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Surface enhanced Raman spectroscopy and cultural heritage biodeterioration: Fungi identification in earthen architecture from Paraíba Valley (São Paulo, Brazil)

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ABSTRACT

In this work, Surface Enhanced Raman Spectroscopy (SERS) was employed in the taxonomic identification of fungi found in biofilms formed on earthen architecture walls (adobe, wattle and daub, and rammed earth) of historical buildings in the region known as Paraíba Valley (or São Paulo Historical Valley), which are representative of the first phase of the Brazilian coffee cycle (1820–1880). Very few studies are reported in the literature where SERS-based techniques are used in fungi identification, most of them focused on clinical diagnosis. In the present investigation, pure colonies isolated from biofilms on earthen walls previously identified by classic taxonomy and molecular biology were selected. The genera were *Trichoderma*, *Cladosporium*, *Aspergillus*, *Neurospora*, *Fusarium* and *Penicillium*. The fungi were cultured on solid potato dextrose agar, extracted with ethyl acetate and the extracts were applied on dried Au nanoparticles. The SERS spectra exhibited bands in the 600–1800 cm^{-1} region which are characteristic of each genus, except *Penicillium*, as revealed by PCA statistical analysis. This work reports the use of a facile to prepare SERS-active substrate in the identification of microbial communities on earthen architecture walls and is the first step of an investigation aiming at the fast identification of fungi species from biofilms formed on earthen architecture buildings without the need of isolating the pure cultures.

1. Introduction

Filamentous fungi play an important role in biotechnology and also as biodeterioration agents. The methods used in their identification were originally only at the biological level, as in classical taxonomy, through the observation of their macro and micro morphological characteristics. Later, molecular biology allowed DNA base pairs sequencing and, more recently, mass spectrometry hyphenated techniques and infrared absorption spectroscopy were introduced, aiming at to identify the chemical signatures which could be used as chemotaxonomic markers [1].

Another approach involves chemical speciation coupled to high spatial resolution and cytoplasm spectrochemical characterization, which can lead to the understanding of the hyphae growing processes. Prusinkiewicz et al. adopted such approach and used FTIR, Raman spectroscopy and Surface Enhanced Raman Spectroscopy (SERS) to obtain information on *Aspergillus nidulans* and *Curvularia protuberata* biochemistry. In the SERS measurements, Au nanoparticles were synthesized by adding $HAuCl_4$ directly to the culture media containing *A. nidulans* [2].

The literature on microorganisms identification by Raman spectroscopy was recently reviewed [3] and the authors compiled a wide range of applications, including medical diagnosis [4], which benefited from the fingerprinting characteristics of the technique for both whole-organism [5] or metabolites [6]. Raman spectroscopy was used, for example, in the differentiation of taxons from distinct macromycetes genera, based on the lipid and protein signatures observed in the spectra collected from basidiospores [7]. The authors used linear discrimination to assign 90% of the spectral features to the correct genus but it was not possible to achieve the identification at the species level.

The capability of Raman microscopy to allow a fast characterization and identification of individual spores of a variety of micromycetes species was demonstrated by Goshal et al. [8], where the authors compiled a reference library of Raman spectra of micromycetes

