1-1-2015

Multivariate Analysis of Fungal Volatile Metabolites for Aflatoxigenic Fungi Detection

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Multivariate analysis of fungal volatile metabolites for aflatoxigenic fungi detection

By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Chemistry
in the Department of Chemistry

Mississippi State, Mississippi

May 2015

Multivariate analysis of fungal volatile metabolites for aflatoxigenic fungi detection

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Pages in Study: 179

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My research focuses on the development of a novel method for the fast detection of aflatoxin-producing fungi from the volatile organic compounds that they produce. Aflatoxins have received great attention because of their demonstrated potent carcinogenic effect in susceptible laboratory animals and their acute toxicological effects in humans. Traditional detection and quantification techniques are considered time-consuming, high cost, and require technical professionals.

The `odor' or so called volatile metabolites released by a fungus is the key for fast detection. Several researchers have reported that diverse fungi species have unique volatile metabolite patterns. This study focuses on answering several questions: Is it possible to discriminate aflatoxins-producing fungi from other fungi based on volatile metabolites? What are the key discriminating biomarkers related to each fungus? Does the growth environment have an effect on the production of volatile metabolites? What chemicals are consistently emitted by a fungus under varied conditions?

To answer these questions, one toxigenic and one nontoxigenic *A. flavus* isolate were studied to evaluate the microbial volatile organic compound (MVOC) profiles. The results described in chapter two of this dissertation indicate that MVOC production is time-dependent and that aflatoxigenic and non-aflatoxigenic strains have different MVOC expression patterns. Chapter three describes the effects of experimental parameters on fungal volatile metabolites. The identity and quantity of MVOCs can be affected by many factors including SPME fiber type, fungal growth media, and growth temperature. A CAR/PDMS coated fiber performed better than the other SPME fibers by collecting a larger variety and quantity of MVOCs. Fungi grown on the chemical defined liquid media produced much larger quantities of MVOCs compared to the other media. The highest MVOC production results were found at 30 ℃.

The fungi discrimination study was extended in chapter four by including 3 toxigenic and 3 non-toxigenic isolates using multivariate analysis. The results indicate that volatile patterns vary even at the fungal isolate level and that discrimination of aflatoxin-producing fungi from non-toxigenic fungi is possible.

Key words: *Aspergillus flavus*, microbial volatile organic compounds, multivariate analysis

DEDICATION

I dedicate my dissertation work to my loving parents, Shaohua Sun and Lishi Gao who always support and encourage me to improve myself through all my walks of life. I am honored to have them as my parents.

This dissertation is also dedicated to my memory of my grandfather, Shenglin Sun. I miss him every day, but I am sure he must be happy to see the completion of my dissertation. I am thankful to his lessons, inspiration, and affection.

I also dedicate to my great grandmother, Guiying Yang who took care of me since I was born until I went to college. I regret I was not with her before she died. This dissertation is dedicated to her support and love.

This dissertation is also dedicated to my grandmother, Yuhuan Yang who is fighting with Spinocerebellar Atrophy. I hope she will be alright soon.

I dedicate this work to my love, my girlfriend, Ting Zhang who supported and consoled me when I felt disappointed and frustrated.

In the end, I dedicate all my professors, friends and relatives for their support, encouragement, education, criticism during my journey to obtain Ph.D.

ACKNOWLEDGEMENTS

I would like to gratefully and sincerely thank Dr. Todd Mlsna for his guidance, understanding, patience, and most importantly, his friendship during my graduate studies at Mississippi State University. He encouraged me to not only grow as an analytical chemist but also an independent thinker. That is the reason why I came to the United States. I do what I believe and what I want to do. Dr. Mlsna' intellectual heft is matched only by his genuinely good nature and down-toearth humility, and I am truly fortunate to have had the opportunity to work with him. I realized that a good scientist is not only a good researcher but also has a believe and dream to make this world better.

I would also like to thank Dr. Richard Baird for his assistance and guidance in microbiology work. I thank his encouragement and advice for continuing my PhD study and job hunting.

I also appreciate to my committee members, Drs. David Wipf, Dongmao Zhang, Joseph Emerson, and Steven Gwaltney for their time, valuable criticisms, and suggestions for my Ph.D study. Especially, I learned a lot in depth when I defended my proposal. I realized the importance of thinking in depth for a successful analytical chemist.

I would also like to thank Dr. Deb Mlsna for her kindly assistance with excellent advice and detailed review during the preparation of this dissertation. I always admire the passion she put in the education and her teaching skills for enhancing the students' knowledge on chemistry.

