Chapter 3

Discrimination of Fungi by MALDI-TOF Mass Spectrometry

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Traditionally, fungal identification has largely been based on the subjective micro- and macroscopic examination of morphological and culture characteristics. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) was used to generate reproducible mass spectral "fingerprints" for 76 fungal species, particularly from the medically important genera, *Penicillium* and *Aspergillus*. The mass spectra contain abundant mass signals and allow unambiguous discrimination between species. Species identification error rates were determined to be 0% and 1.4% using resubstitution and cross-validation methods, respectively. The ability of MALDI TOF MS to differentiate fungal strains was additionally examined for the aflatoxin producing species, *Aspergillus flavus*. Identification error rates for 40 tested *A. flavus* cultures from five unique strains were determined to be 0% and 5% using resubstitution and cross-validation methods, respectively. Analysis of dematiaceous (dark-pigmented) fungi has been observed to yield poor MALDI-TOF mass spectra. Results demonstrate this was due to the presence of melanin in the cell wall of fungal spores and hyphae. Strategies to overcome this limitation are presented. These results indicate that MALDI-TOF MS data may be a useful diagnostic tool and alternative to available immunodiagnostic and molecular

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methods for the objective identification of environmental, industrial and clinically important fungal species.

Introduction

Fungi are a diverse group of heterotrophic eukaryotes that disseminate a variety of bioaerosols into the environment. Fungal spores, hyphae, and fragments can be ubiquitous in indoor, outdoor, and occupational environments (*1–3*) and are among the most common bioaerosols that humans inhale (*2*). Fungal bioaerosols contain proteins, secondary metabolites, mycotoxins, β(1,3)-D-glucan, chitin, and volatile organic compounds (*2*, *4*, *5*) that may be a burden to public health, particularly in indoor and occupational contaminated environments (*1*, *5*). Personal exposure has been associated with exacerbations of adverse health effects ranging from allergic rhinitis, asthma, hypersensitivity pneumonitis, dermatitis, invasive aspergillosis, to death (*5–10*). Some dimorphic fungi may even act as invasive pathogens in patients that are immunocompetent (*11*). Viable and nonviable methodologies have been traditionally used to detect and quantify fungi associated with adverse health effects; however, many of these techniques have been confounded by bias and subjectivity (*2*). Due to the health and economic impacts associated with fungal exposure, a number of new molecular and proteomic technologies have been developed that have improved the identification and characterization of medically important fungi.

Traditional viable and non-viable methods of fungal identification rely on the subjective identification of micro- and macroscopic morphological culture and spore characteristics. As such, these evaluations rely on the taxonomic judgment of a trained mycologist and are therefore subject to observer bias. A number of studies have shown that morphological similarities exist between numerous genera, species and strains, and as a result, fungal misclassifications are a confounding variable associated with viable and nonviable analyses (*12–14*). Emerging immunodiagnostic and molecular technologies for rapid identification of microorganisms, including mass spectrometry-based techniques, particularly those based on matrix-assisted laser desorption/ionization (MALDI) (*15*, *16*) time-of-flight mass spectrometry (TOF MS) (*17*) are particularly promising. Mass spectrometry-based techniques have provided a rapid (MS analysis takes minutes) and sensitive alternative method for identifying as few as 10^3 fungal cells.

A number of studies have been presented in the literature on the analysis of intact cells by MALDI-TOF MS. Although significantly more attention has been paid to bacteria (*18–29*), recent studies have focused on the identification of medically important fungi (*30–36*). Of particular interest for rapid identification are so called "fingerprint" methods (*24*, *37*, *38*) that utilize pattern recognition to correlate an unknown mass spectrum with a known organism from a library of spectra.

Early experiments in our laboratory focused on utilizing MALDI-TOF MS data coupled with biostatistical analysis to discriminate between *Mycobacterium* species (*28*). Discrimination was possible at both the species and strain level (*29*). The results of our research and that of other groups (*26*) suggested that care must

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be taken to carefully define both the culture conditions and mass spectrometry parameters in order to achieve highly reproducible mass spectrometry fingerprints from microorganisms. Any changes to the experimental conditions may alter the appearance of the MALDI-TOF mass spectrum and affect the results of the identification. Among the variables to be considered in MALDI TOF analysis are culture time, nutrient media, microorganism concentration, and cell lysis. Similarly, mass spectrometry parameters such as choice of matrix, desorption laser fluence, and delayed extraction settings must be maintained. Consistency in experimental parameters is the key to reproducible fingerprint mass spectra.