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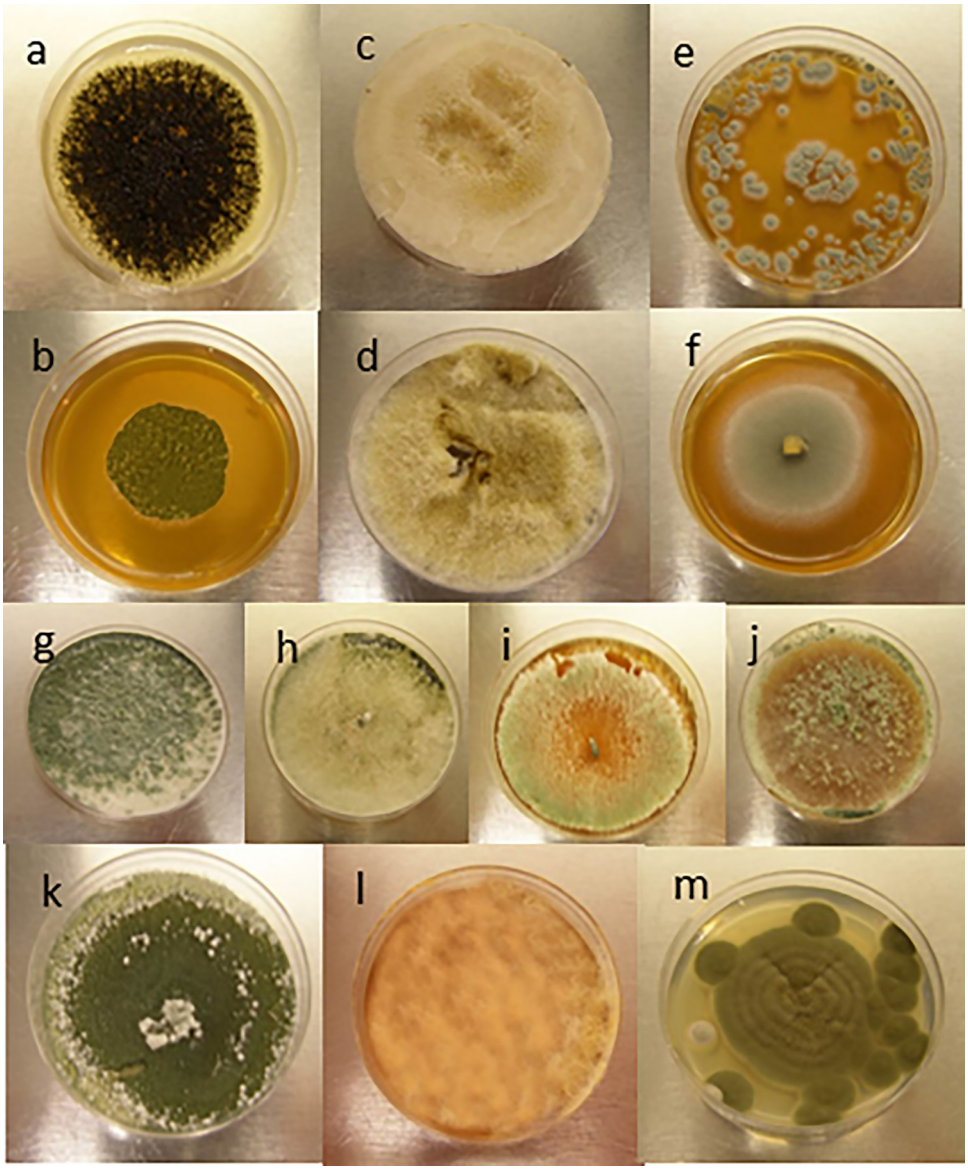

Fig. 1. Fungi isolate used in the SERS measurements: a) *Aspergillus carbonarius*; b) *A. parasiticus*; c) *Fusarium aff. incarnatum*; d) *F. equiseti*; e) *Penicillium aff. flavigenum*; f) *P. chrysogenum*; g) *Trichoderma atroviride*; h) *T. koningiopsis;* i) *T. aff. atroviride*; j) *T. harzianum*; k) *T. longibrachiatum*; l) *Neurospora sitophila*; m) *Cladosporium uredinicola*.

typically associated with humid environments. The spatial resolution in Raman microscopes is diffraction limited to the micrometric level, which is enough to the investigation of individual cells with the further advantages that minimal sample preparation is required and small volumes are employed.

