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Research Article

APPLICATION OF FOURIER TRANSFORM MID-INFRARED SPECTROSCOPY FOR IDENTIFICATION OF *ASPERGILLUS* SPECIES ISOLATED FROM COFFEE BEANS

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ABSTRACT

In mycology, one of the challenges is to differentiate similar species. Morphology-based methods are often limited, thereby increasing the demand for new field explorations to obtain better and more reliable results. Fourier transform infrared (FT-IR) spectroscopy is proposed as an alternative method for the classification of highly related fungi such as *Aspergillusochraceus* and *Aspergilluswesterdijkiae* or *Aspergilluscarbonarius* and *Aspergillusniger*. However, methodologies for sample preparation are time consuming and laborious. A traditional method for sample preparation was adapted by reducing the time and maintaining the quality of analysis and a chemometric model was developed. The fungi were grown in Sabouraud medium for 4 days, the mycelium was blended with KBr and analyzed by Fourier transform mid-infrared transmittance (FT-MIR) spectroscopy. Partial least squares regression was applied to the species comparison models and a prediction test was used to evaluate the models. The coefficient of determination for calibration and root mean square error of calibration were 0.94 and 0.23, respectively. In the external validation, only one *A. westerdijkiae* was not correctly classified. Results suggest that adaptation of the methodology accelerated the process of sample preparation, maintaining reliability.

Keywords: *Aspergillus*; coffee beans; spectroscopy; identification.

INTRODUCTION

*Aspergillus*is one of the most economically important genera of microfungi; hence, rigor and stability of its taxonomy are of significant practical concern[1]. Some *Aspergillus* species are well known as potential producers of ochratoxin A (OTA), a mycotoxin capable to produce nephrotoxic, teratogenic, immunotoxic, and carcinogenic effects on animals[2] and considered a potential carcinogen in humans by the International Agency for Research on Cancer[3,4]. Recent results indicate that *Aspergilluswesterdijkiae*, *Aspergillussteynii*, *Aspergillusochraceus*, *Aspergillusniger*and *Aspergilluscarbonarius*are responsible for the formation of OTA in coffee beans[5-7].

Several studies suggest that *A. ochraceus* is the main species producing OTA in coffee beans, being found in higher numbers than other species of the section*Circumdati*[8]. However, Frisvadet al.[9] and Fungaro et al.[10] observed specific features of *A. ochraceus* and proposed a subdivision of *A. ochraceus* into two species, *A. ochraceus* and *A. westerdijkiae.* Through genotype analysis[10] and taxonomic reorganization[9], it was concluded that *A.*

westerdijkiae is strongly related to the presence of OTA in coffee beans produced in Brazil.

The taxonomy of *Aspergillus* section *Nigri* has been extensively studied and was recently reviewed. Taxonomic studies using molecular methods led to the separation of the *A. niger* complex into two species, *A. niger* and *A. tubingensis*[11].

The systematics of *Aspergillus* is complicated by the existence of morphological divergence among isolates of the same species. Morphology- based markers are often limited, leading to new field exploration to obtain better and more reliable results. Modern approaches such as polyphasic profiling of microorganisms provide relevant results for phylogenetic and taxonomic issues; however, they can be time-consuming and unsuitable for routine use[12]. Infrared spectroscopy has been recognized as a valuable alternative for characterization of fungi[13].

Infrared spectroscopy is a technique based on the measurement of fundamental molecular vibrational modes[14]. Fourier transform infrared (FT-IR) spectroscopy is generally used to determine the chemical composition of organic compounds. The technique relies on the fact that infrared radiation is absorbed by molecular bonds such as C–H, O–H, N–H, C═O and C–C. The energy absorbed by the sample results in bending, stretching, and twisting of the bonds leading to characteristic transmittance and reflectance patterns[15-17]. The result is presented in form of an FT-IR spectrum and each spectral band is characterized by its frequency and intensity, and the overall spectrum represents "the molecular fingerprint" of the molecules[18, 19]. These spectral profiles give information about important macromolecules present in cells, such as proteins, lipids, nucleic acids and carbohydrates[14].

