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The Effects of Fungal Pigments on Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectral Peak Formation

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The Effects of Fungal Pigments on Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass
Spectral Peak Formation

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Thesis submitted to the
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at West Virginia University
in partial fulfillment of the requirements
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in
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ABSTRACT

The Effects of Fungal Pigments on Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectral Peak Formation

Amanda Dawn Buskirk

Fungi are ubiquitous microorganisms that commonly cause diseases with high mortality rates in immunocompromised hosts. In order to successfully treat the infection and subsequently decrease mortality rates, it is necessary to rapidly identify the causal organism(s) and promptly begin the proper treatment. Common identification methods, such as viable culture characterization, are often time consuming or not species-specific, as is the case with many molecular or immunodiagnostic assays. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used to discriminate between many clinically relevant microorganisms. Recently, MALDI-TOF MS has been used to discriminate between monilaceous fungal species, however, darkly pigmented fungi yield poor mass spectra with only a few peaks of low relative abundance. Darkly pigmented fungi are of interest because they are common indoor contaminants, and frequently a cause of disease in immunocompromised patients. In this study, the effect of dark fungal pigments on the ionization of standard peptide and protein solutions during MALDI-TOF MS is investigated. Solutions of human serum albumin and angiotensin II were spiked with varying concentrations of both synthetic melanin and fungal pigments extracted from *Aspergillus niger*, *A. fumigatus*, and *A. terreus*. Serum albumin and angiotensin II $[M+H]^+$ signals were suppressed in a concentration dependent manner. Examination of the MALDI sample deposit under magnification showed significant heterogeneity, with regions of highly concentrated pigment appearing black. MALDI-TOF mass spectra acquired from darkly pigmented regions of the sample deposit yielded poor or no $[M+H]^+$ ion signal from the standard. In contrast, non-pigmented regions within the sample deposit and liquid hyphal negative control extracts of *A. niger*, *A. fumigatus*, and *A. terreus* produced no such inhibition. Gas chromatography analysis of each fungal pigment extract revealed the presence of putative cell wall components including aliphatic chains and fatty acids. Field emission scanning electron microscopy (FESEM) showed *A. niger* and *A. fumigatus* extracts retain original spore morphology, while the *A. terreus* extract was homogeneous and morphologically similar to synthetic melanin. This study demonstrates that synthetic and fungal pigmentation derived from darkly pigmented fungi inhibits the desorption/ionization process during MALDI-TOF MS, however these fungi may be successfully analyzed by MALDI-TOF MS when culture methods that suppress pigment expression are employed.

To my dad, who's constant love, support, and encouragement has carried me further than he will ever know.

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TABLE OF CONTENTS

ABSTRACT	ii
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF ABBREVIATIONS	viii
CHAPTER I: GENERAL INTRODUCTION/LITERATURE REVIEW	1
<i>Overview of Fungi</i>	1
<i>Methods of Fungal Identification</i>	3
<i>MALDI-TOF MS</i>	6
<i>Microbial Discrimination/Identification using MALDI-TOF MS</i>	7
<i>Limitations of MALDI-TOF MS Microbial Fingerprinting</i>	11
<i>MALDI-TOF MS Fingerprinting of Fungi</i>	12
<i>Fungal Pigmentation</i>	13
<i>Melanin Production</i>	15
CHAPTER II: MATERIALS AND METHODS	18
<i>Reagents</i>	18
<i>Fungal Culture</i>	18
<i>Preparation of Fungal Culture Samples for MALDI-TOF MS Analysis</i>	19
<i>Preparation of Synthetic Melanin Samples for MALDI-TOF MS Analysis</i>	19
<i>Isolation of Melanin Ghosts</i>	19
<i>UV Spectroscopy</i>	20
<i>Gas Chromatography/Mass Spectrometry</i>	21
<i>Field Emission Scanning Electron Microscopy</i>	21
<i>MALDI-TOF MS Analysis</i>	21
<i>Statistical Analysis</i>	22
CHAPTER III: RESULTS	23
<i>MALDI-TOF MS Suppression by Darkly Pigmented Fungi</i>	23
<i>Synthetic Melanin Suppression of MALDI-TOF MS</i>	24
<i>Characterization of Isolated Fungal Pigments</i>	26
<i>Suppression of MALDI-TOF MS by Isolated Fungal Pigments</i>	29
<i>Fungal Culture Conditions Effect Melanin Production</i>	35
<i>Inhibition of Melanin Production via Tricyclazole</i>	37
CHAPTER IV: DISCUSSION	42
REFERENCES	47
CURRICULUM VITAE	52

LIST OF TABLES

Table	Page
1. <i>TABLE 1: Fungal Pigments</i>	15

LIST OF FIGURES

Figure	Page
1. DHN-melanin production pathway.....	17
2. Fingerprint spectra of monilaceous fungi.....	23
3. Fingerprint spectra of darkly pigmented fungi.....	24
4. Spectra of HSA spiked with synthetic melanin.....	25
5. Fingerprints of <i>Aspergillus spp</i>	26
6. FESEM images of pigment extracts.....	27
7. GC/MS spectra of synthetic melanin and <i>A. niger</i>	28
8. UV/VIS spectrum of <i>A. niger</i>	29
9. Spectra of HSA spiked with <i>A. niger</i> pigment extract.....	30
10. Spectra of HSA spiked with <i>A. fumigatus</i> pigment extract.....	31
11. Spectra of HSA spiked with <i>A. terreus</i> pigment extract.....	32
12. Graph of effect of fungal pigments on HSA peak height.....	33
13. Light microscopy images of MALDI target spot deposition.....	34
14. MALDI Q-TOF mass spectra of angiotensin II spiked with <i>A. fumigatus</i> pigment extract.....	35
15. Fingerprints of <i>Aspergillus spp</i> grown in submerged cultures.....	36
16. 2D canonical distribution of fungi grown in submerged cultures.....	37
17. Appearance of cultures grown on MEA and MEA with tricyclazole.....	38
18. Fingerprints of <i>A. niger</i> 7 days vs. 14 days, MEA vs. MEA with tricyclazole.....	39
19. Fingerprints of <i>A. fumigatus</i> 7 days vs. 14 days, MEA vs. MEA with tricyclazole.....	40
20. Fingerprints of <i>A. terreus</i> 7 days vs. 14 days, MEA vs. MEA with tricyclazole.....	41

LIST OF ABBREVIATIONS

ACN	Acetonitrile
CHCA	α -cyano-4-hydroxycinnamic acid
CSF	Cerebral spinal fluid
CT	Computer tomography
DDI	Deionized water
DHN	Dihydroxynapthalene
DOPA	Dihydroxyphenylalanine
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FESEM	Field emission scanning electron microscopy
GC/MS	Gas chromatography/Mass spectrometry
HSA	Human serum albumin
IA	Invasive aspergillosis
ITS	Internal transcribed spacer
MALDI	Matrix-assisted laser/desorption ionization
MEA	Malt extract agar
MEB	Malt extract broth
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MRI	Magnetic resonance imaging
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MS	Mass spectrometry
MWCO	Molecular weight cutoff
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
Q-TOF MS	Quadrupole time-of-flight mass spectrometry
TCA	The Citric Acid Cycle
TFA	Trifluoroacetic acid
TOF	Time-of-flight
UV	Ultraviolet
VIS	Visible

Chapter I

General Introduction/Literature Review

Overview of Fungi

Fungi are a diverse kingdom of spore-forming microorganisms. Fungal spores emerge from the reproductive structures (e.g. hyphae and conidial head) of germinating fungi (1). These spores are released into the environment upon environmental disturbances including wind, vibrations, humidity, and water exposures (2). Because of their small size and aerodynamic nature, these spores may be carried for long distances in the wind current until they deposit onto a suitable substrate under appropriate environmental conditions. These substrates include nutrients such as carbohydrates, sugars, and high water content, with environmental temperatures commonly within the range of 25-37°C. This means that fungi can grow on many surfaces especially in indoor environments that are prone to water infiltration, contain cellulose-based building materials, and lack adequate ventilation (1, 2).

Because of the ability to easily propagate large cultures of fungi, as well as the ease in manipulating the culture conditions to produce desirable products, many fungal products have been utilized in industry, agriculture, and pharmaceuticals. *Saccharomyces cerevisiae*, an ascomycetes yeast, has been used in the fermentation process to produce alcohol, yogurt, and bread (3). Several fungi, such as members of the genera *Aspergillus*, *Chaetomium*, and most commonly known *Penicillium*, are used to produce antimicrobial drugs from metabolites and toxins produced during fungal growth (4). Furthermore, mycotoxins from the fungi *Aspergillus terreus* and *Penicillium citrinum* have been used to produce lovastatin and compactin, respectively, which are drugs used to lower cholesterol in patients (5).

Despite the many beneficial uses of fungi, personal exposure to spores, hyphae, and fungal fragments can result in adverse health effects such as induction and exacerbation of allergy and asthma, as well as causing mycoses or toxicoses in immunocompromised patients (6-9). Eduard et al. reported that the total number of spores within contaminated buildings may exceed levels of 10^7 spores/m³, and those spores are commonly inhaled by humans during natural and occupational fungal exposures (2, 10). Health

effects occurring in patients following fungal exposures range from fatigue, respiratory symptoms, gastrointestinal problems, as well as neurocognitive symptoms, dizziness, and vision problems (11). It has been estimated that as much as 10% of the U.S. population has allergy to common fungal agents (11). In addition, different climatic regions of the world support varying assemblages of fungal species and the concentration of these fungi are dependent upon the season (12). In 2002, it was estimated that in the U.S. alone, 10,000 lawsuits related to mold in households were pending in the court systems. Insurance companies had paid out over 2 billion dollars in Texas alone due to mold related claims, illustrating the economic impact of unwanted fungal growth (11).