Among other plasmonic techniques, Surface Enhanced Raman Spectroscopy (SERS) based techniques [9] are becoming tools of choice in biomedical studies [10]. The very large enhancement in the intensity of selected bands in the Raman spectra (10^4–10^8) originates mostly from the amplification of the electric field of both incident and scattered electromagnetic radiation, in resonance with localized surface plasmon (LSP) absorption associated with nanostructured metal surfaces or particles (typically Ag, Au and Cu) [11].

Regarding the use of SERS in studies focused on fungi or fungi metabolites, Szeghalmi et al. [12] reported on the growth of fungal hyphae (*Aspergillus nidulans*) on commercial nanostructured gold substrate, highlighting the potential of SERS in life sciences. The same type of SERS active substrate was employed by He et al. [13] to discriminate among five bacillus spores (*B. cereus* ATCC13061, *B. cereus* ATCC 10876, *B. cereus* sp., *B. subtilis* sp. and *B. stearothermophilus sp*.); SERS was used to enhance the Raman signal from the spores and PCA was applied considering the 900–1200 cm^{-1} spectral window. Martin-Sanchez et al. [14] used silver nanoparticles to investigate the black stains formed in Lascaux Cave and found them to be melanine from the fungus *Ochroconis* sp. Functionalized Ag nanoparticles were synthetized to target *Candida albicans*, *Candida glabrata*, *Candida krusei* and *Aspergillus fumigatus* [15] and, in recent papers, SERS spectra were processed using chemometrics in the characterization of clinically relevant fungi (*Aspergillus fumigatus ss.*, *A. fumigatus* complex species and *Rhizomucor pusillus*) within a clinical diagnosis perspective [16] and in dermatophyte fungi identification at genus and species-level [17].

As it can be concluded from some investigations cited above, the Raman spectra reported in microorganism studies present a complex structure, and their interpretation is frequently made using chemometrics. Multivariate exploratory methods such as Principal Component Analysis (PCA) are among the most important and popular chemometric tools for performing an easier visualization of data trends or patterns and for finding outliers. Briefly, this method performs a

dimensionality reduction by calculating linear combinations from the original variables, known as principal components following the equation $\mathbf{D} = \mathbf{CS}^{\mathbf{T}} + \mathbf{E}$ where D is a matrix containing the original dataset, C stores the new coordinates for each component, and S^T contains the loadings, which supplies information about the importance of each variable on data behavior; residual variance is contained in matrix E [18]. PCA does not distort the variation data since its main characteristic is the rotation process done to performing a simpler analysis.

Although earth is the oldest construction material and its use is widespread, it is also highly susceptible to the action of environmental factors and microorganisms [19]. Earthen architecture is almost the only constructive technique used to build the stylish farm houses and small villages in the area between São Paulo and Rio de Janeiro known as Paraíba Valley, which experienced an extraordinary economic growth during the so-called first phase of the Coffee Cycle, that lasted from 1820 to 1880 approximately. The buildings in such area are representative of the 19th century Brazilian earth architecture and suffer from lack of regular maintenance and damage caused by weathering factors, in special high humidity and temperatures, which create the ideal conditions for a broad range of biological activity.

The objective of this work is the development and use of a SERS-based methodology for the identification of different fungi genera from the spectral signatures produced by extracts of micromycete colonies collected from biofilms formed on deteriorated walls of colonial-style buildings in the cities of Areais and São José do Barreiro, both located in the Paraíba Valley.

2. Experimental

The fungi samples were isolated colonies (Fig. 1) from earthen architecture substrates collected from walls in farm buildings located at the Paraiba Valley in São Paulo State, Brazil. All species had been previously identified through classic taxonomic methods and molecular biology [20], as shown in Table 1. The isolates were grown and held in Petri dishes containing potato dextrose agar (PDA) medium.

Sampling was carried out in 2 and 7-day-old culture grown, when the fungi could be distinguished by the absence or presence of conidia, respectively. Mycelium was collected from the Petri dishes containing each fungi isolate, by scratching the PDA surface with a sterile spatula and 10 mg of each culture were deposited in Eppendorf tubes and extracted with ethyl acetate during 24 h (ethanol 80% was unsuccessfully tested); the supernatant was then collected and filtered before the essays with Au nanoparticles (AuNPs).

