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# Genus- and species-level identification of dermatophyte fungi by surface-enhanced Raman spectroscopy

Evelin Witkowska [a], Tomasz Jagielski [b], Agnieszka Kamińska [a,*]

[a] Institute of Physical Chemistry, Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw, Poland
[b] University of Warsaw, Faculty of Biology, Institute of Microbiology, Department of Applied Microbiology, I. Miecznikowa 1, 02-096 Warsaw, Poland

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ABSTRACT

This paper demonstrates that surface-enhanced Raman spectroscopy (SERS) coupled with principal component analysis (PCA) can serve as a fast and reliable technique for detection and identification of dermatophyte fungi at both genus and species level. Dermatophyte infections are the most common mycotic diseases worldwide, affecting a quarter of the human population. Currently, there is no optimal method for detection and identification of fungal diseases, as each has certain limitations. Here, for the first time, we have achieved with a high accuracy, differentiation of dermatophytes representing three major genera, i.e. *Trichophyton*, *Microsporum*, and *Epidermophyton*. Two first principal components (PC), namely PC-1 and PC-2, gave together 97% of total variance. Additionally, species-level identification within the *Trichophyton* genus has been performed. PC-1 and PC-2, which are the most diagnostically significant, explain 98% of the variance in the data obtained from spectra of: *Trichophyton rubrum*, *Trichophyton menatgrophytes*, *Trichophyton interdigitale* and *Trichophyton tonsurans*. This study offers a new diagnostic approach for the identification of dermatophytes. Being fast, reliable and cost-effective, it has the potential to be incorporated in the clinical practice to improve diagnostics of medically important fungi.



## 1. Introduction

Dermatophytes are among the oldest microorganisms that are known to cause infections in humans [1,2]. The causative dermatophytes included species of three anamorphic genera, namely *Trichophyton*, *Microsporum*, and *Epidermophyton*, incidence of which has been on the rise since the mid-20th century, are the major pathogens globally [3].

The identification of dermatophytes has relied almost exclusively upon conventional culture-based methods, including examination of gross colony morphology and microscopic features of conidia and other hyphal structures, supported by mating experiments and biochemical tests, such as urease test or nutritional assays [4–6]. All these methods are laborious, time-consuming (in the case of dermatophytes a 4-week incubation period is common [7]), and lack sufficient discriminatory power and reproducibility. The inconsistency and ambiguity of the speciation results for dermatophytes are due to their phenotypic variability and pleomorphism, strongly influenced by the phase of growth and culture conditions.

Much progress has been achieved with the introduction of molecular, DNA-based identification techniques, most of which involve PCR or its variations as the core element. Over the last two decades, several PCR-based and post-PCR modalities (e.g. PCR-restriction fragment length polymorphism (RFLP), PCR-sequencing, probe hybridization), targeting different genetic loci (e.g. ribosomal RNA genes, genes coding for chitin synthase 1, DNA topoisomerase II, and β-tubulin), have been developed for identification of dermatophytes at the species and strain level, significantly increasing the reliability of the analysis and reducing its turnaround time from weeks to hours [8,9].

Yet, the molecular diagnostic approaches are not free from limitations. One is the paucity of sequence databases and contamination of the existing databases with sequencing errors, making the identification either impossible or doubtful. Another problem is the lack of a reliable and objective cutoff of nucleotide identity for species demarcation. PCR methods offer the possibility of identifying fungi directly in clinical samples. This, however, may be complicated by the heterogeneity of the collected sample. The fungus detected may not represent an infectious agent, but a transient contaminant or autochthonous colonizer.

Recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as an attractive alternative to both phenotype- and DNA-based methods for identification of fungi, including dermatophytes [10,11]. MALDI-TOF is an automated system, which provides fast and accurate identification through an

* Corresponding author.
*E-mail addresses:* ewitkowska@ichf.edu.pl (E. Witkowska), akamin@ichf.edu.pl (A. Kamińska).

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acquisition of unique protein spectra produced from extracts of microbial cells. The disadvantages of this technology are the up-front capital cost of the equipment and further maintenance costs, inadequate reference spectra libraries available for dermatophytes, and culture-conditions-dependent changes in protein expression, potentially altering the MALDI-TOF MS profiles [11]. Overall, the search for an optimal diagnostic method for dermatophytes, delivering an unambiguous result in a short time, at a low operating cost, and easily reproducible at the intra- and inter-laboratory levels continues.