Furthermore, because a biological specimen of interest may be a pathogen or aeroallergen, biosafety is of paramount concern. For this reason, our laboratory avoids "whole cell" methods where the microorganism of interest is deposited directly on the sample stage and introduced to the mass spectrometer. Rather, microorganisms are handled and extracted in a biological safety cabinet and extract preparations are subsequently analyzed. Early experiments in our laboratory demonstrated that the data acquired from extracts that mimic the solvent composition of the MALDI matrix solution produce high-quality results, similar to "whole cell" spectra (*28*). In addition, our previous experiments (*35*) demonstrated that for fungal analysis, a bead-beating cellular disruption step during the chemical extraction increases both the number of peaks and the signal-to-noise ratio observed in MALDI-TOF fingerprint mass spectra. Similar approaches to cell lysis, such as exposure to acid, ultrasonication, and corona plasma discharge, have been previously applied by other researchers with success (*30*).

In this study, we have applied MALDI-TOF MS fingerprinting methods to an extensive library of fungal isolates covering more than sixty-eight species in thirty-five genera to evaluate our methodology for fungal discrimination using a library covering a broad range of genera. Furthermore, we have previously observed that certain dematiaceous (dark-pigmented) fungi yield poor MALDI-TOF fingerprint mass spectra (*34*). Here we present data supporting the hypothesis that fungal melanins, which have both photo- and chemoprotective properties in fungi, have a suppressive effect on MALDI-TOF fingerprint mass spectra.

Experimental

Reagents

Angiotensin II (human), insulin oxidized B chain (bovine), cytochrome C (equine), albumin (bovine serum), α-cyano-4-hydroxycinnamic acid (CHCA) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Fairlawn, NJ). Malt extract agar was purchased from Difco (Sparks, MD). Tricyclazole (5-methyl-1,2,4-triazolo[3,4-*b*][1,3] benzothiazole) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Deionized water was produced by a Millipore Synthesis A-10 (Billerica, MA).

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Fungal Culture

Fungal Fingerprinting

Seventy-six fungal isolates from a variety of genera (Table I) were sub-cultured from NIOSH or American Type Culture Collection (ATCC, Manassas, VA) stock sources and grown for 14 days at 25 °C on malt extract agar (MEA). To ensure reproducibility, eight independent cultures were performed for each isolate, for a total of 608 individual fungal cultures. Conidia and hyphae from one culture plate (~10^8 cells) were transferred to 100 µL of 0.1 mm zirconium beads (Biospec, Bartlesville, OK) and 1 mL 50/50 acetonitrile/4% trifluoroacetic acid. The samples were subjected to three one-minute bead-beating cycles. The resulting solutions were centrifuged at 8,800 x g for 10 minutes and the supernatant taken for MALDI-TOF MS analysis.

Table I. List of fungal isolates cultured for MALDI analysis

Genus	*No. of Species/Strains*	*No. of cultures*
Acremonium	1	8
Alternaria	2	16
Aspergillus	18	144
Aureobasidium	1	8
Candida	1	8
Chaetomium	1	8
Cladosporium	3	24
Cochliobolus	1	8
Cryptococcus	1	8
Curvularia	1	8
Emericella	1	8
Epicoccum	1	8
Eurotium	2	16
Exserohilum	1	8
Fusarium	3	24
Geotrichum	1	8
Hansenula	1	8
Memnoniella	1	8
Mucor	1	8
Myrothecium	1	8

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Table I. (Continued). List of fungal isolates cultured for MALDI analysis

Genus	*No. of Species/Strains*	*No. of cultures*
Neosartorya	1	8
Paecilomyces	3	24
Penicillium	12	96
Pithomyces	1	8
Phoma	1	8
Rhizopus	2	16
Rhodotorula	1	8
Saccharomyces	1	8
Scopulariopsis	1	8
Stachybotrys	3	24
Stemphylium	1	8
Talaromyces	1	8
Trichoderma	2	16
Ulocladium	2	16
Wallemia	1	8
TOTAL	**76**	**608**

