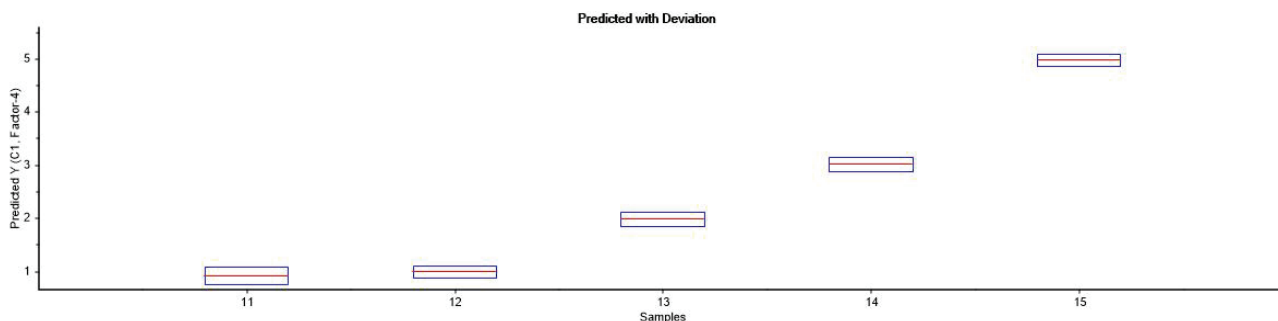


Figure 9- Predicted with deviation plot of unknown grass species of Panicoideae subfamily.

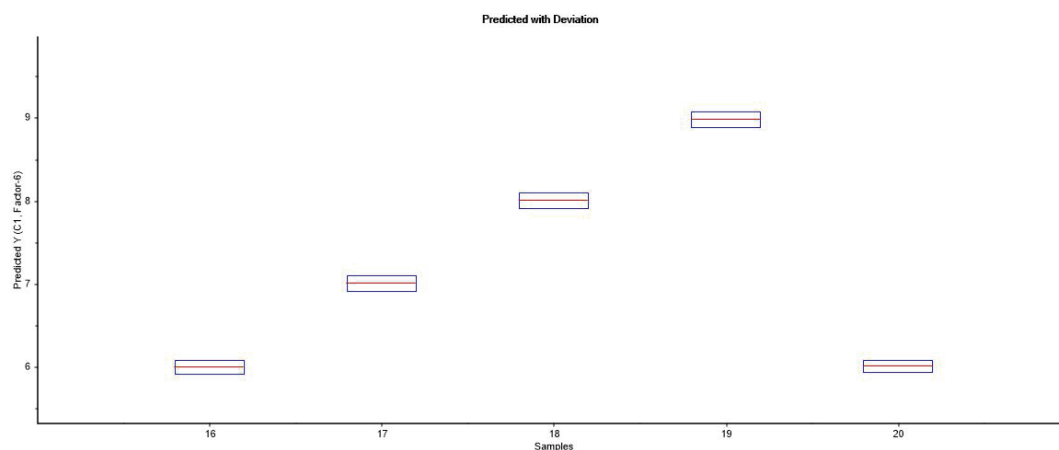


Figure 10- Predicted with deviation plot of unknown species of Chlorodoideae subfamilies.



18 belongs to *Saccharum spontaneum*; and sample number 19 belongs to *Dicanthium annulatum*.

7. Conclusion

In this preliminary study, ATR FT-IR spectroscopy in tandem with chemometrics has been used successfully for the differentiation of selected grass species. Since the spectra obtained from grass stains were dominated by noise, the ethanol extract of the grass stains were preferred for the analysis. Another benefit of using ethanol extract lies in the fact that ethanol does not degrade DNA and the sample can be successfully used for subsequent DNA analysis. Although the sample must be extracted for better results, ATR FT-IR spectroscopy performs as non-destructive technique, which gives excellent discrimination and classification results when combined with the chemometric tools such as PLSR. Samples of three subfamilies of panicoideae, chlorodoideae, and bambusoideae were successfully differentiated. Further discrimination between species of each individual subfamily was also achieved. The classification model used in this study was validated with 10 unknown samples and the validation ability of the constructed model was established by zero occurrences of false classification. By adding more species and more samples of different subfamilies in the training data set, the applicability of the current classification model will be strengthened.

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Conflict of interest

None declared

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