Currently, there is no method which is optimal in the case of detection and identification of dermatophytes. In the novel diagnostic methods the criteria such as resolution, differentiation capacity, accuracy, reproducibility and duration of the analysis should be taken into account. The cost, easiness of performance and personnel demands are also very important. For this reason, we present the SERS-based method of dermatophytes detection and differentiation, which meets the above criteria and most importantly, is can omit time-consuming culture procedures, as the analysis can be made directly from skin scrapings [12].

Surface-enhanced Raman spectroscopy (SERS) is an optical spectroscopy method with high sensitivity and chemical specificity. The phenomenon of SERS is explained by the combination of an electromagnetic (EM) mechanism and a chemical mechanism related to charge transfer (CT) between a substrate and an adsorbed molecule [13]. Both mechanisms can increase bands intensities by 7 to 14 orders of magnitude, in relation to normal Raman signals [14,15], with the possibility of single molecules detection [16]. One of the key advantages of using SERS spectroscopy is the ability to reduce fluorescence background compared to normal Raman so it is more suitable for biological samples analysis even in aqueous environment. Additionally, the SERS technique offers nondestructive, reliable, and fast detection of samples which leads to varied practical applications of this technique [17–20]. Recently, this technique has been extensively applied to the microbial studies, especially bacterial cells [21–24]. However, only few reports have addressed the use of this technique to analysis of fungal cells [25–27]. In our previous work we proved that it is possible to differentiate four fungal species causing different types of human infections [12]. It should be highlighted, that it was the first report in which the SERS spectra of different human fungal pathogens at very high resolution have been presented and analyzed.

In the present paper we demonstrate the SERS-PCA-based method for the detection and identification of three different fungal pathogens (i.e. *Microsporum canis*, *Epidermophyton floccosum*, *Trichophyton interdigitale*), all causing skin infections. Additionally, as *Trichophyton* sp. is the most popular dermatophyte, we decided to show that SERS method is suitable for differentiation of four fungal pathogens, all belonging to one genus – *Trichophyton*. This analysis is very important, as all *Trichophyton* species are closely related and thus difficult to distinguish by other known methods.

## 2. Materials and Methods

### 2.1. Chemicals and Materials

Six strains representing different dermatophyte species, namely *E. floccosum*, *M. canis*, *T. interdigitale*, *T. mentagrophytes*, *T. tonsurans*, and *T. rubrum* were included in the study. The strains, housed by the Department of Applied Microbiology, University of Warsaw, Poland, were originally either purchased from the Centraalbureau voor Schimmelcultures (CBS; Utrecht, the Netherlands), and from the American Type Culture Collection (ATCC; Manassas, USA) or kindly provided by collaborating laboratories. Species identity of the strains included in the study was established by conventional, culture-based methods, exploiting morphological criteria, and confirmed with PCR-sequencing of two marker loci within the rDNA cluster, that is internal transcribed spacer (ITS) 1/2 regions and D1/D2 domains of the 26S rRNA gene, as described previously [28].

The chitin, galactomannan, and glucan were obtained from Sigma-Aldrich (Darmstadt, Germany) and used as received without further purification.

### 2.2. SERS substrates and SERS Measurements

Ag-coated SERS platforms were purchased from Silmeco ApS (Copenhagen, Denmark). The SERS measurements were performed using the Renishaw inVia Raman system equipped with a 300 mW diode laser emitting a 785 nm line which was used as an excitation source. The laser light was passed through a line filter and focused on a sample mounted on an X–Y–Z translation stage with a 50× objective lens (numerical aperture 0.75) that focused the laser to a spot size of ca. 2.5 μm. The Raman-scattered light was collected by the same objective through a holographic notch filter to block the Rayleigh scattering. A 1200 grooves per mm grating was used to provide a spectral resolution of 5 $cm^{-1}$. The Raman scattering signal was recorded by a 1024 × 256 pixel RenCam CCD detector.

The spectroscopic maps were acquired by collecting SERS spectra over the previously defined range at each point on a grid with 3 μm spacing using an automated microscope stage.

Typically, 30 SERS spectra for each fungal culture/species were acquired for 20 s each, by using this mapping mode. The laser power measured at the sample was 5 mW.