To date, research efforts have focused on understanding the health effects associated with fungal exposures, such as allergic sensitization, toxicoses, invasive infections, and exacerbation of existing pulmonary conditions such as allergy and asthma (1, 6-9, 13, 14). Immunocompetent individuals rapidly clear inhaled fungal spores and fragments with no damage to the lungs (15). However, in immunosuppressed, neutropenic individuals, fungi can begin to germinate within 6-8 hours after exposure (1, 16). Once the fungus begins to grow within the airways of these patients, invasive infection most commonly ensues and is associated with high mortality rates ranging between 50-99%, even after strong antifungal treatments (1, 11, 16). Due to increasing numbers of immunosuppressed patients, fungal infections have placed a significant economic burden on health care. Invasive aspergillosis (IA), most commonly caused by *Aspergillus fumigatus*, is one of the predominant diseases associated with immunocompromised patients. It was reported to occur in 17-26% of patients after lung transplants, 5-15% after allogenic bone marrow transplants, 5-24% of those with acute leukemia, and 2-13% of patients after heart transplants in the U.S. (11). The average age of patients with invasive aspergillosis is 53 years old, and 58% of patients are men. In 1996 alone, there were over 10,000 hospitalizations for invasive aspergillosis, with greater than 19% mortality in those individuals. These cases resulted in over 170,000 days of hospitalization, costing the U.S. over 630 million dollars, further illustrating the economic burden of fungal exposures (11).

Methods of Fungal Identification

Successful treatment of fungal infections is dependent upon rapid identification of the causal fungus followed by the administration of appropriate antifungal agents (17, 18). Samples must first be obtained from the patient to identify the causal organism(s) and may be obtained from the tissue, blood/serum, bronchoalveolar lavage fluid (BAL), or cerebrospinal fluid (CSF). Obtaining tissue samples or biopsies from a patient that is immunosuppressed is an invasive procedure that may be detrimental to the patient, thus this method is often avoided. Therefore, fluids are most commonly used to determine the contaminating organism(s). Viable culture is the most widely used method for identifying fungal infections from the patient (19). This method involves culturing the causal fungus on differential media selective for specific fungi and then macro and microscopic examination of the culture by a trained mycologist. Morphology, hyphal fragmentation, colony color, and spore emergence from the hyphae are colony characteristics that can be used to identify fungal colonies. Major drawbacks with these identification methods are that the laboratory requires a trained mycologist and a long culture incubation time (24-72 hours for most fungi, with some requiring weeks for growth). Further, many fungi may have similar morphological characteristics that may lead to misidentification (20).

While the 'gold standard' for positive fungal identification in clinical samples commonly requires positive culture identification, as well as visualization of fungal masses via chest x-rays, computer tomography (CT scans), or magnetic resonance imaging (MRI), other techniques have also been used in conjunction with these methods (1, 19). Most of these are immunoassays or enzyme-linked immunosorbent assays (ELISAs), which use monoclonal and polyclonal antibodies that have been developed for detecting fungal components or metabolites that may be present in the serum of the patient. In these cases serum is tested by direct or indirect immunoassay formats for the presence of previously identified and characterized antigens isolated from a number of known fungal pathogens (21-26). Some common monoclonal antibodies include those against β -glucans, ergosterol, glycoproteins, mannans, or other cell wall components. In addition, putative diagnostic markers have been identified for species-

specific antigenic proteins in common fungal pathogens, such as Asp f 1 from *Aspergillus fumigatus*, the causal organism of IA (27, 28).

Since IA is a disease primarily associated with immunosuppressed patients, and is associated with high mortality rates, it is of major concern in the medical community. IA is difficult to diagnose in the early stages, however, at later stages antifungal therapy often fails resulting in mortality rates as high as 99% (1, 11, 29, 30). There are two commercially available immunoassays for *Aspergillus* identification, both with varying specificity and sensitivity. These assays, the Platelia *Aspergillus* enzyme immunoassay and the Pastorex *Aspergillus* latex agglutination test, detect galactomannan, a carbohydrate cell wall component, associated with *Aspergillus*, and utilize the same rat monoclonal antibody, EBA-2 (26). In the Pastorex assay, latex beads are coated with the EBA-2 antibody and the patient serum is added to the beads. If the sample is positive for galactomannan, then the beads will agglutinate. The Platelia assay is a sandwich ELISA, in which a 96-well polystyrene plate is first coated with the EBA-2 antibody, the patient serum is added, and an enzyme-linked EBA-2 detection antibody is added to the well. Specific substrate for the enzyme-linked detection antibody is then added to the well and a color change is observed in a positive sample (22). Both of these techniques can yield a false positive if the patient has been treated with β -lactam antibiotics. These antibiotics are isolated from *Penicillium*, and because the EBA-2 antibody specifically recognizes the (1 $\rightarrow$ 5)- β -D-galactofuranoside side chains of the galactomannan moiety, which are found in *Aspergillus*, *Penicillium*, and *Paecilomyces* fungi, false positives may occur. A false positive may also arise if the origin of the patient infection is from *Penicillium* or *Paecilomyces* for the same reason (21, 30, 31). Therefore, these assays are limited to only identifying known antigens. Patient samples containing antigens from an unknown pathogen may also result in false negatives. Though ELISAs can be completed rapidly in comparison to viable culture and morphological characterization, many of the antibodies developed to date are not species-specific. Therefore, despite the possibility of rapidly identifying the presence of fungus in the patient sample, more species-specificity is needed.

Polymerase Chain Reaction (PCR) is a molecular technique that has recently been used for detecting pathogenic and non-pathogenic fungi. This method involves using commercially available primers directed toward common sequences within the fungal genome, typically from the 18S rRNA subunit, though others, such as primers directed towards the genes encoding ergosterol, β -glucans, β -tubulin, or other cell wall components may also be used (19, 32). The latter primers can identify a positive fungal infection but are not species-specific, as many cell wall components are conserved in most fungi, regardless of the species origin (32). Internal transcribed spacer regions (ITS regions) are also targets for PCR amplification, as they are often conserved among species (19, 32). Primers directed toward the ITS regions are species-specific in most cases, offering as high as 98% identification, with low levels of false positives (19). Real-time PCR for fungal identification has also been used. This technique offers the ability to quantitate the number of fungal specific gene copies, which has been proposed to be correlated to disease progression. This information would be invaluable for disease prognosis, as well as for identifying the proper course of prophylactics to use for the best patient outcome (19).

Though PCR offers increased sensitivity for fungal identification, approaching 100% sensitivity and specificity when done in conjunction with other methods, there are many drawbacks to this technique (23, 24). The major issues surrounding PCR for fungal identification are the ubiquitous nature of fungi. Fungi may contaminate samples at the isolation stage, DNA extraction stage, the sample PCR preparation stage, as well as cross-contamination between PCR runs, and therefore are common areas yielding false positives. The presence of fungal components in the DNA extraction solutions, many of which may contain enzymes that have been isolated from different fungi, may also be a source for false positives. Also, many of these solutions are not compatible with common methods for fungal decontamination, such as UV irradiation, phenol precipitation, or filtration (32). Although using PCR using primers directed toward the rRNA subunits can detect the target from a fraction of one organism, interference with the larger amounts of human DNA in the patient samples has been reported. Some of the primers may interact with human DNA, preventing the fungal target from being amplified in high enough amounts to be analyzed. Furthermore, molecular techniques have not been standardized and have only recently been

realized by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (26, 32).

Commercially available nucleic acid hybridization probes have also been employed for fungal identification (19, 33). In this method, species-specific chemiluminescent single stranded DNA probes are bound to a chip, the organism is cultured and a small amount removed and lysed to release the nucleic acids, which are then denatured and added to the chip. The complementary ribosomal RNA released from the isolated fungus will then hybridize to the probes and activate the chemiluminescence. The degree of hybridization can be measured in relative light units using a luminometer. This technique has high sensitivity and specificity for certain fungi tested, such as *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Coccidioides immitis*, however, cross reactivity with the *B. dermatitidis* probe has been reported (19). Despite the potential of this technique, the lack of species-specific DNA probes highlights a major drawback in the field of fungal research. Over 70,000 fungal species have been identified, and over 1.5 million species are thought to exist; however, only a fraction of these fungi have been sequenced (34, 35). Further research needs to be completed with this technique to further identify species-specific probes, as well as to increase the commercial availability to realize the potential of this method.

MALDI-TOF MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a more recent analytical method that can be used to identify an analyte present in a sample, and has been used to discriminate between clinically relevant microorganisms (36, 37). MALDI is a specific type of ionization method for mass spectrometry that utilizes samples mixed with a matrix, or an H^+ donor which co-crystallizes with the sample on a target chip. Commonly, the target chip is made of an electrical conductor, such as stainless steel or gold (38). Once the sample is loaded into the mass spectrometer, a UV laser (commonly 337-nm N_2 or 355-nm Nd:YAG) is fired at the sample spot. Upon laser excitation of the sample, matrix and co-crystallized analyte are desorbed in a supersonic plume of ions and neutrals

into the gas phase in the vacuum of the mass spectrometer where transfer of protons from the sample matrix to the analyte occurs (39, 40).

Time-of-flight mass spectrometry (TOF MS) is the method used for detecting and analyzing samples (41, 42). There is a short source region near the sample plume, which consists of a backing plate and an extraction grid. Upon laser excitation of the sample, the potential energy (PE) of the short source region is zero. A positive voltage is then applied to the backing plate, creating an electric field, thereby increasing the PE "behind" the sample. Ions are accelerated to a constant kinetic energy by the high electrical potential (typically as high as 20kV) into a vacuum tube, or field-free drift region. A vacuum tube is used to lessen the potential for collisions within the field free region, as such interactions may alter the velocity of the ions, thereby altering mass resolution of the ions. Mass resolution is the ability to distinguish between peaks within a spectrum based on analyte peak widths as well as relative separation of peaks. The analytes travel through the vacuum tube at a rate that is proportional to their mass to charge ratio, i.e. larger, heavier molecules will travel slower than smaller, lighter molecules, while an increase in the number of charges on an ion will allow it to travel faster than an ion with fewer charges. The ions then impact a detector at the end of the vacuum tube, creating a cascade of electrons which are amplified and detected as electrical current (41, 42). The spectra are recorded based on mass/charge (m/z) and the relative abundance of the analyte (proportional to the number of hits reaching the detector at a specific m/z). Therefore, most protein-containing samples can be analyzed if the protein content is above that of the detectable limit of the instrument. Each protein mixture yields a spectrum that is representative of the molecules within the sample and is unique to individual analytes.