Suppression of Fungal Melanin Production in Culture

Eight replicate cultures of *Aspergillus niger* were grown for 7 days at 25 °C on malt extract agar and malt extract agar supplemented with 1% ethanol containing 50 µg/mL tricyclazole. Conidia and hyphae from one culture plate (~10^8 cells) were transferred to 100 µL of 0.1 mm zirconium beads (Biospec, Bartlesville, OK) and 1 mL of 50/50 acetonitrile/4% trifluoroacetic acid. Samples were subjected to three one-minute bead-beating cycles. The resulting solutions were centrifuged at 8,800 x g for 10 minutes and the supernatant taken for MALDI-TOF MS analysis.

Preparation of Melanin "Ghosts"

Melanin "ghosts" were produced from *Aspergillus niger* according to the method published by Wang, et al. (*39*). In brief, *A. niger* was grown on malt extract agar for ten days at 25 °C. Sporulating fungal cultures were harvested from the culture plate using sterilized deionized water (DI). The resulting suspension was centrifuged at 1000 x g for ten minutes, and the supernatant discarded. The pellet was resuspended in sodium citrate buffer containing 10 mg/mL of cell lysing enzymes from *T. harzianum* and 2 mg/mL cellulase from *T. reesei* and incubated overnight with agitation at 30 °C. The sample was then centrifuged at

1000 x g for ten minutes and the supernatant discarded. The sample was washed with phosphate buffered saline (PBS) and centrifuged for ten minutes at 1000 x g. The pellet was resuspended in 4 M guanidine isothiocyanate and incubated at room temperature over night with agitation. The sample was washed with PBS as described above and the pellet resuspended in 1 mg/mL of proteinase K in DI and incubated overnight at 37°C with agitation. The sample was washed with PBS and the pellet resuspended in 6 M HCl and boiled for one hour. The sample was then washed with PBS a final time and resuspended in PBS. The resulting final melanin extract was dialyzed against DI H_2O for ten days at 4°C using 3500 Da molecular weight cutoff (MWCO) membrane tubing (Spectra/Por® , Laguna Hills, CA, USA) . The sample was then lyophilized and the resulting powder was used for further experiments.

The *A. niger* melanin ghosts were diluted with 50/50 ACN/DI and mixed with an equal volume of 0.5 mg/mL HSA. The final concentration of melanin ghosts was approximately 0.1 mg/mL. The resulting solution was mixed with an equal volume of 10 mg/mL α-cyano-4-hydroxycinnamic acid and 1 μL aliquots deposited on a gold sample stage (Bio-Rad, Hercules, CA, USA) and allowed to air dry prior to MALDI analysis.

Mass Spectrometry

MALDI-TOF MS samples were prepared by mixing supernatant 1:1 with 10 mg/mL α-cyano-4-hydroxycinnamic acid in 50/50 acetonitrile/0.1% trifluoroacetic acid. 1 μL of the resulting solution was deposited on a gold sample stage (Bio-Rad, Hercules, CA) and allowed to air dry. Each sample was analyzed in duplicate, for a total of sixteen composite MALDI-TOF mass spectra per isolate. MALDI-TOF mass spectra were acquired using a Ciphergen PBS-IIc linear time-of-flight mass spectrometer (Bio-Rad, Hercules, CA) with a flight path of 0.8 m, capable of mass resolution ($m/\Delta m$) of 500-1000 and a mass accuracy of ± 1000 ppm. Spectra were acquired over the m/z range 0-100 kDa, with the delayed extraction parameters set to optimally focus the 10-20 kDa range. Composite mass spectra are the average of 100 laser shots taken from 20 distinct positions across the sample deposit. These positions were held constant for all samples. N_2 laser (337-nm) intensity was maintained just above the threshold for ion production (laser step 140-160). Mass spectra were externally calibrated using a set of peptide and protein calibrants that covered the range of 1-66 kDa.