### 2.3. Fungi Culture and SERS Sample Preparation

The fungi were cultured on Sabouraud dextrose agar (SDA; Biocorp, Poland) in Petri dishes at 30 °C for up to 2 weeks, depending on the species and until colonies were clearly visible. Afterwards, randomly picked colonies were suspended in 100 μL of 0.9% NaCl solution and homogenized in a microtube with pellet pestle rods with motor to obtain fragmented mycelium. Then the samples were centrifuged for 5 min at 13,000 rpm at room temperature. Finally, the supernatant was discarded and the pellet of mycelial debris was resuspended in 20 μL of 0.9% NaCl solution. Centrifugation was repeated 3 times to obtain a homogenous solution, after which ca. 5 μL of such solution was applied onto the SERS substrate and the spectra were recorded.

### 2.4. Chemometric Analysis

The PCA was applied for all spectra collected for *M. canis*, *E. floccosum* and *T. interdigitale* and for four species of the *Trichophyton* genus. The analysis allowed to investigate the spectral variations and to find the most significant modes contributing to the variance accounted for the PCs. The PCA was performed for the pre-processed Raman spectra in order to (i) evaluate the spectral differences between the fungal species (ii), identify the individual fungal species, and (iii) develop a model for identification of fungal pathogens from clinical samples.

### 2.5. PCA - Spectral Data Analysis

Prior statistical analysis, the pre-treatments of raw SERS data e.g., baseline correction, smoothing, and normalization were performed sequentially, to minimalize and/or eliminate the influence of noise and forescence-background.

SERS spectra were prepared for principal component analysis (PCA) using a two-step approach. First, using a built-in OPUS software (Bruker Optic GmbH 2012 version) the spectra were smoothed with Savitzky-Golay filter, the background was removed using baseline correction (concave rubberband correction; no. of iterations 10, no. of baseline points 64), and then the spectra were normalized using a Min-Max normalization. All the data were transferred to the Unscrambler@ software (CAMO software AS, version 10.3, Norway), where the PCA was performed.

## 3. Results and Discussion

### 3.1. SERS Investigation of Spectral Changes of Fungi

In this study, the SERS technique was employed for the detection and identification of six in total dermatophyte species, namely *Microsporum canis*, *Epidermophyton floccosum*, *Trichophyton interdigitale*, *T. rubrum*, *T. menatgrophytes*, and *T. tonsurans* using Simceco SERS substrates (Silmeco ApS, Copenhagen, Denmark), the commercially available platforms.

First, the SERS spectra of three different fungal species belonging to three different genera were measured (i.e. *Microsporum canis*, *Epidermophyton floccosum*, *Trichophyton interdigitale*). Additionally, we performed the Raman measurements for major components of fungal cell walls, i.e. chitin, galactomannan, and 1,3-β-glucan to make the bands assignments possible. Fig. S1 presents the normal Raman spectra for these biomolecules. Every fugal SERS spectrum in Fig. 1 showed bands detectable for some constituents of the fungal cell wall, e.g. ca. four hundred and eighty centimetre$^{-1}$ (chitin), ca. one thousand and five centimetre$^{-1}$ (galactomannan) and ca. one thousand, one hundred centimetre$^{-1}$ (1,3-β-glucan). Additionally, every spectrum showed bands at ca. 850, 890, 960, and 1034 cm$^{-1}$ assignable to tyrosine residues [29], C—H equatorial bending vibrations for β-glucan [30,31], C—N stretch [26], and phenylalanine [32], respectively. The strong peak at ca. one thousand, four hundred and fifty centimetre$^{-1}$, characteristic for all dermatophytes, could represent $CH_2$ bend in proteins and lipids [33].

As seen in Fig. 1, the spectral images exhibit also many differences. Firstly, only *T. interdigitale* SERS spectrum showed an intense band at ~480 cm$^{-1}$, which could be assigned to chitin. Secondly, for *M. canis*, a peak at ~730 cm$^{-1}$ was the most intense compared with two other species and was attributed to the C—N stretching mode of the adenine of the flavin derivatives [34] or to the adenine containing molecules [35]. Thirdly, spectra for *E. floccosum* exhibited a very strong band at about 1130 cm$^{-1}$, representing ═C—O—C═ (unsaturated fatty acids in lipids) [36,37] or galactomannan. A detailed analysis of the SERS spectra of analyzed fungi cells is presented in Supplementary Materials.