Microbial Discrimination/Identification using MALDI-TOF MS

MALDI-TOF MS has been widely used for the discrimination and identification of numerous microbes because the spectra produced by MALDI-TOF MS analysis are unique to each microbe, synonymous to a human fingerprint. Thus, these spectra are referred to as microbial "fingerprints". The majority of experiments done using MALDI-TOF MS for the discrimination of microbes have focused on

bacterial fingerprinting. In 1994, the first experiment using MALDI-TOF MS for bacterial discrimination was able to detect water soluble surface proteins of bacteria, which allowed for the differentiation between gram positive and gram negative organisms (43). Further studies were done to show the ability of MALDI-TOF MS to distinguish between multiple organisms of different species using whole cell bacterial preparations to obtain unique fingerprints for each bacterium (44-46). Briefly, a small aliquot of bacteria were placed on a target spot, allowed to air dry, and then overlaid with matrix before analyzing with the mass spectrometer. Using this technique, many clinically relevant bacteria have been analyzed, including *Escherichia coli*, *Pseudomonas* (45), *Staphylococcus*, *Mycobacterium* (44), and *Bacillus anthracis* (46). The ability to differentiate these organisms is due to the presence of unique ions at different m/z ranges for each species. A complete list of bacteria which have been analyzed using MALDI-TOF MS is shown in a recent publication by Giebel *et al.* and includes 22 clinically relevant bacterial genera (47).

MALDI-TOF MS has also been used to differentiate between different strains of the same organism (48, 49). For example, this technique has been useful in discriminating between pathogenic, multi-drug resistant strains of normal microbiota, such as *Staphylococcus* and methicillin resistant *Staphylococcus aureus* (MRSA) (48). In this study, each strain yielded a unique distinct spectrum, however, when analyzing large numbers of clinical isolates and laboratory strains, the authors state that a distinctive spectrum representative of MRSA could not be obtained. The authors noted, however, that the MRSA strains could be differentiated from each other, and this could be used to identify the origin of clonal strains.

Several studies have focused on the use of MALDI-TOF MS for bacterial identification in clinical laboratories (50-54). In these studies, bacteria had been recovered from blood, cerebrospinal fluid (CSF), pus, biopsy, the respiratory tract, stool, and wounds. Samples were identified using standard phenotypical methodology by using differential growth mediums and conditions. In parallel, a single colony of the bacterium were deposited on a MALDI-TOF MS target plate and overlaid with matrix and then analyzed. (53). Of the 1660 isolates, from 45 genera and 109 species obtained, MALDI-TOF MS was able to

correctly identify 95.5% of the organisms, the same as done by standard routine identification, with 84.1% of the samples being identified at the species level. Forty-six of the isolates were unable to be fingerprinted using MALDI-TOF MS, however, this number was not statistically different from the standard routine identification methods, which failed to identify thirty-two of the isolates. Furthermore, this study reported that the lack of identification by MALDI-TOF MS was related to the lack of reference spectra for comparison of those species. For example, for the bacterial species that contained more than 5 reference spectra, greater than 85% of those bacterial species were identified using MALDI-TOF MS. This highlights the importance of having a large database library for direct comparison of unknown samples. The authors also reported a cost/time of identification comparison analysis, whereby they compared the standard protocols for bacterial identification with MALDI-TOF MS discriminate analysis. They reported that mass spectrometry identification took approximately 8.5 minutes and cost 1.43 euros/sample. When compared to gram staining, which takes 6 minutes and costs 0.6 euros/sample, API system identification which takes 1080-2880 minutes and costs 4.6-6.0 euros/sample, antibiotic susceptibility testing which takes 1080-2880 minutes and costs 6.6-7.4 euros/sample, Phoenix system identification and susceptibility testing which takes 300-1200 minutes and costs 12.65 euros/sample, and the Vitek system which takes 300-480 minutes for identification and costs 5.9-8.23 euros/sample and in conjunction with susceptibility testing costs 10.38-12.71 euros/sample, MALDI-TOF MS is the most cost and time efficient method for comparable bacterial identification (53).

Further studies have examined the potential use of MALDI-TOF MS for bacterial identification in positive blood cultures. In a study done by Christner et al., 304 positive blood cultures (reported positive by an automated incubation system), of which 277 contained a single bacterium, were analyzed using MALDI-TOF MS fingerprinting (51). The authors report 95% correct bacterial identification, with 94% of those samples being identifiable on the same day as the blood samples showed positive culture. Another study of 1,371 isolates obtained from clinical samples were identified using conventional methods and analyzed via MALDI-TOF MS in parallel, reported 95.1% correct identification rate. In this study 63 isolates were unable to be identified, however, the authors reported possible reasons for the

misidentification/inability to identify as being initial conventional misidentification in 7 isolates, and the remaining 56 isolates were predominantly misidentified as a result of human error in taxonomic assignment of the reference spectra in the database, recent changes in taxonomy of a given species, or different levels of precision in identification between conventional identification methods and MALDI-TOF MS (50). A study done in 2010 reported 95.1% correct species identification of 327 isolates taken from blood, pus, respiratory tract, stool, wounds, CSF, and urine (54).

Another study, which focused on bacterial identification in positive blood cultures, reported the ability of MALDI-TOF MS to identify false positives (as labeled positive by standard conventional protocols), and reported 83.3% species level identification of gram-negative bacteria, 96.6% genus level in a sample population of 318 positive blood cultures (47, 52). In gram-positive bacteria, species identification was 31.8% and 65.7% at the genus level when compared to conventional methods. The comparable difference between identification in gram-positive and gram-negative bacteria may be due to the sample processing done by the authors, as there were multiple wash steps before analysis. Further, gram-positive bacteria have a thicker cell wall, which strongly binds surface proteins and may be harder to lyse. Therefore, the amount of protein obtained from gram-positive bacteria may have been lower than that obtained from the gram-negative bacteria which may have lysed during the washing steps. These are speculations, as the authors did not report sample extraction optimization.

As shown by Hettick et al., bacterial samples with thicker cell walls, such as *Mycobacterium* spp., are sufficiently discriminated from each other when harsher protein extraction steps are used for sample preparation (55). In this study, several different species of *Mycobacterium*, including *M. fortuitum*, *M. kansasii*, *M. intracellulare*, *M. avium*, *M. bovis* BCG, and *M. tuberculosis* H37Ra, were subjected organic/acid extraction and analyzed via MALDI-TOF MS. Using linear discriminant analysis of 77 peaks identified among all species of the sample, 100% of the spectra were correctly identified.

In addition to fingerprinting and discriminating bacteria, MALDI-TOF MS has been used to identify the presence of viruses. Because the standard methodology for viral identification is relatively quick and has a low false identification rate, MALDI-TOF MS has not been as extensively examined as

an alternative method of viral identification, but has been used for detection. Specifically, 6 different viruses have been fingerprinted including, Hepatitis B and C, Norovirus, Avian influenza viruses, Newcastle disease virus, and the Sindbis virus (47). Commonly, specific proteins or peptides are targeted for specific viral identification. These include the capsid protein (56), fusion proteins (57, 58), and glycoproteins associated with the viral envelope (59). In addition, DNA has also been used to specifically identify some viruses, including determining the molecular weight of specific restriction fragments from complementary DNA (60-62).

Limitations of MALDI-TOF MS Microbial Fingerprinting

Though multiple studies have demonstrated the ability of MALDI-TOF MS to discriminate between numerous clinically relevant microbes, there are also several limitations to this technique. The major limitation is the reference library or database (53). In order to correctly identify clinical pathogens by MALDI-TOF MS, a representative comparison spectrum must be present in the database. If no reference spectrum is present to compare the sample spectrum to, then no identification can be made. This limitation is not one of the instrument, but rather of the compiled database. Therefore, a large, extensive database of all clinically relevant organisms must be compiled for MALDI-TOF MS to correctly identify or discriminate the clinical organism.

In addition, the culture conditions may alter the spectrum of the sample organism when compared to the database. The length of time the culture is grown, as well as the temperature, and the culture medium have been shown to alter the protein content of many microbes (63). The method for protein extraction, where applicable, may also alter the proteins present in the sample. Though intact cell mass spectrometry may not be altered by protein extraction protocols, results from our laboratory show that bead beating samples, both bacterial (*Mycobacterium*) and fungi (*Aspergillus* and *Penicillium*), increase the number of peaks in a spectrum, presumably due to the release of intracellular proteins due to bead beating (49, 64, 65). In addition, the choice of solvent used for the elution of extracellular proteins must be kept uniform, as different proteins may or may not solubilize in the presence of different organic

and/or acidic solvents. Therefore, samples comprising the reference database must be treated in the same manner as the unknown sample in order to accurately identify the unknown microbe.

Furthermore, studies have shown that a minimum of 1×10^4 microbial cells must be present within the sample to yield a spectrum with an adequate number of peaks with a high enough relative abundance above the background noise (66). The number of required microbial cells may differ between each individual mass spectrometer used, as each commercially available instrument has differing sensitivities, as well as between microorganisms, as the concentration of soluble proteins may differ between species.

Another limitation of MALDI-TOF MS is the reproducibility of the technique. Reproducibility within a laboratory is often very high (75% and higher) (48, 67-70), however, when the same samples are sent to other laboratories for the same analysis, the reproducibility may decline (as low as 23%) (71). The lack of reproducibility between laboratories is attributed to the differences in sensitivity of the different mass spectrometers used in each different laboratory. To address this issue, many mass spectrometer companies have been designing 'biotypers', which are essentially mass spectrometers that use a commercial database consisting of a library of spectra from common microorganisms for reference and discrimination (72). These instruments can be used to identify pathogenic organisms from human samples within hours.