Data Analysis

Initial data analysis was performed using the Biomarker Wizard (Bio-Rad, Hercules, CA) software suite. Spectra were baseline corrected and normalized to total ion current. "Clusters" of peaks common to a given isolate were generated by selecting all peaks with signal-to-noise (S/N) greater than 5 that occurred in each spectrum from that isolate. The mass tolerance for each cluster was set to 0.3% of the m/z. Linear discriminant functions were analyzed using SAS/STAT software, Version 9.1 of the SAS system for Windows (SAS Institute, Cary, NC). The intensity values were first tested for their distribution, and were found to be

log-normal, so a natural log transformation was utilized on the intensity values. A stepwise variable selection method using the "PROC STEPDISC" procedure, which selects a subset of the variables of interest using a stepwise discriminant analysis, keeping the most significant variables from iterative F-tests, was then performed to select a subset of variables that could serve as predictor variables for class membership. Using this new subset of significant peaks, "PROC DISCRIM" was utilized to determine the classification error rate. This was done by calculating a discriminant function that allowed each data point to be compared to all others for cross-validation classification. This process was performed iteratively for each data point individually. This procedure was followed by the "PROC CANDISC" procedure, a procedure that reduces the number of dimensions to find linear combinations of the variable set that also summarizes between-class variation, to perform a canonical discriminant analysis on that subset of variables. This creates new variables by taking linear combinations of the original variables and aids in determining the true underlying dimension of the data space. The canonical functions generated allow the calculation of canonical scores, which can be used to discriminate among the various isolates. Using the same subsets of variables, cluster analysis was then performed using the "PROC CLUSTER" procedure, which uses distances between data points to form hierarchical clusters, and a dendrogram was generated using the "PROC TREE" procedure, a procedure that uses the data set from "PROC CLUSTER" to produce a dendrogram.

Results and Discussion

Fungal Fingerprinting

Seventy-six fungal isolates from thirty-five genera were independently cultured in order to perform MALDI-TOF MS fingerprinting (Table I). Included in this library were twelve species derived from the genera *Penicillium* and *Aspergillus*, as well as five unique strains of the aflatoxin producing species, *A. flavus*. Six hundred and eight independent cultures were analyzed in duplicate by MALDI-TOF MS, resulting in a database of 1216 individual mass spectra. Representative spectra from five species are presented in Figure 1. In general, fingerprint mass spectra from fungi were characterized by several abundant signals in the region of 5-25 kDa, however, several fungi exhibited signals as high in *m/z* as 40-45 kDa. Many fungi exhibited a strong peak at approximately 8.5 kDa, a peak which has been tentatively identified as ubiquitin, an abundant protein that has been suggested to be a biomarker for the eukaryotic kingdom (*30*).

Initial statistical analysis of the complete database of fungal fingerprint mass spectra identified 1422 peaks of S/N greater than 5. The "STEPDISC" stepwise variable selection method within the SAS/STAT software identified a subset of 181 significant peaks to utilize for discrimination within the dataset. Both resubstitution and cross-validation methods were used to test how well this subset of 181 peaks could discriminate between the 68 species present in the database. The resubstitution method resulted in a 0% error rate, whereas the cross-validation method resulted in a 1.47% error rate. In resubstitution, the discriminant function is fitted to the dataset and then applied to each observation.

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In contrast, cross-validation deletes the observation, fits the discriminant function to the remaining dataset, and then applies the function to the deleted observation. Resubstitution tends to underestimate classification error, whereas cross-validation is unbiased and preferred for large datasets (*40*). The ability to match an acquired fingerprint mass spectrum to one of 68 species in the database with 0-1.5% error underscores the utility of the methodology for fungal identification.

In order to further illustrate the utility of this methodology for species discrimination, several subsets of the full database were queried in more detail. If just the subset of the database corresponding to the species within the genus *Aspergillus* is examined, discrimination on the basis of the raw statistical data (S/N > 5) provides an error rate of 0 or 18% (resubstitution and cross-validation, respectively), however, when just the significant peaks are utilized, both methods return 0% error rates for classification. The discriminatory power of this method can be illustrated by plotting canonical variables for this subset of the database. Figure 2 presents a three-dimensional plot of the first three canonical variables for each species of *Aspergillus*.

The three canonical variables for each independent culture shown in Figure 2 are plotted individually and represented by a circle. There are eight observations for each species, one for each individual culture. In each case, the eight observations cluster tightly together in three-dimensional space and are spatially resolved from the other *Aspergillus* species. These data demonstrate that the mass-abundance data derived from the MALDI-TOF MS fingerprint spectrum of each independent culture are highly reproducible and distinct from other similar species of the same genera.