All spectral bands observed, and their assignment, for each of the three species analyzed, are listed in Table 1.

The reproducibility of the recorded fungal SERS signals is a crucial parameter for further analytical and biomedical applications of this technique. Therefore, the reproducibility of the SERS signals for *E. floccosum*, *M. canis*, and *T. interdigitale* was assessed (Fig. S2, Table S1). For example, Fig. S2 shows the SERS spectra of *E. floccosum*, recorded from different spots within the same sample. To obtain statistically valid results, the strong signal at 1182 cm$^{-1}$ was chosen to calculate the average standard deviation (Av. SD). The Av. SD of the intensity of this vibration in the 30 SERS spectra recorded for the same platform was 6.2%. The reproducibilities of the SERS signals of each dermatophyte species are presented in Table S1.

**Table 1**
Major spectral bands observed in *T. interdigitale*, *M. canis* and *E. floccosum* [22,23,29,37–45].

| Assignment | Range | *T. interdigitale* | *M. canis* | *E. floccosum* |
|---|---|---|---|---|
| Chitin | 475–490 | ++ | − | − |
| Guanine, tyrosine | 640–675 | ++ | + | ++ |
| Adenine | 713–740 | + | ++ | + |
| Cytosine, uracil | 745–790 | − | + | + |
| Tyrosine residues | 850–850 | + | + | + |
| β-Glucan | 885–905 | + | + | + |
| C—N stretching, C═C deformation | 930–990 | + | + | + |
| Phenylalanine, galactomannan, C—C aromatic ring stretching | 1000–1010 | + | + | + |
| Phenylalanine | 1025–1040 | + | + | + |
| C—C skeletal vibration of acyl backbone in lipids (gauche conformation), C—O stretching in carbohydrates | 1078–1110 | + | + | + |
| ═C—O—C═ (unsaturated fatty acids in lipids), galactomannan | 1129–1145 | + | + | ++ |
| Aromatic amino acids | 1150–1195 | − | + | + |
| Amide III (random), thymine | 1213–1295 | ++ | + | + |
| Amide III (protein) | 1315–1345 | ++ | ++ | ++ |
| Galactomannan | 1400–1415 | − | + | + |
| $CH_2$ deformation | 1440–1475 | ++ | ++ | ++ |
| Amide II | 1510–1560 | − | + | + |

++: strong, +:present, −:absent.

### 3.2. Statistical Classification

In order to prove that SERS is a reliable method for dermatophyte identification, the PCA was performed (Fig. 2). PCA analysis was conducted in the whole spectral region between 400 and 1600 cm$^{-1}$ for *E. floccosum*, *M. canis*, and *T. interdigitale*. In the first step we found that the two principal components (PC-1 and PC-2) were the most diagnostically significant and explained 66% and 31% of the variance in the analyzed data set (Fig. 2A). The differences among the three fungal species were due to the bands described and specified in section 3.1, and were clearly illustrated by loading plots presented in Fig. 2B. The loading provides information on the variables (wavenumbers of the spectrum) that are important for group separation. The loading plot of PC-1 in Fig. 2B calculated for the three species indicated the most important

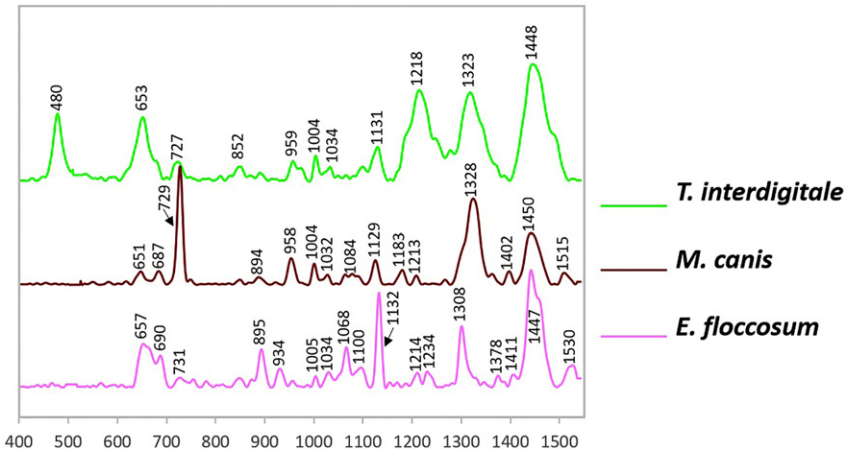


**Fig. 1.** The average SERS spectra of *T. interdigitale*, *M. canis*, and *E. floccosum* acquired from aqueous fungal cell solutions on SERS platforms. Experimental conditions: 5 mW, 785 nm excitation. The SERS spectra were baseline corrected, normalized and shifted vertically for better visualization. Each spectrum was averaged from 30 measurements in different places of the SERS platform.