Gold nanostructures were used as substrate for achieving the enhancement effect. To accomplish this, colloidal gold was synthetized following a modified Lee-Meisel procedure [21,22]. Briefly, a 0.05 mol L^{-1} $HAuCl_4$ solution was prepared in *ca.* 95.0 mL of milli-Q water, heated until boiling under continuous stirring. Then, 3.0 mL of 1% (by mass) sodium citrate solution was slowly added. As the solution turned red, stirring and heating were stopped and volume was brought to 100 mL. The resultant colloid was concentrated by centrifugation and supernatant removal to 10% of its original volume. Thereafter, 5 µl of this suspension were deposited over quartz substrates in a laminar flow cabinet and left to dry. A similar drop casting procedure was applied for the liquid extracts.

Table 1
Fungi species identified [20] in biofilms formed on rammed earth, wattle-and-daub and adobe walls from historical buildings in the cities of Areais and São José do Barreiro (Paraíba Valley).

Species	Substrate
Aspergillus carbonarius	Rammed earth
Trichoderma atroviride	
Fusarium equiseti	
Trichoderma aff. atroviride	
Penicillium aff. flavigenum	
Penicillium chrysogenum	
Aspergillus parasiticus	
Trichoderma longibrachiatum	
Trichoderma koningiopsis	Wattle-and-daub
Neurospora sitophila	
Trichoderma harzianum	
Fusarium aff. incarnatum	Adobe
Cladosporium uredinicola	

A Renishaw Raman microscope (inVia Reflex) was employed and the 785 nm line of a solid state laser (Renishaw) was used to acquire the spectra (2 10^4 W/cm^2). The spectrometer is fitted with a thermoelectrically cooled CCD detector (Andor 600 × 400 pixels) and coupled to a Leica DM2500 M microscope. A ×50 objective was used to collect the light scattered by the samples and the spectra were recorded by continuously scanning the spectral window from 500 to 1850 cm^{-1} (SynchroScan mode) with 10 s of exposure time. For each sample the mean of 3 to 5 spectra was used. Additionally, normal Raman spectra were acquired directly from the mycelia.

Data processing was performed in Matlab environment Version 7.8 and chemometric analysis were run using the PLS Toolbox 8.5.2 (2017) by Eigenvector Research, Inc. Data were pre-processed with a smoothing procedure (symmetric Savitsky-Golay filter, 5 points window) and with Savitzky-Golay first derivative to correct the base line and the offset on the *y* axis of the spectra.

3. Results and discussion

There are cases reported in the literature where it was possible to obtain Raman spectra directly from microorganisms [23,24], however, initial attempts (λ_0 = 785 nm) made by exposing the hyphae surfaces of the fungi colonies resulted in very poor signal-to-noise ratio (spectra not shown); when higher laser power was used to improve the spectra quality, local heating damaged the samples, observed as changes in the pattern when sequential spectra were obtained. Another approach was to prepare the PDA culture medium in the presence of the AuNPs and in this case, although the metal sol did not interfere with the fungi grow, acceptable SERS signal was obtained only for high laser power and because of the risk of sample damaging such procedure was discarded.

When the ethyl acetate extracts were applied over dried AuNPs the SERS spectra obtained after solvent evaporation exhibited good signal-to-noise ratio as shown in Figs. 2 and 3. These spectra must correspond to the metabolites produced by the fungi in the culture medium and, therefore, have the potential to discriminate different fungi genus. Band assignment of individual components is not straightforward in complex mixtures such as the extracts here investigated and, therefore, statistical treatment has to be applied in an attempt to identify the fungi genus and species. Band shifting and changes in relative intensities when compared to ordinary Raman data makes the assignment based only in group frequencies unreliable.