Infrared spectroscopy has already been used by several authors in the identification of microorganisms, highlighting the applications of this technique[13, 20-23]. However, methods of sample preparation are time consuming and elaborate[12, 14, 24]. Thus, our goal was to optimize a traditional technique of sample preparation, making it faster and easier and also to develop a satisfactory chemometric model for a correct identification of *Aspergillus* species.

MATERIALS AND METHODS

Biological material

Forty fungi isolates obtained from the surface of coffee beans from Londrina, PR, (2011 harvest) were analyzed. The isolates are maintained in the culture collection of LabMicro (Laboratory of Microbiology and Molecular Biology, Federal University of Paraná, Paraná, Brazil). Microscopic and macroscopic characteristics were observed[9, 25, 26]and the isolates were identified to genus level.

Four standard *Aspergillus* strains, all producers of OTA, were obtained: *Aspergillusochraceus* 40013 (ATCC 22947) from National Institute of Health Quality Control, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. *Aspergillusniger* UEL 1, *Aspergilluscarbonarius* UEL 187 and *Aspergilluswesterdijkiae* UEL 91 were kindly provided by the Laboratory of Molecular Biology at UEL (State University of Londrina), Paraná, Brazil.

Molecular identification

For confirmation of species, partial β-tubulin and calmodulin genes and internal transcribed spacer (ITS) region were sequenced using specific .

.primers. Primers Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') were used to amplify the β – tubulin gene; primers CL1 (5'-GARTWCAAGGAGGCCTTCTC-3') and CL2A (5'-TTTTGCATCATGAGTTGGAC-3') were used to amplify the calmodulin gene and primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS region[27, 28, 29].

Sample preparation for FT-IR spectroscopy

For spectroscopic analyses, strains validated by sequencing were chosen. The strains were cultivated in SDA medium (40g/L glucose, 10g/L peptone and 10g/L agar) for 4 days, at 28° C in a BOD, in triplicate. The resulting mycelium was scraped off with a Kole cable and transferred to Eppendorf tubes.

FT-IR spectroscopy

Immediately after removal, mycelia were blended with dried KBr powder in a 1:100 ratio (w/w) and pressed into tablets with a hydraulic press (CrushIR, PIKE Technologies, USA). Spectra were recorded in transmittance mode at 64 scans, spectral resolution of 4 cm^{-1}, and spectral range of 4000-400 cm^{-1}, using a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, USA).

Analysis of FT-IR spectra

Chemometric analysis was performed with The Unscrambler software (version 9.7, Camo Software AS, Oslo, Norway). The FT-IR spectra were truncated to the region 4000-750cm^{-1}, for removal of interferents; this region included most of the biochemical information[14]. The region 2393-2283 cm^{-1}, relating to CO_2

contamination, was removed. KBr was used as a background and KBr spectrum subtracted during the spectra manipulation.Several mathematical pre-processing methods were assessed and the best was selected (data not shown). Spectra were normalized and the primary derivative was applied to data, with Savitzky-Golay five-point smoothing followed by multiplicative scatter correction (MSC), to obtain good accuracy in the mathematical model.

In the present study, partial least squares (PLS) analysis was the classification method used. With the advantage that all data available can be used to determine the calibration model, the cross-validation method was adopted to evaluate the established models[30-32]. In cross-validation of a calibration model, one or more samples are removed from the set of calibration samples. Calibration models are then prepared with the remaining samples for different numbers of principal components, with the prediction of sample removal determined for each model. This process is repeated until all the samples are assigned to correct groups[33]. Thus, all spectra from calibration models served for both calibration and internal validation.

Coefficient of determination for calibration (R^2), coefficient of determination for cross-validation (r^2), root mean square error of calibration (RMSEC) and root mean square error of cross-validation (RMSECV) were used to evaluate the PLS model parameters.

To prove that the mathematical model obtained could be used to predict the species in the study, external validation was performed with nine new fungal isolates. In this external validation, the mathematical model was used to predict which group of fungi each isolate belonged to. Spectra for the external validation were collected 15 days after the other.