Similarly, the error rates for species classification were calculated on the subset of the database comprised of twelve species belonging to the genus, *Penicillium*. In this case, both resubstitution and cross-validation methods returned 0% error rates for species identification within the *Penicillium* subset using both the raw MALDI-TOF MS data (S/N > 5) and the stepwise-selected significant variables. The three-dimensional canonical discriminant data for the twelve species of *Penicillium* are presented in Figure 3. Similar to the *Aspergillus* data presented in Figure 2, the eight independent cultures of each species of *Penicillium* cluster tightly together and are distinct from other species within the genus. It should be noted that although a few clusters in Figure 3 appear not to be resolved, this figure utilizes only three canonical variables for ease of visualization. More canonical variables exist and can be used to unambiguously discriminate these species.

In addition to discrimination of species within a genus, the MALDI-TOF MS fingerprint method may be used to discriminate between strains of the same species. Five strains of *Aspergillus flavus* were independently cultured and analyzed by MALDI-TOF fingerprint analysis. Although the MALDI-TOF fingerprint mass spectra for the five species were very similar in terms of observed *m/z* (*34*), reproducible variations in the relative abundance of observed *m/z* ratios allows unambiguous discrimination. Figure 4 presents the three-dimensional canonical discriminant data for the *A. flavus* subset of the database. It is important to note that this discrimination is possible based solely on the differential

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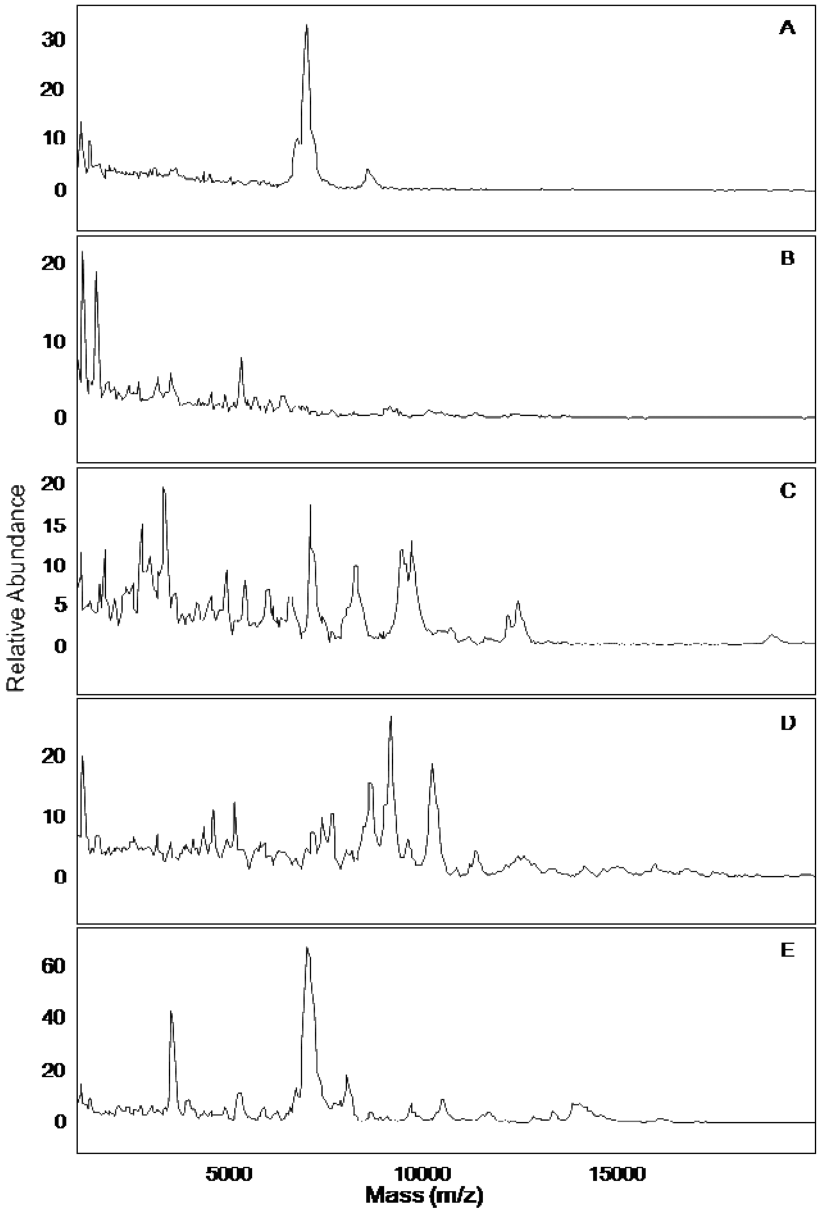