Several spectra were acquired for each fungi type and they exhibited some degree of variability, as shown in Fig. 2, but all of them show an intense band at *ca.* 730 cm^{-1} assigned to adenine symmetric ring breathing of DNA bases; adenine is known to exhibit a strong SERS enhancement and such mode is the most intensified one [25,26]. Other strong features show up at *ca.* 1300 cm^{-1} and between 1400 and 1650 cm^{-1} irrespective of the fungi genus, although changes in relative intensities and band positions are clearly noticed.

In Fig. 3, all the spectra recorded for all the fungi genus here investigated are plotted together, after smoothing, baseline correction and intensity offset correction.

Exploratory PCA analysis were run for all the pre-processed SERS spectra of the fungi extracts and near 70% of the data variance was described by the first three principal components with their respective scores and loadings depicted in Figs. 4 and 5. While no trends associated with the species classification were observed, the SERS signals were able to show patterns related to the genus. Thus, *Trichoderma*

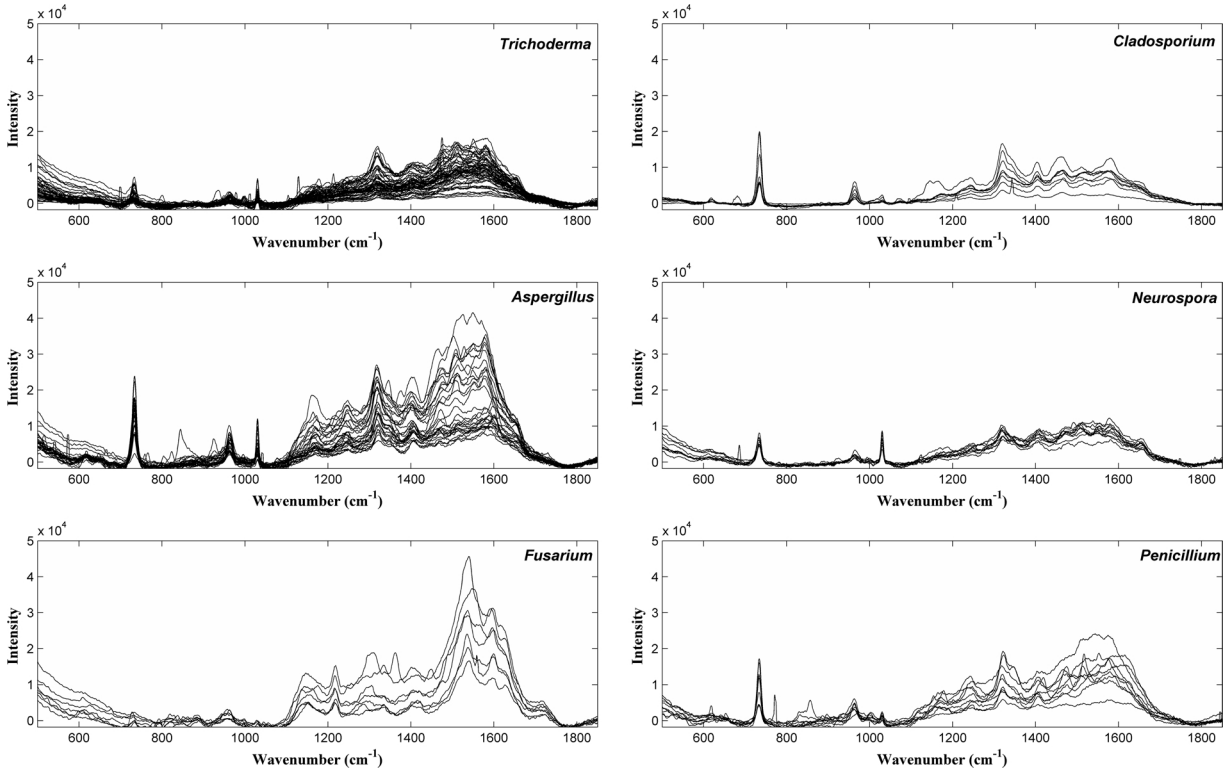

Fig. 2. Surface Enhanced Raman Spectra Raman (SERS) obtained for each isolate and at different spots on the dried AuNPs using the 785 nm laser line.