RESULTS AND DISCUSSION

Morfological and molecular identification

Among 40 *Aspergillus* isolates, 30 species were identified as belonging to section *Nigri,* being29 *A. niger* and 1 *A. carbonarius;* ten isolates from section*Circumdati*were identified as *A. westerdijkiae*.

Twelve selected strains (Table 1) and the standard strains were used to evaluate the accuracy of FT-MIR spectroscopy transmittance methodology to discriminate *A. niger, A. carbonarius, A. ochraceus* and *A. westerdijkiae.* Due to the unequal number of strains from the species of interest, repetitions of cultures were performed to ensure that each group had at least four representatives. A uniform number of samples from each group is necessary, to ensure that the mathematical model can satisfactorily represent each group.

Upon learning of the role of *A. ochraceus* in OTA production, we decided to perform repetitions of the standard strain of *A. ochraceus*, because no isolates of this species had been found.

FT-IR analysis: sample preparation

The advantages of FT-IR spectroscopy for the discrimination and identification of fungi are well known, the technique is non-destructive, rapid and does not require the use of chemical reagents[34,35].

Several methods of sample preparation for the discrimination and classification of fungi by FT-IR spectroscopy have been described. However, many of them are time consuming and difficult to perform[14, 24]. Therefore, in addition to developing a model to differentiate fungi samples correctly, we sought a rapid, easy and reliable sample preparation method. Our simplified preparation method consist of scraping mycelia from solid medium after 4 days of growth and grinding the mycelia into a powder.

Powdered mycelia is a smooth and homogeneous mixture that allows a representative reading of the entire organism, rather than a heterogeneous mixture of larger and chemically distinct structures such as hyphae[36]. Fungal mycelia have a more specific and complex biochemical composition than spores, which are metabolically inactive[14].

Tralamazza et al.[12], also used powdered mycelia and obtained very satisfactory results, correctly identifying 100% of the tested isolates to discriminate *A. niger* from *A. ochraceus* and *A. westerdijkiae*; however, their sample preparation method required more steps, including lyophilization and grinding with liquid nitrogen.

Naumann[36], developed a technique for sample preparation using powdered mycelia, however, subjected to FTIR attenuated total reflection (ATR) measurements. By internal validation, the method classified 99% of the spectra correctly. The author chose ATR spectroscopy because it is a fast and easy method for powders, where the sample is simply laced onto the ATR crystal, while weighing and mixing small amounts of sample powder with KBr powder, to prepare KBr pellets, is an elaborate procedure.

Meanwhile, the use of KBr pellets eliminates the need for further sample preparation; hence, the variability that might arise in sample preparation is avoided and scattering effects, potentially significant in transmission and reflectance measurements, are minimized[37].

FTIR analysis: spectrum analysis

Spectra were screened for ranges that showed differences among the tested species (*A. niger*, *A. carbonarius*, *A. ochraceus* and *A. westerdijkiae)*. Spectra were normalized and the infrared absorption bands shown in Figure 1 are typical of the biological samples.

The spectra of *A. niger* and *A. carbonarius* are closer to each other than to the other spectra, likewise, the spectra of *A. ochraceus*and *A. westerdijkiae* are highly similar (Figure1). The standard spectrum reflects the morphological and physiological analyses because *A. niger* and *A. carbonarius*both belong to section *Nigri*, which comprises fungi characterized by black or nearly black conidia[38]. *Aspergillusochraceus*and *A. westerdijkiae* belong to section *Circumdati,* comprising fungi of the genus *Aspergillus* with conidia ranging from yellow to ocher[11].

It is possible to roughly distinguish regions with different band patterns known to be characteristic of certain chemical compounds: polysaccharides (1185-900cm^{-1}); proteins, lipids, and phosphate compounds (1485-1185 cm^{-1}); amides (1800-1485 cm^{-1}); and lipids (2996-2800 cm^{-1})[12,39].