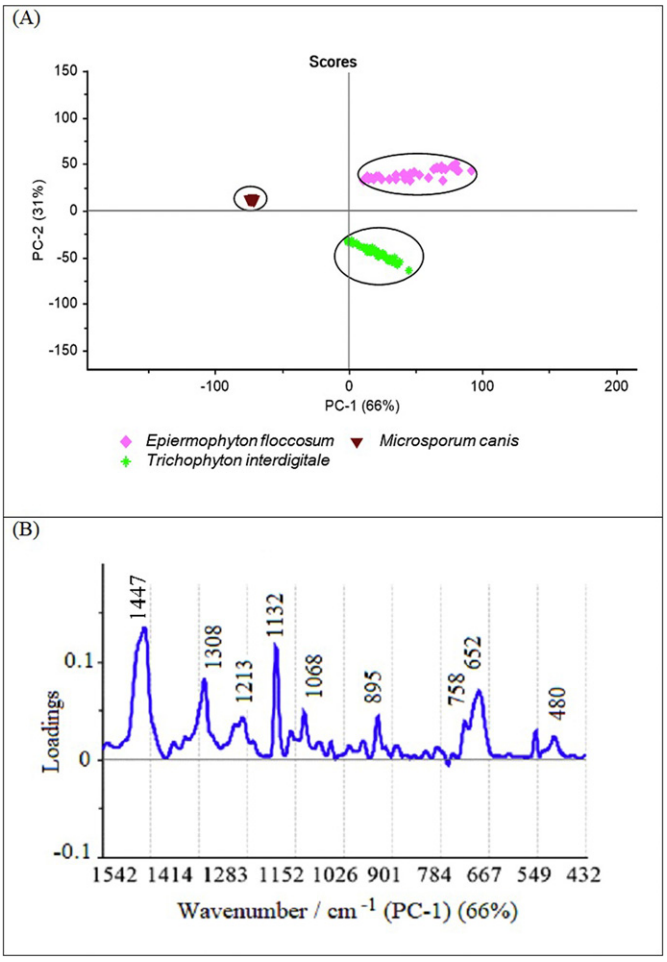


**Fig. 2.** (A) The score plots of PC-1 versus PC-2 component for differentiation of *E. floccosum*, *T. interdigitale*, and *M. canis*. PCA was calculated for the whole region (400–1500 $cm^{-1}$). Each spot in Fig. 2 (A) represents one SERS spectrum for analyzed pathogenic fungal cells. (B) PC-1 loading plot in the fingerprint region.

diagnostic variables in the analyzed data set. Variables with high loading values are the most important for diagnostic purposes. Unlike the SERS spectrum, the loading spectrum contains positive and negative bands, and their corresponding frequencies can be correlated with some of the major variations in the molecular structure of the analyzed fungal pathogens. On the basis of the SERS spectra analysis and loadings plots from PC-1 the most important variation among these three fungi (*E. floccosum*, *M. canis*, and *T. interdigitale*) was found to be associated with the bands at ca. 480, 652, 895, 1068, 1132, 1206, 1308 and 1447 $cm^{-1}$, corresponding to the fungal cell wall's constituents (Fig. 1; Table 1).

To investigate the intra-genus discriminatory potential of the SERS method, spectra of four species of the *Trichophyton* genus (*T. interdigitale*, *T. rubrum*, *T. mentagrophytes*, and *T. tonsurans*) were analyzed.

The obtained spectra shared many features (Fig. 3). The universally observed bands were at ca. 480, 650, 730, 850, 960, 1004, 1130, 1323, 1450 $cm^{-1}$. However there were some easily noticed differences too. It refers mainly to the bands intensities, but additional peaks in some cases can be also detected, e.g. 550 and 1080 $cm^{-1}$ in the case of *T. tonsurans*, 1061 $cm^{-1}$ for *T. rubrum* and 1100 for *T. interdigitale*.