showed to be described by the variables with positive loadings of components 1 and 2 corresponding mainly to symmetric ring breathing of adenine (730 cm^{-1}) and CH_2 deformation of purines (around 1300 cm^{-1}) as shown by the PC1 loading plot (Fig. 5), and CH_2 deformation of lipids for PC2, albeit with low loading values, as can be seen in Fig. 5.

Fusarium and *Cladosporium* genus were observed in opposite locations on the PC2, with the former described by PC2 negative loadings; the bands in the 1000-1050 cm^{-1} range are probably due to lipids, saccharides and phenylalanine.

More than 95% of samples of *Aspergillus* are located at the negative side of PC1 and positive side of PC3, influenced by vibrational modes of lipids, nucleic acids and peptides, while on PC2 this genus does not exhibit a clear behaviour.

Particularly, *Neurospora* samples seems not to be strongly influenced by PC1 vs. PC2 but instead, the region between 1200 and 1650 cm^{-1} on the negative loadings of PC3 and the band at 1050 cm^{-1} on PC2 (also negative values) permitted a separation from the whole dataset. The SERS response of *Penicillium*, however, seems not to be specific enough to allow its discrimination considering the experimental conditions used. Although positive loadings of PC2 seems to separate it from the

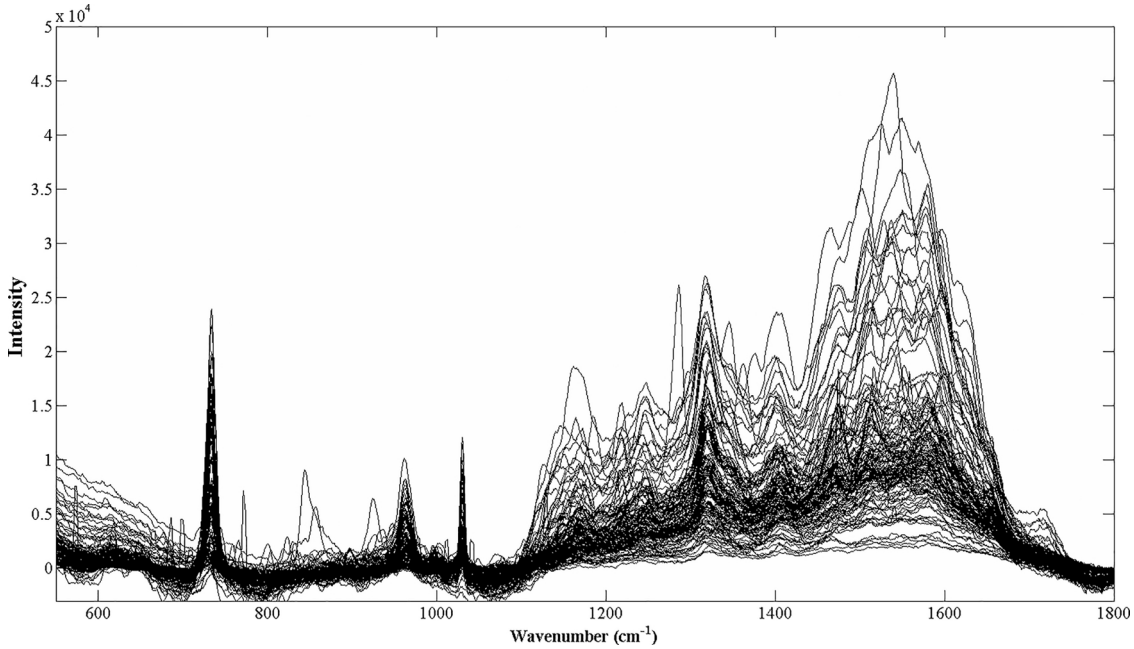

Fig. 3. Preprocessed SERS spectra (785 nm) from all the six fungi isolates.