All species showed a peak at 3660 cm^{-1} (Figure1), caused by associated water molecules, *A. ochraceus* and *A. westerdijkiae* had the highest peaks.

Aspergillusochraceus and *A. westerdijkiae* showed the highest peaks for the band at 1542 cm^{-1},designated “amide II” (Table 2). Further, there is a difference in the amplitude of this band between *A. niger* and *A. carbonarius*. The bands at 1400 and 1458 cm^{-1} are attributed to lipids. While these two peaks are distinct in the *A. ochraceus* and *A. westerdijkiae*spectra, *A. carbonarius*and *A. niger*show a single peak in this region.

Overall, all of the peaks related to proteins, lipids and water were stronger in *A. ochraceus* and *A. westerdijkiae*spectra.

FT-IR analysis: PLS

Pre-processing of FT-IR spectral data has become an integral part of chemometric modeling. The objective of pre-processing is to remove physical phenomena in the spectra to improve the subsequent multivariate regression, classification model, or exploratory analysis[40]. For example, normalization compensates for differences in absorption due to sample size variations or sample thickness[41]. The use of combinations of pre-processing methods is abundant in literature and, in principle; any sequence of pre-processing methods is possible[40].

Generally, first or second derivatives are used, but the decision regarding which one to apply is usually made by trial and error, because it depends on the kind of identification problem and the group of organisms being studied. A positive effect of calculating derivatives is the elimination of baseline shifts that may have been induced by nonspecific light scattering[41].

Several pre-processing methods, including MSC, attenuate the effect of light scattering. With particulate systems, it is generally assumed that the information removed from the spectra by the application of these empirical methods is essentially the manifestation of the underlying physics of light scattering, and there is no significant loss of chemical information. Thus, the performance of multivariate regression models is improved by estimating chemical information from the corrected spectra[42]. In this paper, normalization, first derivative with five-point smoothing and finally MSC were adopted, because this combination produced a more accurate final model than other types of pre-processing.

Figure 2 shows the score plot of the first two principal components (PCs) or latent variable of PLS, in the analysis of 22 spectra. It shows four groups (*A. carbonarius, A. niger, A, westerdijkiae* and *A. ochraceus*) in the two-dimensional space.

Groups 1 and 2 correspond to isolates from section *Nigri* and groups 3 and 4 consist of isolates from section *Circumdati*. We note that there is a wide separation between the two sections, showing that the model was able to discriminate between sections *Circumdati* and *Nigri*correctly.

The greatest proximity was observed in the isolates of groups 3 and 4, and there was no clear separation between these groups. Discrimination of *A. ochraceus* and *A. westerdijkiae* occurred only in 2004[9]; prior to that both species were considered as *A. ochraceus*. Group 2 (*A. niger*) is better defined than group 1 (*A. carbonarius*) and there is separation between these groups.

The performance of the PLS calibration models was evaluated by R^2, r^2, RMSEC and RMSECV. A good model should have a low RMSEC and RMSECV (with a small difference) and a high coefficient of determination (closer to 1)[43].

With three PCs, high R^2 (0.94) and r^2 (0.86) values and low RMSEC (0.23) and RMSECV (0.37) values were observed, showing that the model could correctly discriminate the tested groups. A larger number of PCs can be used to improve calibration of the mathematical model, albeit within a limit to avoid errors resulting from over-fitting.

Over-fitting is harmful because it incorporates not merely predictive features of data into the model, but also noise, which results in degraded model performance in the prediction stage. The consequences of over-fitting are likely to be much more severely disturbing for multivariate calibration models, and this is the very reason why component selection is regarded as a critical step in multivariate predictive modeling[44]. Regression methods with fewer PCs can provide better predictions of future observations. Ramadan et al.[45], to avoid over-fitting, also used three PCs.

Cuadradoet al.[46], proposed criteria for assessing results of models by using R^2 values. According to these authors a model shows excellent precision when R^2 values are 0.90 or higher; good precision when R^2 values are between 0.70 and 0.89; and good separation between low, medium, and high values when R^2 values are between 0.50 and 0.69.