MALDI-TOF MS Fingerprinting of Fungi

MALDI-TOF MS has been used to fingerprint and discriminate between fungi. Fourteen medically important fungal genera have been fingerprinted to date. The first study to fingerprint fungi was published in 2000, where various species of *Penicillium*, as well as *Scytalidium dimidiatum* and *Trichophyton rubrum* were characterized. In most studies examining the fingerprints of fungi, methods to lyse the spores of fungi are used to obtain a fingerprint with a greater number of peaks/ increased relative abundance (64, 65, 73). These methods include acidic lysing, as done with *Saccharomyces cerevisiae* (74), or bead beating in an organic/acidic solvent, as done with several species of *Aspergillus* and

Penicillium (64, 65). In the studies done by Hettick et al., MALDI-TOF MS was able to discriminate between 12 species of *Penicillium* and 12 species of *Aspergillus* with a 0% error rate using discriminant analysis. MALDI-TOF MS was also able to correctly discriminate between 5 different strains of *A. flavus* 95% of the time.

Several studies have demonstrated the utility of MALDI-TOF MS for fingerprint profiling of multiple species of fungi; however, one limitation has been identified by several groups (64, 73, 75). Darkly pigmented fungi, such as *Aspergillus niger*, *Stachybotrys chartarum*, and *Alternaria alternata* could not be fingerprinted using this technique. These samples produce a mass spectrum containing a paucity of distinct peaks preventing identification. Many factors may explain the difficulty in analyzing these darkly pigmented fungi. Although the precise mechanism(s) by which the MALDI desorption/ionization event occurs is the subject of much discussion and active research (39), it is possible that the unique properties of fungal pigments could interfere in a number of fundamental ways with the MALDI process. One plausible explanation for the observed inhibition of MALDI-TOF ionization is that melanin in fungal samples (particularly at high concentrations) directly competes with the organic acid matrix for irradiating photons. Following the MALDI laser pulse, ionization in the dense plume above the sample deposit will only occur where there is a sufficient population of electronically excited matrix molecules. If a photoexcitation/pooling mechanism for MALDI is assumed (76), mobile excitons in the bulk sample deposit could be quenched by fungal pigments.

Fungal Pigmentation

Darkly pigmented fungi are dark in color because of the high levels of intracellular pigment. Fungi are common plant pathogens, and therefore produce pigmentation to protect themselves from environmental stresses such as ultraviolet light, as well as to aid in the buildup of turgor pressure which the fungi use to penetrate plant tissues (77-79). In submerged conditions, however, most fungi do not produce pigments, presumably because the nutritional needs are altered in these environments and the

stresses necessary to induce pigment formation are not induced (29). The most common pigment associated with fungi is melanin (78, 79).

Melanin is a large, complex macromolecule that is formed through the oxidation of phenolic or indolic compounds. Typically, melanins appear black, brown, or green in color (79, 80). Several types of melanins have been identified (Table 1) (78, 79). Each type of melanin is produced through the polymerization of different biomolecules, and thus potentially different biosynthetic pathways (79). Phaeomelanins are derived from tyrosine and cysteine, allomelanins are formed from nitrogen-free precursors, and eumelanins are a product of the polymerization of tyrosine, quinones, and free radicals (Table 1) (80). The predominant types of melanin found in fungi are phaeomelanins and eumelanins. In many pathogenic fungi, eumelanins are the most common form of melanin present, though multiple types of melanin may exist simultaneously (78-82). There are also two different associated forms of melanin, including cell-associated or vesicle-associated melanins (78). Commonly, in darkly pigmented fungi, the melanins produced are intercalated within the spore wall of the organism and help to provide stability and strength to the spore wall coating. These melanins are covalently bound to carbohydrates and lipids that make up the cell wall (16, 78).

The exact structure of melanin has not been identified due to the properties of this macromolecule which make it difficult to use common biochemical analyses. These properties include: insolubility in organic or aqueous solvents, resistance to degradation by strong acids and alkaline agents, and resistance to bleaching by strong oxidizing agents. Melanins are negatively charged and hydrophobic (78, 80, 82, 83). More importantly, fungal melanins have photoprotective properties whereby they absorb UV light and dissipate it as heat (84-86). This specific property may be at least partially responsible for the inability to acquire unique fingerprints in darkly pigmented fungi. Therefore, it is being hypothesized that the presence of dark pigmentation in darkly pigmented fungi prevents proper ionization of the proteins/peptides (analytes) within the sample preparation, thereby inhibiting peak formation within the spectrum.

Fungal Melanins				
<i>Types of fungal melanins</i>	<i>Chemical metabolism</i>	<i>Color</i>	<i>Family Occurrence</i>	<i>Ref.</i>
Eumelanin	formed through the polymerization of tyrosine, quinones, and free radicals	Black, dark green, dark brown	Various fungi	(78, 80, 84)
L-3,4-Dihydroxyphenylalanine (DOPA)	a subtype of eumelanin	-----	-----	(78)
Phaeomelanin	formed through the polymerization of tyrosine, cysteine, and dihydroxyphenylalanine (DOPA)	Red, yellow	Various fungi	(78, 80)
Allomelanin	formed from nitrogen-free precursors	Black, brown	Various fungi	(80, 87)
Catechol	a subtype of allomelanin formed through phenol oxidation of catechol	Black, dark brown	Basidiomycetes	(78, 87)
1,8-Dihydroxynaphthalene (DHN)	formed from acetyl coenzyme A and malonyl coenzyme A in the pentaketide biosynthesis pathway	Black, dark green, dark brown	Ascomycetes, Ascomycetous imperfecti fungi, and Dithideaceous species	(78)
γ-glutaminy-3,4-dihydroxybenzene	formed through oxidation of γ -glutaminy-4-hydroxybenzene in shikimic acid pathway	Black, dark brown	Basidiomycetes	(78, 88)

Table 1: Common types of fungal melanins.

Melanin Production

Eumelanins are divided into subgroups including L-3,4-dihydroxyphenylalanine (L-DOPA) melanin and 1,8-dihydroxynaphthalene (DHN) melanins (77). Both types of melanin have been identified in fungi, and may exist within the same organism. Though multiple types of melanins are associated with

fungi, it has been reported that the predominant forms of fungal-associated melanins are DHN-melanins (89). The DHN-melanin synthesis pathway begins with glucose, a common sugar which is used by cells in the citric acid cycle (TCA). Before entering the TCA cycle one molecule of glucose is converted into fructose 6-phosphate, followed by conversion into one molecule of fructose-1,6-diphosphate. This is then converted into glyceraldehyde-3-phosphate and then pyruvate (Figure 1). Pyruvate is converted into two molecules of acetyl coenzyme A (acetyl-CoA) which is the initial molecule for the pentaketide synthesis pathway. In this pathway, acetyl-CoA is converted into 1,3,6,8-4-hydroxynaphthalene (1,3,6,8-4HN). A tetrahydroxynaphthalene reductase then converts 1,3,6,8-4-hydroxynaphthalene into scytalone. Tricyclazole, an antifungal agent, can block the activity of this reductase producing a shunted pigmented byproduct called flaviolin. Following conversion to scytalone, a trihydroxynaphthalene reductase converts scytalone into vermelone. Again, this conversion may be specifically blocked by tricyclazole, producing another shunted pigmented byproduct called 2-hydroxyjugalone. Vermelone is then converted by an unknown convertase into dihydroxynaphthalene (DHN) which then is polymerized to produce DHN-melanin (90). Thus, it is being hypothesized that due to the electro- and biochemical nature of melanins present in darkly pigmented fungi, specifically DHN-melanins and/or DHN-melanin-like pigments, are interfering with the proper ionization of the analytes present in the MALDI-TOF MS sample, thereby inhibiting the formation of unique, discriminate fingerprint spectra from these species.

Figure 1: Dihydroxynaphthalene melanin (DHN-melanin) production pathway. Figure adapted from Lee et al. (90).

In this study, it is shown that synthetic melanin, as well as fungal pigment extracts from *Aspergillus niger*, *A. fumigatus*, and *A. terreus*, inhibit MALDI-TOF MS analysis of the 66,000 m/z peak formation of human serum albumin in a concentration dependent manner. Additionally, heavily pigmented areas within a MALDI-TOF target spot directly inhibit mass spectral formation when compared to an area within the same spot that contains less pigment. Finally, when darkly pigmented fungi, such as *A. niger*, are cultured under conditions that inhibit pigment formation, a unique fingerprint spectrum is obtained using MALDI-TOF MS.

Chapter II

Materials and Methods

Reagents

Acetonitrile (ACN) (HPLC grade), sodium citrate, and hydrochloric acid (6N) were acquired from Fisher Scientific (Fairlawn, NJ). Tricyclazole was acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Malt extract agar (MEA) was acquired from Difco (Sparks, MD). Deionized water (DDI) was produced with a Synthesis A-10 (Millipore, Billerica, MA). All other reagents were acquired from Sigma Aldrich (St. Louis, MO) and all reagents were used without further purification.

Fungal Culture

Fungal isolates were obtained from either the American Type Culture Collection (ATCC, Manassas, VA) or the Fungal Genetics Stock Center (FGSC, Kansas City, MO). *Aspergillus terreus* (ATCC 1012), *A. fumigatus* (AF293; FGSC A1100) and *A. niger* (FGSC A1143) were subcultured from stock sources and grown for 7 or 14 days at 25°C on malt extract agar (MEA) nutrient media in 100 mm culture plates. To ensure reproducibility, each fungal species was cultured eight times independently.

For submerged culture preparation, approximately 1×10^2 spores were inoculated into 50 mL of malt extract broth (MEB) and grown for 4 days in a C24KC refrigerated incubator shaker (Edison, NJ) at 25°C. Cultures were transferred to 50 mL conical tubes and centrifuged at 41 x g for 5 minutes. The supernatant was retained and frozen at -80°C. The mycelial mass was washed 3 times with phosphate buffered saline (PBS) to remove residual MEB and forced through a 70 µm mesh filter (BD, Bedford, MA) using a syringe plunger in 20 mL of PBS. This solution was centrifuged at 41 x g for 5 minutes and the supernatant collected, lyophilized and the dry weight recorded for quantification. The resulting dry mass was suspended in 1 mL of ACN/4%TFA (50:50). To ensure reproducibility, each fungal species was cultured eight times independently. For tricyclazole mediated melanin inhibition, cultures were grown for

7 or 14 days at 25°C on MEA plates supplemented with 50 µg/ml of tricyclazole in 1% ethanol. To ensure reproducibility, each fungal species was cultured eight times independently.