Due to these changes almost each exhibits its characteristic spectrum. The multivariate technique PCA was applied to obtain SERS data in order to improve the sensitivity of differentiation. The two principal components, PC-1 and PC-2, were calculated in the whole recorded spectral region (400–1500 $cm^{-1}$), what together explained 82% of

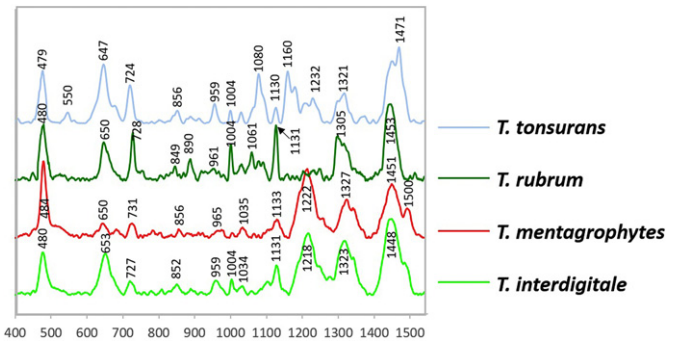


**Fig. 3.** The average SERS spectra *of T. tonsurans*, *T. rubrum*, *T. mentagrophytes*, and *T. interdigitale* from aqueous fungal cell solutions onto SERS platform. Experimental conditions: 5 mW, 785 nm excitation. The SERS spectra were baseline corrected, normalized and shifted vertically for better visualization. Each SERS spectrum was averaged from 30 measurements in different places of SERS platform.

variability and allowed for the differentiation between the species of the same genus. A score plot (Fig. 4A) showed that spectra could be divided into four groups (clusters) corresponding to *T. tonsurans* (azure), *T. rubrum*, (green), *T. mentagrophytes* (red), and *T. interdigitale* strains (light green). By analyzing these plots (Fig. 4B) the most important for diagnostic purposes variables could be noted. The wavenumbers of ca. six hundred and forty seven centimetre$^{-1}$ and 727 $cm^{-1}$ had the largest weights in the negative direction. Other wavenumbers of ca. 1457, 1410, 1327, and 1218 $cm^{-1}$, which also have an important contribution to PC1, have the same direction as the most intensive one at ca. four hundred and seventy eight centimetre$^{-1}$. Taking into account the weight of each of these PC1 loadings it is obvious that especially two bands at ca. four hundred and seventy eight centimetre$^{-1}$ and 1218 $cm^{-1}$ had the main contributions to PC1 and point the most significant changes between these dermatophytes. It should be highlighted that, the peak at 479–480 $cm^{-1}$ recorded in SERS spectrum (Fig. 3) is the same mode that was calculated during the PCA analysis (Fig. 4 and Fig. S5), since the peak frequency precision for our experimental setup is $\pm 1.5$ $cm^{-1}$. The PCA results were consistent with the SERS spectra depicted in Fig. 3, which showed intensive bands due to the amide III (random) or thymine vibrations (1218 $cm^{-1}$) and collective vibration mode of the macromolecule skeleton (478 $cm^{-1}$) of chitin [46].

As mentioned above, in the 400–520 $cm^{-1}$ region one of the most prominent marker band at ca. four hundred and eighty centimetre$^{-1}$ was present (Fig. S5). The PCA scores calculated for the region of the chosen marker gave equal PC-1 and PC-2 values of 98% of total variance. This demonstrated the excellent separation of the studied dermatophytes into four separated clusters corresponding to the *T. tonsurans*, *T. rubrum*, *T. mentagrophytes*, and *T. interdigitale*, respectively (Fig. S5A).

## 4. Conclusions

The results obtained in this study demonstrate that SERS is a powerful technique for the detection and identification of pathogenic fungi not only at strain but also at genus level. The standard biochemical and genetic speciation assays are often laborious, technically demanding, and not always producing consistent results. SERS spectroscopy coupled with PCA analysis provide an attractive approach for discrimination between human fungal pathogens with high accuracy. In this study we have shown for the first time, label-free, simultaneous, and rapid identification of six important human fungal pathogenic species, i.e. *M. canis*, *E. floccosum*, *T. tonsurans*, *T. rubrum*, *T. mentagrophytes*, and *T. interdigitale* within which four species belong to one genus - *Trichophyton*.