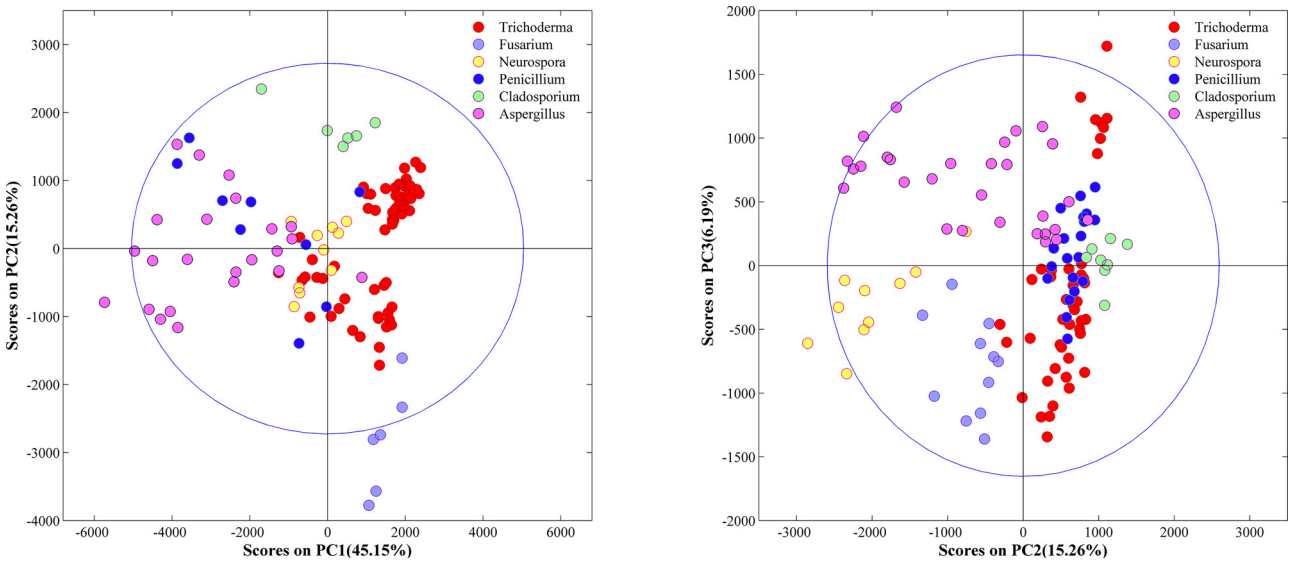

Fig. 4. Score plots PC1 vs. PC2 (left) and PC2 vs. PC3 (right) for the SERS spectra of six fungi genera investigated (*Trichoderma*, *Fusarium*, *Neurospora*, *Penicillium*, *Cladosporium* and *Aspergillus*).

other samples, still there is an overlapping with *Trichoderma* on the rest of PCs.

Secondary metabolites were successfully used in the fungal chemotaxonomy for some genera here investigated such as *Aspergillus*, *Fusarium* and *Penicillium*; the metabolites profile are frequently obtained using mass spectrometry hyphenated techniques [27]. In this work, it was demonstrated that the SERS spectra of ethyl acetate extracts on AuNPs combined with PCA can be used as a fast procedure to differentiate fungi colonies, that have interest from the cultural heritage point of view.

4. Conclusions

SERS was used to discriminate fungi from five out of six different genus found in biofilms formed on historical buildings made using earthen technology (adobe, wattle and daub and rammed earth); the method was not sensitive to *Penicillium*. It has to be remembered that the spectra here reported were obtained from ethyl acetate extracts containing the microorganisms metabolites and further investigations are necessary to clarify if it is the best solvent to be used in the proposed protocol or if there are others which could be more efficient in extracting the metabolites from the culture medium, thus improving the identification procedure.