Figure 1. MALDI-TOF MS fingerprint spectra of selected fungi from the dataset. (A) C. herbarum; (B) A. alternata; (C) P. variotii; (D) A. brassicola; (E) C. albicans.

expression of shared *m/z*, rather than a difference in observed m/z as is the case when differentiating between species/genera. Resubstitution and cross-validation methods produce error rates for strain identification of 0 and 5% respectively.

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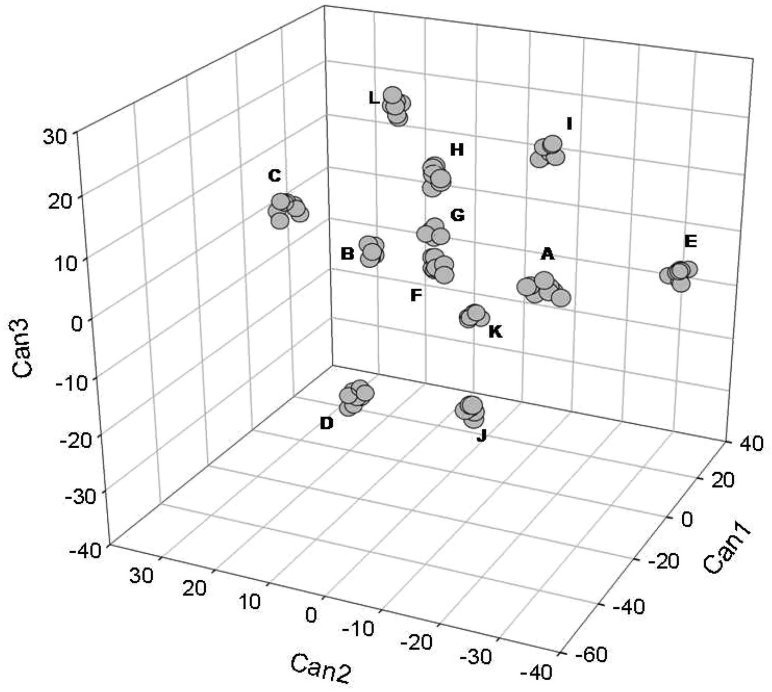

Figure 2. Three-dimensional canonical discriminant plot for MALDI-TOF MS data derived from Aspergillus species from the whole fungal dataset. (A) A. candidus; (B) A. chevalieri; (C) A. flavus; (D) A. fumigatus; (E) A. nidulans; (F) A. niger; (G) A. parasiticus; (H) A. repens; (I) A. sydowii; (J) A. terreus; (K) A. ustus; (L) A. versicolor.

Suppression of MALDI-TOF MS Signal by Fungal Melanin

While constructing the library of MALDI-TOF MS fingerprint mass spectra from 76 fungal isolates, it was noted that certain species yielded very poor fingerprint spectra in which very few or no peaks were observed. Examination of the database demonstrated that this phenomenon was unique to dematiaceous fungal species, including *A. niger* and *Stachybotrys chartarum*. Dematiaceous fungi are of particular interest from an occupational safety and public health standpoint as they are common contaminants of water damaged cellulose-based building materials. Examination of the literature indicated that previous attempts by other laboratories to produce MALDI-TOF MS spectra from *A. niger* had also been unsuccessful. Valentine and coworkers attempted to fingerprint *A. niger* in 2002 and stated in their manuscript, "*The analysis of some of the fungal samples, e.g., A. niger, were particularly challenging. A. niger was difficult to analyze by MALDI under all circumstances including pretreatment. Biomarkers were not easily detected for this fungal species… Further efforts to analyze this fungal species are needed.*" (*32*).