To confirm the quality of the model, we performed an external validation; in which, we used the FT-IR spectra of nine samples as input, and obtained their classification using the PLS model. In this validation, the model correctly classified 88.88% of the samples (Table 3), with a prediction root mean square error of 0.35.

Discrimination of highly related species such as *A. niger* and *A. carbonarius* confirms the robustness of the model. However, the model did not correctly differentiate between *A. ochraceus* and *A. westerdijkiae*: one isolate from group 4 (*A. ochraceus*) was predicted as belonging to group 3 (*A. westerdijkiae*).

The section *Circumdati* is especially well known for its production of ochratoxin A. This toxin is named after the producer *A. ochraceus* and has raised much interest because of its role in the contamination of coffee. Interestingly, some *A. ochraceus* strains have now been re-identified as *A. westerdijkiae*; hence, many of the species reported as *A. ochraceus* may indeed be *A. westerdijkiae*[47].

This difficulty in differentiating *A. ochraceus* and *A. westerdijkiae* is also observed when morphological, physiological and molecular biological analyses are performed; hence, we can say that the FT-IR spectra contain no significant differences that help differentiate these two groups.

CONCLUSION

In this study, we developed a chemometric model on the basis of FT-MIR spectra transmittance that was able to correctly distinguish between highly related species such as *A. niger* and *A. carbonarius*. However, due to the chemical similarity between *A. ochraceus* and *A. westerdijkiae*, the differentiation of these two species was not possible in all cases.The adaptation of traditional method of sample preparation proved to be very fast, being completed in 4 days, and easy to apply, with few processing steps. However, we believe that further tests should be performed with a number of additional strains for accurate classification of fungal species.

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Table 1: Strains used in the FT-IR spectroscopy analysis and their GenBank accession numbers

Strains	Sequenced genes	GenBank accession No.	Identification
102 3 F1	ITS	KJ599601	*A. westerdijkiae*
107 3 F3	ITS	KJ599602	*A. westerdijkiae*
104 2 F4	ITS	KJ599603	*A. westerdijkiae*
165 1 B1	β-tubulin	KJ599623	*A. westerdijkiae*
189 1 A3	β-tubulin	KJ599618	*A. westerdijkiae*
144 3 K2	β-tubulin, calmodulin	KJ599604/KJ599576	*A. carbonarius*
177 2 F1	β-tubulin, calmodulin	KJ599619/KJ599597	*A. niger*
136 1 C1	β-tubulin, calmodulin	KJ599607/KJ599598	*A. niger*
132 3 L1	β-tubulin, calmodulin	KJ599618/KJ599599	*A. niger*
187 2 H1	β-tubulin, calmodulin	KJ599619/KJ599596	*A. niger*
149 2 G1	β-tubulin, calmodulin	KJ599605/KJ599594	*A. niger*
144 3 I1	β-tubulin, calmodulin	KJ599606/KJ599595	*A. niger*

Table 2: Characteristic infrared absorption frequencies typical of microorganisms and their biomolecular attribution[14, 19]

Frequency (cm^{-1})	Molecular bond	Biomolecular attribution
4000-3500	O—H	Hydroxyl groups
3200-2800	CH_2, CH_3	Lipids
	N—H	Proteins
1780-1700	C═O	Fatty acids
1695-1625	C═O, C—N	Proteins (amide I)
	N—H	
1560-1525	C—N	Proteins (amide II)
	N—H	
1480-1400	CH_3, CH_2	Lipids
	C═O	
1300-1200	P═O	Nucleic acids
1200-900	C—O—C, C—O, P═O, C—C/C—O	Ribose, glycogen, nucleic acids
900-700	C—H	Aromatic groups

Table 3: Prediction of groups of strains

Strain	Identification	Actual group	Predict group	Correspondence
1	*A. carbonarius*	1	1	Yes
7	*A. carbonarius*	1	1	Yes
2	*A. niger*	2	2	Yes
8	*A. niger*	2	2	Yes
16	*A. niger*	2	2	Yes
3	*A. westerdijkiae*	3	3	Yes
11	*A. westerdijkiae*	3	3	Yes
4	*A. ochraceus*	4	3	No
4R	*A. ochraceus*	4	4	Yes