The data here reported has shown the potential of SERS

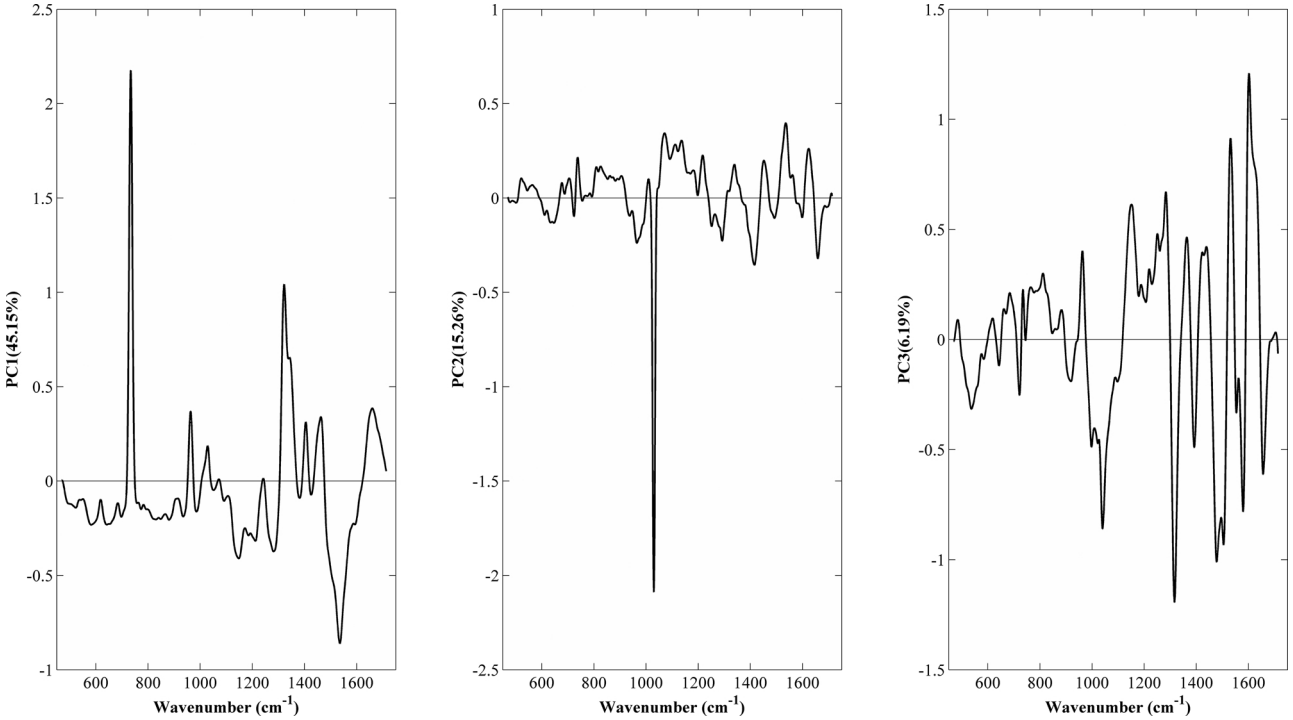

Fig. 5. Loading plots for PC1 (blue), PC2 (green) and PC3 (red) for the SERS spectra of six fungi genera investigated (*Trichoderma*, *Fusarium*, *Neurospora*, *Penicillium*, *Cladosporium* and *Aspergillus*).

spectroscopy as an alternative and fast method for the discrimination of fungi genus and, taking into account that metabolic activities play a key role among the critical factors involved in fungi growing and reproduction it can be envisaged that the SERS-based techniques will be a powerful tool in the understanding of fungi response to different environments.

In this study, SERS was successfully used in the differentiation of filamentous fungi genera which are associated with cultural heritage biodeterioration.

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