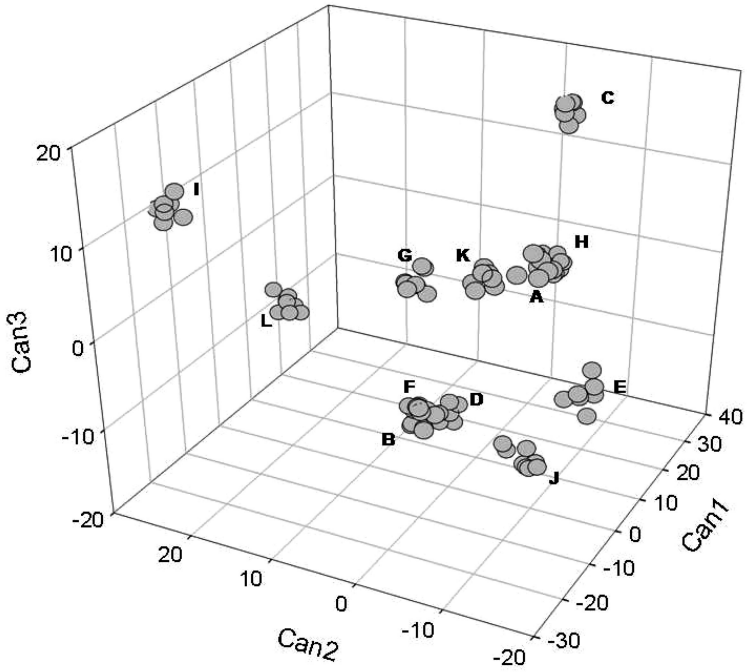

Figure 3. Three-dimensional canonical discriminant plot for MALDI-TOF MS data derived from Penicillium species from the whole fungal dataset. (A) P. aurantiogriseum; (B) P. brevicompactum; (C) P. citrinum; (D) P. chrysogenum; (E) P. expansum; (F) P. fellutanum; (G) P. jensenii; (H) P. melinii; (I) P. purpurogenum; (J) P. roqueforti; (K) P. simplicissimum; (L) P. variable.

Dematiaceous fungi contain eumelanins, a class of high molecular weight polymers of dihydroxynapthalene or dihydroxyphenylalanine, as a component of their cell wall (*41*). Melanins help provide stability and strength to the spore wall. Melanins are dark pigments that have photo- and chemo-protective properties (*42*, *43*) and may contribute to the virulence of several pathogenic fungi (*44*). Melanin absorbs UV radiation and dissipates the energy through ultrafast internal conversion. Based on this information, we hypothesized that fungal melanin in the cell extracts of dematiaceous fungi was interfering with the MALDI desorption/ionization process (*45*).

The exact chemical makeup of the pigment present in *A. niger* is still a matter of active research. Some investigators have dubbed this pigment "aspergillin" and suggested it is a polymer of high-molecular weight melanins (*46*). For the purposes of this discussion we shall refer to the black pigment isolated from *A. niger* using the generic term "melanin." In the present investigation, black pigment was purified from *A. niger* using a series of protease and strong acid digestions to remove all proteinaceous material. These treatments left behind structural melanin "ghosts". Addition of purified melanin to pure protein and peptide standards resulted in strong suppression of the MALDI-TOF MS $[M+H]^+$ signals

for those standards in all cases. This observation strengthened the hypothesis that fungal melanin from *A. niger* suppressed MALDI ionization (*45*). We further demonstrate that blocking *A. niger* melanin synthesis by adding tricyclazole, a polyketide melanin pathway inhibitor, to the culture media (*47*) results in dramatically improved MALDI-TOF MS fingerprint mass spectra (Figure 5). Figure 5A presents the MALDI-TOF MS fingerprint mass spectrum of *A. niger* under conventional (and melanin producing) conditions. The MALDI-TOF MS fingerprint mass spectrum of melanin-deficient *A. niger* (Figure 5B), in contrast, yields a high number of very abundant *m/z* signals that may be used for discrimination.