Preparation of Fungal Culture Samples for MALDI-TOF MS Analysis

Conidia and hyphae derived from one culture plate of *A. terreus*, *A. fumigatus*, and *A. niger* ($\sim 10^8$ spores/mL) were transferred to 100 µL of 0.1 mm zirconium beads (Biospec, Bartlesville, OK, USA) and 1 mL of ACN/4% trifluoroacetic acid (TFA) (50:50). The samples were then subjected to three-one minute bead beating cycles. Samples were cooled on ice between bead beating cycles. The resulting solution was centrifuged at 21,600 x g for ten minutes, and the supernatant fluid retained. A 10 µL aliquot of supernatant fluid was combined with 10 µL of α -cyano-4-hydroxycinnamic acid (CHCA, 10 mg/mL) in 50/50 ACN/0.1% TFA and duplicate 1.5 µL aliquots were deposited on a gold sample stage (Bio-Rad, Hercules, CA) and allowed to air dry prior to MALDI-TOF MS analysis.

Preparation of Synthetic Melanin Samples for MALDI-TOF MS Analysis

Samples were prepared by dissolving varying concentrations of synthetic melanin over the range of 0.001 mg/mL to 20 mg/mL in 50/50 ACN/DDI. A 10 µL aliquot of synthetic melanin solution was combined with an equal volume of either human serum albumin (7.5 pmol/µL) or angiotensin II (10 pmol/µL). A 10 µL aliquot of the resultant mixture was combined with 10 µL of CHCA (10 mg/mL in 50/50 ACN/0.1% TFA) and duplicate 1.5 µL aliquots were deposited on a gold sample stage (Bio-Rad, Hercules, CA) and allowed to air dry prior to MALDI-TOF MS analysis.

Isolation of Melanin Ghosts

So called “melanin ghosts” were extracted from *A. terreus*, *A. fumigatus*, and *A. niger* using a method modified from Wang et al. (91). Briefly, cultures were grown on MEA for 10 days at 25°C. Sporulating fungal cultures were harvested from the culture plate using sterilized DDI and the resulting suspension was centrifuged at 1000 x g for 10 minutes. The pellet was resuspended in 0.1 M sodium

citrate buffer containing 10 mg/mL of cell lysing enzymes from *T. harzianum* and 2 mg/mL cellulase from *T. reesei* and incubated with agitation overnight with at 30°C. The sample was then pelleted by centrifugation (1000 x g for 10 minutes), washed with PBS and resuspended in 4 M guanidine isothiocyanate and incubated at room temperature (RT) overnight with agitation. The sample was then pelleted, washed with PBS and resuspended in 1 mg/mL of proteinase K in DDI and incubated overnight at 37°C with agitation. The sample was pelleted, washed with PBS and resuspended in 6 M HCl and boiled for one hour. The sample was then pelleted, washed with PBS and dialyzed against DDI for 10 days at 4°C using 3500 molecular weight cutoff (MWCO) dialysis membrane tubing (Spectra/Por®, Laguna Hills, CA). The sample was lyophilized and the resulting melanin ghosts used for further experiments.

Melanin ghosts from each *Aspergillus* species were diluted with ACN/4% TFA and mixed with an equal volume of 7.5 pmol/μL HSA. A 10 μL aliquot of the resulting solution was mixed with 10 μL of 10 mg/mL CHCA and 1.5 μL deposited on a gold sample stage (Bio-Rad, Hercules, CA) and allowed to air dry. Each sample was spotted in duplicate.

UV Spectroscopy

Spectrophotometric characterization of melanin was carried out using a Beckman DU 800 Spectrophotometer (Fullerton, CA) equipped with a deuterium (UV) and a tungsten (Vis) lamp. Data acquisition and processing was carried out using the DU800 Spectrometer software. The analytical wavelength range was 200 to 800 nm. Temperature control was achieved by using a VWR Scientific (Niles, IL) circulating water bath. Fungal pigments were dissolved in 50/50 HNO₃/HF solution with sonication for 5 min. Blanking of the instrument was performed with 50/50 HNO₃/HF before absorbance readings of the melanin were taken.

Gas Chromatography/Mass Spectrometry

Samples were analyzed using an Agilent 6890 gas chromatograph coupled to an Agilent 5975C mass spectrometer (MS) using a 30 m HP5-MS column (Agilent Technologies, Santa Clara, CA). Samples were injected (1 μ L) at a 20:1 split injection ratio into a 300°C inlet. Analytes were eluted from the column using 1.0 mL/min helium and an oven temperature profile as follows: 50°C for 5.0 min then ramped at 5.0°C/min to a final temperature of 300°C. The MS source temperature was maintained at 230°C, the quadrupole temperature was maintained at 150°C.

Field Emission Scanning Electron Microscopy

A small aliquot of lyophilized extracted pigment was deposited on double-stick carbon tape on an aluminum mount and sputter coated with gold/palladium. Images were collected on a Hitachi (Tokyo, Japan) S-4800 field emission scanning electron microscope.

MALDI-TOF MS Analysis

Protein MALDI-TOF mass spectra were acquired using a Ciphergen PBS-IIc linear TOF mass spectrometer (Bio-Rad, Hercules, CA) with a flight path of 0.8 m, capable of mass resolution ($m/\Delta m$) of 1000 and mass accuracy of ± 1000 ppm. Spectra were acquired over the m/z range of 0 to 100 m/z , with delayed extraction parameters set to optimally focus the 10- to 20- m/z range. Composite mass spectra are the average of 90 laser shots taken from 20 distinct positions across the sample deposit. These positions were held constant for all samples used in this study, and 337-nm N_2 laser pulse energy was maintained just above the threshold for ion production. Mass spectra were externally calibrated using a set of peptide and protein calibrants that covered a range of 1 to 66 m/z . Peptide MALDI-TOF mass spectra were acquired using a MALDI-SYNAPTTM MS (Waters, Milford, MA) quadrupole time-of-flight mass spectrometer (Q-TOF MS) capable of mass resolution ($m/\Delta m$) of 14,000 and a mass accuracy of ± 5 ppm. Spectra were acquired over the m/z range of 50 to 2,000 Da. Composite mass spectra are the result of a 60 second acquisition with the N_2 laser (337-nm) operating at 20 Hz, with the laser pulse energy

maintained just above the threshold for ion production. Mass spectra were externally calibrated using tandem mass spectrometry (MS/MS) data covering the mass range 70 to 1570 M/z .

Statistical Analysis

Differences in mean peak heights and the effects of fungal strain and pigment level on MALDI-TOF MS data were statistically determined. Univariate analyses were performed to ensure all data met analytic assumptions. Mixed linear models were used to determine if there were differences in the mean peak height between concentrations within each strain, between pigment levels across all strains, and between strains. Results were considered significantly different at an alpha level of 0.05. All data were analyzed using SAS 9.1 (SAS Institute, Cary, NC).

Chapter III

Results

MALDI Suppression by Darkly Pigmented Fungi

In the initial studies demonstrating the utilization of MALDI-TOF MS in fungal fingerprinting, closely related fungi of several different species belonging to the genera *Aspergillus* and *Penicillium* were differentiated (64, 65). It was observed that MALDI-TOF MS fingerprinting produced unique spectra for each species, and sufficiently allowed for reproducible discrimination between five different isolates of *A. flavus* (64). Unpublished results from additional experiments using different fungi also yielded unique spectra. (Figure 2) (92).

Figure 2: MALDI-TOF MS fingerprint of A) *Eurotium amstelodami*, B) *Penicillium chrysogenum*, C) *Aspergillus versicolor*, D) *Paecilomyces variotii*, E) *Curvularia lunata*, and F) *Chaetomium globosum*.

Despite the utility of MS to differentiate between these monilaceous fungi, several darkly pigmented fungi produced spectra with a paucity of peaks, and therefore prevented subsequent discriminate differentiation (Figure 3).

Figure 3: MALDI-TOF mass spectra of darkly pigmented fungi. A) *Alternaria brassicicola*, B) *Alternaria alternata*, C) *Epicoccum nigrum*, D) *Stachybotrys chartarum*, E) *Pithomyces chartarum*, and F) *Exserohilum rostratum*.

As darkly pigmented fungi are common indoor contaminants that have been implicated in the induction and exacerbation of conditions such as asthma and allergies, it was important to obtain a MALDI-TOF MS fingerprint of these fungi to demonstrate the utility of this discrimination methodology. It was hypothesized that the presence of fungal pigments, such as melanin, in darkly pigmented fungi is preventing ionization of the analytes within the sample preparation by an unknown mechanism.

Synthetic Melanin Suppression of MALDI

In order to determine if melanin is interfering with the MALDI-TOF MS methodology, different concentrations (0.001 mg/mL - 20 mg/mL) of commercially available synthetic melanin were spiked with human serum albumin (HSA) and then analyzed via MALDI-TOF MS. The molar concentration of

melanin in these solutions cannot be determined from the mass of suspended melanin because melanin is a highly disperse polymer, only slightly soluble in the extraction solution, ACN/4% TFA. The MALDI-TOF mass spectrum of control HSA yields a distribution of peaks corresponding to charge states from $[M+H]^+$ to $[M+6H]^{6+}$ (Figure 4A). Addition of synthetic melanin to the sample deposit results in a decrease in peak height in a concentration dependent manner, with the most concentrated melanin samples (> 5 mg/mL) completely suppressing HSA peak formation. A concentration of synthetic melanin of 0.001 mg/ml spiked with HSA resulted in a 16% decrease (Figure 12).

Figure 4: MALDI-TOF mass spectra of human serum albumin spiked with synthetic melanin. A) 2X HSA only, B) 0.001 mg/ml synthetic melanin + HSA, C) 0.01 mg/ml synthetic melanin + HSA, D) 0.1 mg/ml synthetic melanin + HSA, E) 1 mg/ml synthetic melanin + HSA, F) 5 mg/ml synthetic melanin + HSA, G) 10 mg/ml synthetic melanin + HSA, and H) 20 mg/ml synthetic melanin + HSA.