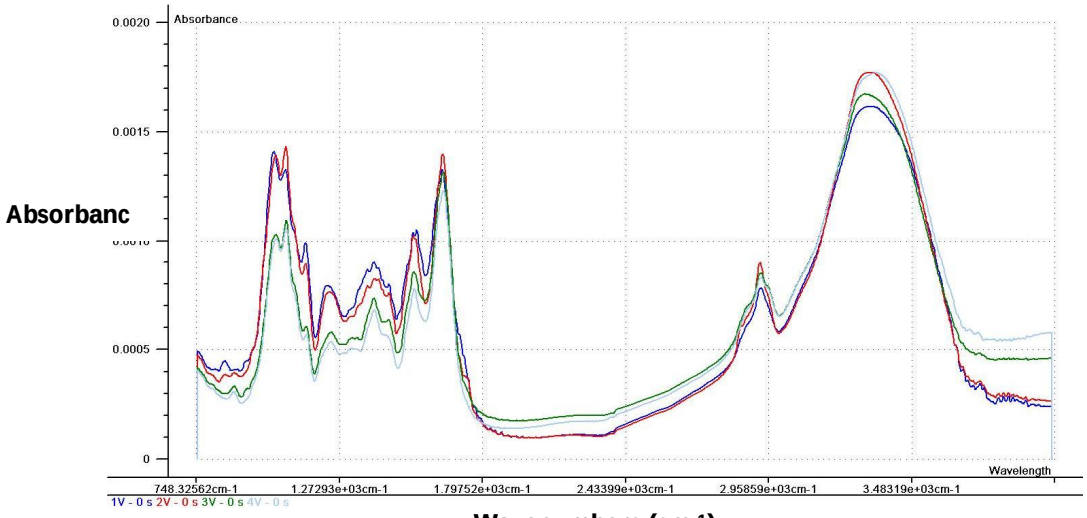

Fig. 1: General view (750-4000 cm-1) of mean absorption spectra normalized and smoothed

Legend: Dark blue – *A. carbonarius*; Red – *A. niger*; Green – *A. westerdijkiae*; Light blue – *A. ochraceus*.

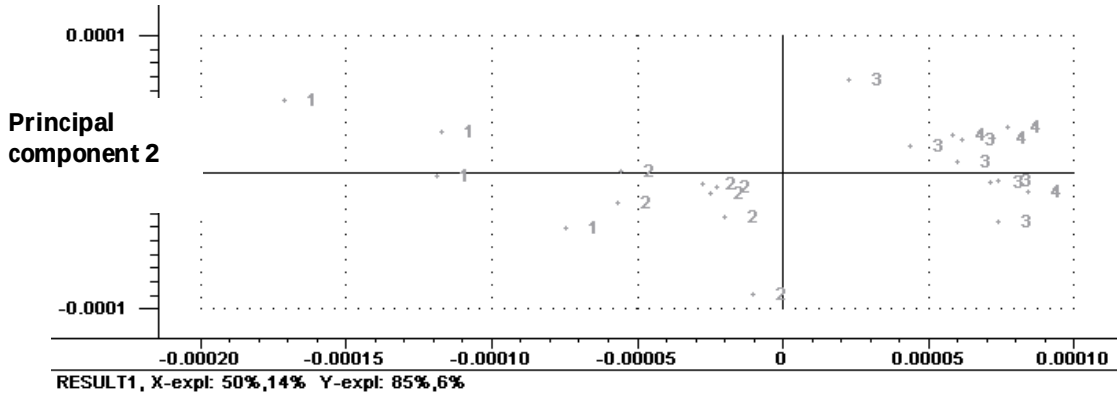

Fig. 2: Score plot of principal component 2 vs. principal component 1

Legend: Group 1 – *A. carbonarius*; Group 2 – *A. niger*; Group 3 – *A. westerdijkiae*; Group 4 – *A. ochraceus*.

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