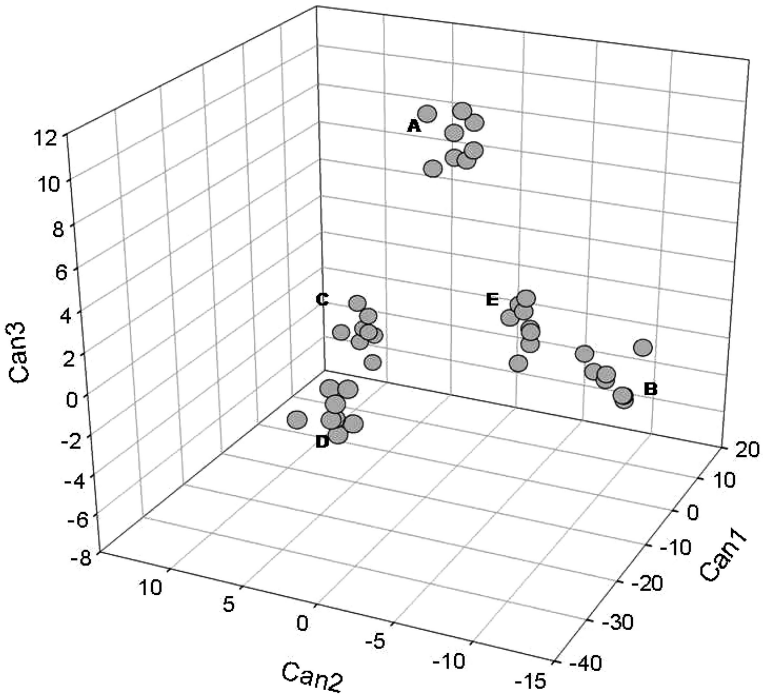

Figure 4. Three-dimensional canonical discriminant plot for MALDI-TOF MS data derived from five strains of Aspergillus flavus. (A) A. flavus NIOSH 15224; (B) A. flavus NIOSH 15417; (C) A. flavus ATCC 16883; (D) A. flavus NIOSH 34689; (E) A. flavus NIOSH PRC86N.

Conclusions

MALDI-TOF MS was used to generate a database of highly reproducible mass spectral "fingerprints" from 76 fungal isolates from 68 different species. Canonical discriminant analysis performed on the MALDI-TOF MS dataset was used to identify each species and/or strain with 98.5-100% accuracy, indicating that the methodology may be utilized for objective identification of fungi that complements traditional subjective identification techniques based on observation

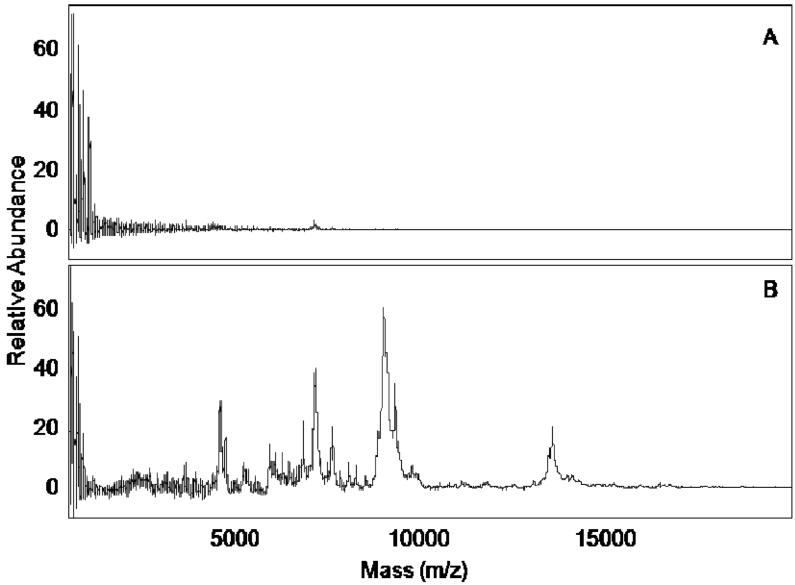

Figure 5. MALDI-TOF MS fingerprint mass spectrum of the dematiaceous fungus Aspergillus niger (A) grown on malt extract agar (B) grown on malt extract agar supplemented with 1% ethanol containing 50 µg/mL tricyclazole.

of colony morphology. In addition, we have confirmed that the presence of fungal melanins in the MALDI sample deposit results in significant suppression of the MALDI process, and consequently yields poor TOF mass spectra. The limitation of poor fingerprint mass spectra produced from dematiaceous fungi may be circumvented by judicious choice of culture conditions which prohibit melanin formation, such as using the polyketide melanin pathway inhibitor, tricyclazole. Blocking melanin formation allows high quality MALDI-TOF MS fingerprint data to be acquired from dematiaceous fungi such as *A. niger*.

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