Characterization of Isolated Fungal Pigments

Because the synthetic melanin examined in the preceding experiments is not chemically identical to that obtained from fungal sources, fungal pigments were isolated from three different species of *Aspergillus* to determine whether these naturally occurring fungal pigments would also suppress analyte $[M+H]^+$ ion formation in MALDI-TOF mass spectra. Specifically, fungal pigments from *Aspergillus niger* (a black fungus), *A. fumigatus* (green), and *A. terreus* (brown) were extracted and characterized prior to MALDI-TOF MS analysis. These pigments were chosen to represent the common phenotypical colors associated with most fungi, and also because of the ability to fingerprint the lighter pigmented fungal samples from *A. terreus* and *A. fumigatus*. Figure 5 shows distinguishable spectra derived from *A. terreus* and *A. fumigatus*; however, samples of *A. niger* produced spectra that lacked peaks.

Figure 5 : MALDI-TOF MS fingerprint of fungi using protocol in Hettick et al. 2008 (64, 65). A) *A. niger*, B) *A. fumigatus*, and C) *A. terreus*.

The pigment preparations were first analyzed and visualized using field emission scanning electron microscopy (FESEM). FESEM analysis suggests that fungal pigments isolated from *A. niger* and *A. fumigatus* maintain the ultrastructural architecture of the fungal spores following melanin extraction (Figure 6A-B). This observation is consistent with previous reports of pigments isolated from other fungal species, including *Cryptococcus neoformans* and the term “melanin ghosts” has been coined (91, 93). In contrast, pigments extracted from *A. terreus* were structurally similar to the commercially available synthetic melanin (Figure 6 C-D).

Figure 6: FESEM images of pigment extracts or synthetic melanin. A) *A. niger* pigment extract, B) *A. fumigatus* pigment extract, C) *A. terreus* pigment extract, and D) synthetic melanin.

In addition to the FESEM, elemental analysis was also conducted to determine the chemical make-up of each sample because different types of melanins contain different concentrations of carbon, nitrogen, hydrogen, and in some cases sulfur. There were no differences between each sample in the

chemical components, as each pigment contained high levels of carbon, oxygen, hydrogen, and sulfur, while none contained nitrogen (data not shown).

Gas chromatography/mass spectrometry (GC/MS) was also conducted on each sample to determine the presence of different functional groups within each sample. GC/MS analysis indicated the presence of long chain fatty acids and aliphatic compounds in each fungal pigment preparation which was not present in synthetic melanin (Figure 7A), most likely due to the presence of cell wall components within the samples (Figure 7B). These results are consistent with DHN-melanins, which are known to be extensively bound to cell wall/structural components of the *A. fumigatus* spore.

Figure 7: GC/MS spectra of A) Synthetic melanin or B) *A. niger* pigment extract. The broad peak present in B indicates the presence of fatty acid chains and aliphatic compounds.

In addition, UV spectroscopy was done on each pigment extract. Each pigment (1 mg/mL) exhibited strong, non-zero, absorption in the 200-800 nm range when analyzed by UV/VIS spectroscopy, and absorption approached 1.0 at 337- nm, the emission wavelength of the nitrogen laser used for MALDI-TOF MS. An example of *A. niger* pigment extract absorption when solubilized in 50/50 hydrofluoric acid/nitric acid is shown in Figure 8. Pigments from other fungi yielded similar spectra.

Figure 8: UV/Vis spectrum of 1 mg/mL *A. niger* in 50/50 hydrofluoric acid/nitric acid.

This observation is consistent with previous studies that demonstrated melanin to have photoprotective properties in fungi, absorbing UV radiation and dissipating energy as heat via rapid internal conversion (84-86). Therefore, it is possible that there is competition between the fungal pigment and the analytes within the sample, whereby the pigments are absorbing photons, preventing ionization of the analytes and subsequently inhibiting the spectrum formation.

Suppression of MALDI-TOF MS by Isolated Fungal Pigments

The isolated pigments from each of these fungi were analyzed for protein content before being spiked into HSA, none was present. In these experiments, a concentration dependent inhibition of the $[M+H]^+$ ion peak height was observed with each fungal pigment extract (Figure 12), similar to that seen with synthetic melanin. The most concentrated sample (20 mg/mL) of *A. niger* pigment reduced the 66 m/z peak abundance by 96%, while 1 mg/mL of pigment reduced the peak height by 8% (Figures 9 and 12). *A. fumigatus* pigment (which did not prevent the acquisition of a unique fingerprint of the fungal sample) reduced the peak height by 89% at 20 mg/mL, while the 0.001 mg/mL sample reduced the peak height by 10% (Figures 10 and 12). Interestingly, the pigment isolated from *A. terreus* reduced the HSA peak height by 96% at 20 mg/mL and by 8% at 1 mg/mL (Figures 11 and 12), which was unexpected

because *A. terreus* is light brown in color when grown on malt extract agar and the fungi itself produces a spectrum that contains multiple peaks of high abundance as shown in Figure 5.

Figure 9: MALDI-TOF mass spectra of human serum albumin spiked *A. niger* pigment preparations. A) 2X HSA only, B) 0.001 mg/ml *A. niger* pigment + HSA, C) 0.01 mg/ml *A. niger* pigment + HSA, D) 0.1 mg/ml *A. niger* pigment + HSA, E) 1 mg/ml *A. niger* pigment + HSA, F) 5 mg/ml *A. niger* pigment + HSA, G) 10 mg/ml *A. niger* pigment + HSA, and H) 20 mg/ml *A. niger* pigment + HSA.

Figure 10: MALDI-TOF mass spectra of human serum albumin spiked with *A. fumigatus* pigment preparations. A) 2X HSA only, B) 0.001 mg/ml *A. fumigatus* pigment + HSA, C) 0.01 mg/ml *A. fumigatus* pigment + HSA, D) 0.1 mg/ml *A. fumigatus* pigment + HSA, E) 1 mg/ml *A. fumigatus* pigment + HSA, F) 5 mg/ml *A. fumigatus* pigment + HSA, G) 10 mg/ml *A. fumigatus* pigment + HSA, and H) 20 mg/ml *A. fumigatus* pigment + HSA.

Figure 11: MALDI-TOF mass spectra of human serum albumin spiked with *A. terreus* pigment preparations. A) 2X HSA only, B) 0.001 mg/ml *A. terreus* pigment + HSA, C) 0.01 mg/ml *A. terreus* pigment + HSA, D) 0.1 mg/ml *A. terreus* pigment + HSA, E) 1 mg/ml *A. terreus* pigment + HSA, F) 5 mg/ml *A. terreus* pigment + HSA, G) 10 mg/ml *A. terreus* pigment + HSA, and H) 20 mg/ml *A. terreus* pigment + HSA.

Figure 12: Effect of fungal pigment suppression of 66 m/z HSA peak height. Data values are reported as the mean $\pm$ the standard error of measure.

Because melanin is poorly soluble under MALDI sample deposit conditions (e.g. 50/50 ACN/4% TFA), the pigment samples analyzed in these experiments were suspensions, not homogenous solutions. Further, MALDI sample deposits are often heterogeneous, leading to poor shot-to-shot and deposit-to-deposit reproducibility (94, 95). Thus, sample deposits used for MALDI-TOF MS analysis which were visualized via light microscopy at 4X magnification and appeared heterogeneous, with regions of high and low pigment levels across the sample deposit (Figure 13).

Figure 13: Light microscopy images of sample spots on a MALDI target chip of A) synthetic melanin, B) *A. niger* pigment extract, C) *A. fumigatus* pigment extract, and D) *A. terreus* pigment extract under 4X magnification. Equal concentration of each pigment were mixed with matrix and spotted.

This observation may account for the HSA peak height inhibition in those samples. Since the synthetic melanin was present more homogeneously throughout the spot, as opposed to the aggregated pigments from *A. niger* and *A. fumigatus*, this presents a greater likelihood that the MALDI UV-laser would fire on a spot where pigment was more concentrated. To examine the effect of this heterogeneity on the acquired MALDI-TOF mass spectrum, a sample of 10 pmol/ μ L of angiotensin II was spiked with an equal volume of 20 mg/mL *A. fumigatus* pigment extract and analyzed by high resolution MALDI-TOF MS (Figure 14). On this instrument, the sample deposit is imaged by a camera and the exact spatial location of the MALDI laser output can be guided in real time. Spectra were acquired from regions of high and low *A. fumigatus* pigment concentration (empirically determined by black coloration). Direct suppression of the angiotensin II $[M+H]^+$ ion (1046 m/z) signal in regions of high pigment concentration is observed, whereas a strong angiotensin II $[M+H]^+$ ion signal is observed from regions of low pigment concentration.

Figure 14: MALDI Q-TOF spectra of *A. fumigatus* pigment extract spiked with 0.5 mg/ml angiotensin II (singly charged peak around 1000 m/z) spike with *A. fumigatus* pigment extract. Top spectrum is taken from an area with no visible pigment. Bottom spectrum is taken from a spot with a high concentration of pigment extract.

Fungal Culture Conditions Effect Melanin Production

The preceding experiments clearly demonstrate that melanin pigments produced by fungi are at least, in part, responsible for the observed suppression of MALDI ionization in fingerprint mass spectra from darkly pigmented fungi. Thus, it was further hypothesized that if melanin production could be blocked in darkly pigmented fungi, problematic species, such as *A. niger*, would yield fingerprint mass spectra similar to their unmelanized relatives. Many fungi, when grown for short durations in liquid culture, do not produce pigments (29, 81). Thus, melanin-free *Aspergillus* cultures were produced by growing each of the three species in liquid media. Unique MALDI-TOF MS fingerprint spectra were acquired from each fungus grown in liquid culture, including *A. niger* (Figure 15).

Figure 15: MALDI-TOF mass spectra of cultures grown under submerged conditions in malt extract broth. A) *A. niger*, B) *A. fumigatus*, and C) *A. terreus*.

The MALDI-TOF mass spectrum for each species grown under submerged conditions contains sufficient information to allow these species to be easily discriminated. Contrast the MALDI-TOF fingerprint mass spectrum for *A. niger* grown under submerged conditions (Figure 15A) with that of *A. niger* grown on MEA (Figure 5A). Specifically, five significant peaks were identified, and the replicate cultures grouped together and were correctly identified with only a 4% error rate. Two canonical functions were used to properly discriminate and group these fungi based predominantly on the peak intensity (Figure 16).