The principal component analysis (PCA) as one of the most robust statistical methods was applied to: (*i*) extract the biochemical information from fungal spectra, (*ii*) show the significant spectral differences among SERS spectral features, and (*iii*) perform the statistical

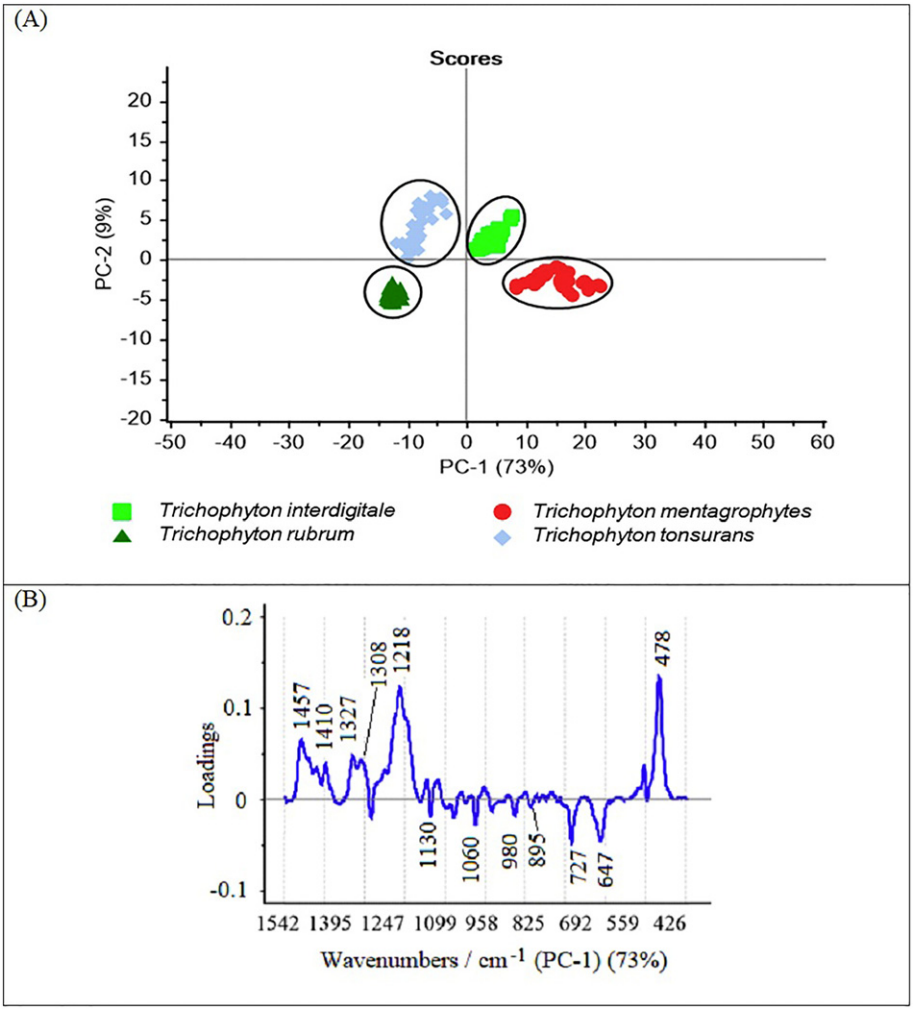


**Fig. 4.** (A) The score plots of PC-1 versus PC-2 component for differentiation of *T. tonsurans*, *T. rubrum*, *T. mentagrophytes*, and *T. interdigitale*. PCA was calculated for the whole region (400–1500 $cm^{-1}$). Each spot in Fig. 4 (A) represents one SERS spectrum for analyzed pathogenic fungal cells. (B) PC-1 loading plot.

classification of analyzed microorganisms. The PCA analysis visualize fungi differentiation in two-dimensional space defined by PC-1 and PC-2. Chemometric calculations demonstrate the impact of the developed SERS strategy up to 98% accuracy.

In conclusion, SERS technique emerges as a rapid and effective method of robust identification of pathogenic fungi. These SERS-based studies should be further extended with higher number of both cultured strains and clinical samples to improve the diagnostic sensitivity and selectivity of this method.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2017.11.008.

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