Figure 16: 2D canonical distribution of fungi grown under submerged culture conditions, $n= 8$ replicate *A. niger* extracts, $f= 8$ replicate *A. fumigatus* extracts, and $t= 8$ replicate *A. terreus* extracts.

Inhibition of Melanin Production via Tricyclazole

Fungi grown in submerged conditions form mycelial masses, a different growth state than sporulation, which has been used in previous studies in our laboratory (64, 65). Because fungi grown under submerged conditions may contain altered protein concentrations and exhibit significant changes to the fingerprint mass spectrum, it makes comparison with sporulating MEA cultures of other *Aspergilli* difficult. Therefore, it was necessary to grow fungi under sporulating conditions and to block melanin production to determine if MALDI-TOF MS fingerprint mass spectra could be acquired from these species. Therefore, each fungal species was grown under sporulating conditions identical to our previously published fungal mass fingerprinting methodology, but in the presence of tricyclazole. Tricyclazole is an antifungal agent that specifically inhibits DHN-melanin formation by inhibiting two reductases critical to the pentaketide DHN-melanin synthesis pathway (96). Therefore, fungi were grown on MEA containing tricyclazole, and then analyzed as above. It is important to note that *A. fumigatus*, when grown on agar containing tricyclazole, appeared light brown in color, similar to *A. terreus*,

however, when grown on MEA only, the color of the culture is dark green. Conversely, when *A. niger* was grown under tricyclazole conditions, a significant color change was not noted (Figure 17).

Figure 17: Appearance of cultures when grown on MEA only (top) or MEA supplemented with 50 µg/mL (bottom) for 7 days. A) *A. niger* grown on MEA, B) *A. niger* grown on MEA with tricyclazole, C) *A. fumigatus* grown on MEA, D) *A. fumigatus* grown on MEA with tricyclazole, E) *A. terreus* grown on MEA, and F) *A. terreus* grown on MEA with tricyclazole.

In general, spectra obtained from cultures grown on agar containing tricyclazole for 14 days contained peaks that were of higher abundance than the spectra obtained from samples grown on MEA only, despite the difference in phenotypical color not being visibly altered in *A. niger* or *A. terreus*. This was evident for cultures of *A. niger*, where peaks were rarely discernable from the background noise of the spectra when the fungus was grown on MEA only, however spectra from *A. niger* grown on agar supplemented with tricyclazole five dominant peaks were present, as shown in Figure 18.

Figure 18: MALDI-TOF mass spectra of *A. niger* cultures grown for 7 days (left) or 14 days (right). A) 7 days *A. niger* MEA, B) 7 days *A. niger* MEA + 1% EtOH, C) 7 days *A. niger* MEA + 1% EtOH + 50 µg/ml tricyclazole, D) 14 days *A. niger* MEA, E) 14 days *A. niger* MEA + 1% EtOH, and F) 14 days *A. niger* MEA + 1% EtOH + 50 µg/ml tricyclazole.

In addition, spectra from *A. terreus* and *A. fumigatus* grown on both MEA and MEA containing tricyclazole contained peaks of matching m/z , however, those grown in tricyclazole conditions appeared as increased peak intensity (Figures 19 and 20). Peak intensity is an important variable for the ability to canonically discriminate between the samples, as shown from the data obtained from the submerged culture identification.

Figure 19: MALDI-TOF mass spectra of *A. fumigatus* cultures grown for 7 days (left) or 14 days (right).

A) 7 days *A. fumigatus* MEA, B) 7 days *A. fumigatus* MEA + 1% EtOH, C) 7 days *A. fumigatus* MEA + 1% EtOH + 50 µg/ml tricyclazole, D) 14 days *A. fumigatus* MEA, E) 14 days *A. fumigatus* MEA + 1% EtOH, and F) *A. fumigatus* 14 days MEA + 1% EtOH + 50 µg/ml tricyclazole.

Figure 20: MALDI-TOF mass spectra of *A. terreus* cultures grown for 7 days (left) or 14 days (right). A) 7 days *A. terreus* MEA, B) 7 days *A. terreus* MEA + 1% EtOH, C) 7 days *A. terreus* MEA + 1% EtOH + 50 $\mu\text{g/ml}$ tricyclazole, D) 14 days *A. terreus* MEA, E) 14 days *A. terreus* MEA + 1% EtOH, and F) 14 days *A. terreus* MEA + 1% EtOH + 50 $\mu\text{g/ml}$ tricyclazole.

Another variable that was examined was culture time. It was hypothesized that the longer the cultures were grown, the more spectra inhibiting melanin would be produced and retained within the fungal spore; therefore, fungi grown for a shorter duration would contain less melanin, and thus produce a unique fingerprint with discernable peaks. In this series of experiments, cultures were grown for seven days under the same conditions as above, and analyzed as previously described. Spectra were obtained from each species from MEA only, control MEA with ethanol, and MEA with the addition of tricyclazole (Figures 18-20 left). Similar results were obtained from samples that were grown under tricyclazole conditions for seven days as were seen for the 14 day culture time. The presence of peaks of similar m/z were observed from each spectra, however, the relative abundance of each peak was increased under tricyclazole conditions when compared to MEA only. The shorter growth duration, with the presence of tricyclazole, did however aid in the visualization of more peaks for *A. niger*.

Chapter IV

Discussion

The necessity for rapid identification of microbial organisms for the successful treatment of patients with active infections has been shown in multiple studies. Current identification techniques, such as viable culture often require relatively long incubation periods, or have the potential for false-positive or false-negative results, as is the case for many current molecular and immunodiagnostic assays. Additionally, molecular and immunodiagnostic techniques have the capability to be rapid, however, there is a general lack of species-specific primers or monoclonal antibodies (19-24, 31). These techniques are used predominantly to detect the presence of a microorganism in a sample, not necessarily the identity, which is often required to determine the proper course of treatment. Relatively recently, MALDI-TOF MS, a rapid, non-biased, discriminate analytical technique has been shown to be a promising technique for fingerprinting microbial species. Specifically, MALDI-TOF MS has been used to identify clinically relevant bacteria and viral components, as well as fungi (43-62). Fungi are ubiquitous microorganisms capable of causing fatal invasive infections in immunocompromised individuals (1, 2). The successful treatment of invasive fungal infections is directly dependent on the rapid identification of the causal fungus and subsequent treatment of the patient with appropriate prophylactics (1).

Though MALDI-TOF MS has shown great promise in the utility of identifying fungi, one common problem has been reported by several groups when attempting to fingerprint darkly pigmented fungi. Darkly pigmented fungi commonly fail to produce a spectrum containing peaks that allow for their discrimination from other fungi (64, 73, 75). Because darkly pigmented fungi have been isolated from contaminated indoor environments, and have been implicated in a vast array of diseases, these fungi are medically important, and their rapid identification is necessary for the successful treatment of disease. Therefore, the purpose of this study was to identify the cause for the inability to fingerprint darkly pigmented fungi, and to examine different methods that would allow for the fingerprinting of these organisms.

One of the many purposes of fungal pigmentation, such as is present in darkly pigmented fungi, is to protect the fungus from UV light (77-79); therefore, it was hypothesized that dark fungal pigments were preventing the analytes in the MALDI-TOF MS preparation from forming peaks in the spectrum potentially by absorbing energy from the UV laser, used to induce ionization of the analyte, during MALDI-TOF MS analysis. In order to address this hypothesis, HSA was spiked with synthetic melanin and then analyzed for the 66 m/z HSA peak. It was determined that the presence of high concentrations of synthetic melanin inhibited the formation of the spectra, as the control HSA only 66 m/z peak height was reduced by 100% for concentrated synthetic melanin samples.

Because the synthetic melanin examined in the preceding experiments is not chemically identical to those obtained from fungal sources, fungal pigments from three different species of *Aspergillus* were isolated to determine whether these naturally obtained fungal pigments would also suppress HSA $[M+H]^+$ ion formation in MALDI-TOF mass spectra. Fungal pigments were extracted from *A. niger*, a darkly pigmented fungus which was unable to be fingerprinted previously by multiple laboratories, *A. fumigatus*, which contains high concentrations of DHN- melanins yet still formed a unique spectrum. *A. terreus*, a fungus which is light brown when grown under sporulating conditions and also produced a unique spectrum. Before MALDI-TOF analysis was conducted with these samples, the pigment extracts were characterized. Pigments extracts from *A. terreus* appeared most like that of the synthetic melanin, whereas *A. niger* and *A. fumigatus* pigments contained cell wall components and maintained the shape of the spores from which they were extracted. In addition, each sample contained similar quantities of carbon, hydrogen, nitrogen, and oxygen, while none contained sulfur. This information helped to identify these pigments as potential eumelanins, as opposed to phaeomelanins, which contain sulfur (80, 84).

Furthermore, 1 mg/mL of each pigment exhibited strong absorption in the 200-800 nm range when analyzed by UV/VIS spectroscopy. Absorption for each pigment approached 1.0 at 337- nm which is the emission wavelength of the nitrogen laser used for MALDI-TOF MS. This is consistent with previous studies that have demonstrated the photoprotective properties of melanin in fungi, whereby melanins absorb UV radiation and dissipate this energy as heat via rapid internal conversion (84-86).

Although the precise mechanism(s) by which the MALDI desorption/ionization event proceeds remains the subject of much discussion and active research (39), it is clear that the unique properties of melanin could interfere in a number of fundamental ways with the MALDI process. One plausible explanation for the observed inhibition of MALDI-TOF ionization is that melanin in fungal samples (particularly at high concentrations) directly competes with the organic acid matrix for irradiating photons. Following the MALDI laser pulse, ionization in the dense plume above the sample deposit will only occur where there is a sufficient population of electronically excited matrix molecules. If a photoexcitation/pooling mechanism for MALDI is assumed (76), mobile excitons in the bulk sample deposit could be quenched by melanin.

To demonstrate that melanin influences MALDI-TOF MS ionization, HSA was spiked with different concentrations of each of these fungal pigment extracts and the 66 m/z peak height was analyzed. The presence of pigments from each fungus directly inhibited the 66 m/z peak height formation, as greater than 89% of the 66 m/z peak height was inhibited in the most concentrated samples of each pigment. Even at 0.001 mg/mL concentration of each pigment, with the exception of *A. niger*, the HSA peak was inhibited by greater than 10%. Surprisingly, the pigment isolated from *A. terreus*, which produces a unique fingerprint spectrum, inhibited HSA peak formation by approximately 23% at the lowest concentration. To understand the influence of melanin on the sample spot, sample spots from each pigment preparation were visualized by microscopy. The results of these studies demonstrated that the pigment in each sample spot was not homogeneously distributed. As shown by the FESEM data, the *A. terreus* pigment extract appeared as crystals of pigment and did not maintain the ultrastructural morphology of the spore wall as observed with *A. fumigatus* and *A. niger*. Therefore, it is possible that the pigment extracted from *A. terreus* was more homogeneously distributed throughout the sample spot, and that the probability of the 90 laser strikes, which are culminated during each MALDI-TOF MS analysis, were present in more of the independent spectra obtained during each laser strike than in those obtained from *A. fumigatus* and *A. niger*. In order to address the hypothesis that the heterogeneity of the spot would alter the mass spectrum, MS equipped with a MALDI source was used to analyze angiotensin II which was spiked with *A. fumigatus* pigment preparation. Using this method, a single spectrum was obtained

from an area within the sample spot, as directed by the user. It was determined that the angiotensin II peak height was greatly reduced in an area containing visible pigment as opposed to an area within the same spot that did not contain pigment. This evidence directly shows the ability of pigment to prevent the formation of peaks within a sample.

In order to determine if darkly pigmented fungi could be fingerprinted if pigmentation was not present, several culture techniques were used to produce fungi which lack pigmentation. The first method used was by growing the cultures under submerged conditions, during which the fungi do not produce pigment. These samples were analyzed and found to produce unique fingerprints which allowed them to be discriminated in canonical plots. Discrimination was based on the presence of 5 unique peaks that were discernable based on their relative intensities. These growth conditions were not the same as those which had been used to acquire previous fingerprinting data, as these conditions induced the formation of hyphae, as opposed to sporulation.

To further explore the influence of demelanizing fungal spores, fungal cultures were grown on MEA agar in the presence of tricyclazole, an antifungal agent known to specifically inhibit the formation of DHN-melanin. DHN-melanin has been reported to be present in *A. fumigatus* and potentially *A. niger*. When grown in the presence of tricyclazole, *A. fumigatus* changes from a dark green color to a light brown color, closer to that of *A. terreus*; however, *A. niger* did not change color. Despite the difference in color changes between the two fungi, the presence of tricyclazole increased the peak intensities for both *A. fumigatus* and *A. terreus*, but more importantly, resulted in the formation of 2 distinct peaks were observed for *A. niger*. Furthermore, pigmentation increases with the culture age, therefore fungi grown for 7 days instead of 14 days formed spectra that contained a greater paucity of peaks with higher relative abundance than those grown for 14 days under all conditions. In addition, *A. niger* produced a unique fingerprint spectrum at 7 days when grown on MEA only which was not observed in 14 day cultures.

In conclusion, fungal melanin suppresses peptide and protein ion signals during the acquisition of MALDI-TOF mass spectra. Suppression of peptide and protein standard ionization was observed upon addition of both commercially available synthetic melanin and melanins purified from three species of

Aspergillus to the MALDI sample. Suppression of peptide and protein $[M+H]^+$ ion signals was observed at melanin concentrations greater than 500 ng/ μ L, a concentration significantly less than the level of melanin obtained from a fungal culture. Ion suppression occurs in a concentration dependent manner, approaching 100% at high melanin concentrations (10 μ g/ μ L). Culture conditions may be manipulated to block fungal melanin synthesis and obtain acceptable MALDI-TOF fingerprint mass spectra from darkly pigmented species such as *A. niger*. MALDI-TOF fingerprint mass spectra were acquired from *Aspergilli* grown under liquid culture conditions, as well as cultures grown on malt extract agar supplemented with 50 μ L/mL of tricyclazole, an antifungal agent used to block DHN-melanin synthesis.

To our knowledge, this is the first report demonstrating the detrimental effects of fungal pigments on the acquired MALDI-TOF mass spectrum, as well as the first report of successful fingerprinting of darkly pigmented fungi such as *A. niger*. This data represents an important step forward for the use of MALDI-TOF MS for the rapid identification of darkly pigmented fungi. In addition, these techniques may be used to aid in the identification of specific biomarkers for medically important fungi. With the identification of species-specific biomarkers via MALDI-TOF MS and subsequent sequencing and identification via MS/MS, recombinant proteins based on these biomarkers may be made and used to immunize mice for the production of monoclonal antibodies. Those antibodies may be exceptionally beneficial in the development of rapid, species-specific immunodiagnostic assays.

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CURRICULUM VITAE

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EDUCATION

M.S. in Biomedical Sciences West Virginia University, Morgantown, WV <i>Advisor: Dr. Donald Beezhold</i>	In progress GPA 3.2/4.0
B.S. in Biology, concentration in Biotechnology Minor in Chemistry Frostburg State University Frostburg, MD	December 2004 GPA 3.4/4.0

AWARDS

West Virginia University Graduate Travel Award	2007
HOPE Science and Technology Scholarship	2001-2004
Academic Excellence Scholarship	2001-2004
Lewis J. Ort Scholarship	2003
Hadley and Smouse Scholarship	2003

POSITIONS HELD

Graduate Researcher , West Virginia University, Morgantown, WV <i>Member of the Allergy and Clinical Immunology Branch at CDC/NIOSH utilizing mass spectrometry to fingerprint mycobacteria and fungal isolates, as well as developing a murine model of dry myco aerosol exposures and characterizing the resulting immune response using histology, flow cytometry, and immunoassays.</i>	June 2005-present
Research Assistant I , Windber Research Institute, Windber, PA <i>Member of the proteomics laboratory working with preparation of breast cancer samples for 2-DIGE and subsequent mass spectrometry analysis searching for unique biomarkers.</i>	March-June 2005
Student Intern , Windber Research Institute, Windber, PA <i>Member of the proteomics laboratory performing western blot analysis on breast cancer samples.</i>	Summer 2004

PRESENTATIONS

110th General Meeting of the American Society for Microbiology , San Diego, CA <i>"Murine Pulmonary CD4 and CD8 T-cell Responses to Aspergillus conidia are Species-Dependent"</i>	May 2010
Van Liere Convocation and Research Day , Morgantown, WV <i>* "Developing and Characterizing a Mouse Model of Dry Myco aerosol Exposures"</i>	April 2009
Joint Statistical Meeting , Denver, CO <i>"Classification of Penicillium Strains Using Discriminant Analysis"</i>	August 2008

- 56th ASMS Conference on Mass Spectrometry and Allied Topics**, Denver, CO **June 2008**
"Discrimination of Penicillium isolates by Matrix Assisted Laser Desorption/ Ionization Time- of- Flight Mass Spectrometry Fingerprinting"
- 56th ASMS Conference on Mass Spectrometry and Allied Topics**, Denver, CO **June 2008**
**"Discrimination of Aspergillus isolates at the species and strain level by MALDI-TOF mass spectrometry fingerprinting"*
- Van Liere Convocation and Research Day**, Morgantown, WV **April 2008**
** "Developing and Characterizing a Mouse Model Of Fungal Exposure To Dry Spores"*
- Van Liere Convocation and Research Day**, Morgantown, WV **April 2007**
**"Optimization of MALDI-TOF Mass Spectrometry Analysis of Mycobacteria"*
- American Academy of Asthma Allergy and Immunology**, Philadelphia, PA **March 2008**
"Identifying fungal isolates using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry"
- 55th ASMS Conference on Mass Spectrometry and Allied Topics**, Indianapolis, ID **June 2007**
** "Investigation of Sample Preparation Strategies for MALDI- TOF MS Analysis of Mycobacterial Proteins"*
- 55th ASMS Conference on Mass Spectrometry and Allied Topics**, Indianapolis, ID **June 2007**
"MALDI-TOF MS Fingerprinting of Fungal Isolates"
- 55th ASMS Conference on Mass Spectrometry and Allied Topics**, Indianapolis, ID **June 2007**
"Identification of Mycobacteria and Mycobacteria Biomarker Proteins by Novel Biological Preparation Combined with Tandem Mass Spectrometry"

NOTE: * *Denotes first author posters*

PUBLICATIONS

- A.D. Buskirk**, B.J. Green, J.M. Hettick, I. Chipinda, B.F. Law, P.D. Siegel, J.E. Slaven, and D.H. Beezhold. "Fungal pigments inhibit the MALDI-TOF mass spectrometry analysis of darkly pigmented fungi". *Analytical Biochemistry*. 2010. Submitted.
- S.P. Templeton, **A.D. Buskirk**, B. Law, B.J. Green, and D.H. Beezhold. "*Aspergillus* Conidia Germination Enhances Murine Airway CD8⁺ T-cell Responses". Manuscript in preparation.
- J.M. Hettick, B.J. Green, **A.D. Buskirk**, J.E. Slaven, M.L. Kashon, and D.H. Beezhold. "Discrimination of fungi by MALDI-TOF mass spectrometry". *Rapid Characterization of Microorganisms by Mass Spectrometry*, P. Demirev and C. Fenselau, Eds. **2010**. In press.
- S.P. Templeton, **A.D. Buskirk**, B.J. Green, D.H. Beezhold, D. Schmechel. "Murine Models of Airway Fungal Exposure and Allergic Sensitization". *Medical Mycology*. **2009**. 48(2) 217-228.
- J.M. Hettick, B.J. Green, **A.D. Buskirk**, M.L. Kashon, J.E. Slaven, E. Janotka, F.M. Blachere, D. Schmechel, and D.H. Beezhold. "Discrimination of *Penicillium* isolates by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting." *Rapid Communications in Mass Spectrometry* **2008**. (16) 2555-2560.
- J.M. Hettick, B.J. Green, **A.D. Buskirk**, M.L. Kashon, J.E. Slaven, E. Janotka, F.M. Blachere, D. Schmechel, and D.H. Beezhold. "Identification of *Aspergillus* isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting." *Analytical Biochemistry* **2008**. (380) 276-281.