My gratitude extends to Dr. Alicia Wood-Jones, Dr. Beth Stokes, and Mary Scruggs from the Department of Biochemistry, Molecular Biology, Entomology, & Plant Pathology for their guidance, advice, and invaluable support with my microbiology work.

I am also thankful to all my undergraduate students, Brad Reese, Patrice Simmons, and Julie Gower for their assistant for my research. I also enjoy teaching them what I know about chemistry. It was a great and valuable experience I have here in this department.

I will never forget chemistry lab teaching experience here and lab coordinator Dr. Patrick Hillesheim and former coordinator Sapna Patel for their patience, guidance. The opportunity of teaching improved my English, presentation skills, and even make me like teaching.

Last, but not the least, I would like to thank all the faculty and staff members of the Department of Chemistry for sharing their knowledge and providing support. I not only learned the cutting-edge technology here, but also understand the importance of caring other, taking responsibility, obtaining new knowledge. I think the latter factors are the key for having a successful career. I would like to express my deep thanks to my former lab colleagues, Drs. Bidhya Kunwar, Shamitha Dissanayake, Sameera Gunatilake, and Rangana Wijayapala, and my present labmates, Akila Karunanayeka, Matthew Essandoh, Jinyan She, Suranga Rajapaksha, and Narada Dewage for the support they provided me with great memories.

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LIST OF ABBREVIATIONS

A.flavus	*Aspergillus flavus*
CA	Cluster analysis
CAR/PDMS	Carboxen/ Polydimethylsiloxane SPME fiber
CAR/DVB/PDMS	Carboxen/Divinylbenzene/ Polydimethylsiloxane fiber
CDA	Chemical defined media
CDL	Chemical defined liquid
CMA	Corn meal agar
CSA	Czapek solution agar
DA	Discriminant analysis
DVB/PDMS	Divinylbenzene/ Polydimethylsiloxane SPME fiber
GC-MS	Gas chromatography mass spectrometry
HS-SPME	Headspace solid phase microextraction
LC-MS	Liquid chromatography mass spectrometry
LDA	Linear discriminant analysis
LSD	Fisher's least significant difference
MEA	Malt extract agar
MVDA	Multivariate data analysis
MVOCs	Microbial volatile organic compounds
NIST	National Institute of Standards and Technology

PCA	Principal component analysis
PLS	Partial least square analysis
PLS-DA	Partial least square discriminant analysis
RI	Retention index
RT	Retention time
TIC	Total ion count
VIPs	Variable importance in projection
VOCs	Volatile organic compounds

CHAPTER I

INTRODUCTION

1.1 Introduction to Aspergillus flavus

The genus *Aspergillus* can be found in the Trichocomaceae family, Eurotiales order, Eurotiomycetes class, Ascomycota division and is a member of the Deuteromycetes fungi kingdom. Approximately 250 species of *Aspergillus* are known.[1] Aspergilli species have no known sexual state or sexual reproduction and are consequently called Fungi Imperfecti because their life cycles are considered to be "imperfect".[2] Reproduction is exclusively accomplished through the production of spores. *Aspergilli* spores can spread through air, soil, water runoff, or through the transportation of contaminated grains. *Aspergilli* growth can occur on a wide variety of different organic materials from live plants to building construction materials.[2]

Aspergillus strains are important to the fermentation and food manufacturing industries where approximately sixty species have been utilized for commercial application in brewing wines and producing vinegar.[3] Many *Aspergilli* are producers of beneficial secondary metabolites, such as antibiotics[4] and other pharmaceuticals.[5] These secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism.

An example of a beneficial secondary metabolite comes from *Aspergillus terreus* which produces lovastatin, a potent cholesterol-lowering drug.[6] Other *Aspergilli* secrete

antibiotics (penicillin[7] and cephalosporin[8]), antifungals (griseofulvin[9]), and anti-tumor drugs (terrequinone A[10]). However, some *Aspergilli* secondary metabolites are toxic to humans. These compounds are referred to as mycotoxins including aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, T-2 toxin, and zearalenone.[11] Within the genus *Aspergillus*, *Aspergillus flavus* (*A. flavus*) is considered to be the most notorious because it produces aflatoxins, which are among the most toxic of any naturally occurring chemicals.[12] *A. flavus* can survive in temperatures ranging from 12 °C to 48 °C, while the optimal growth temperature is from 28 °C to 37 °C.[13]

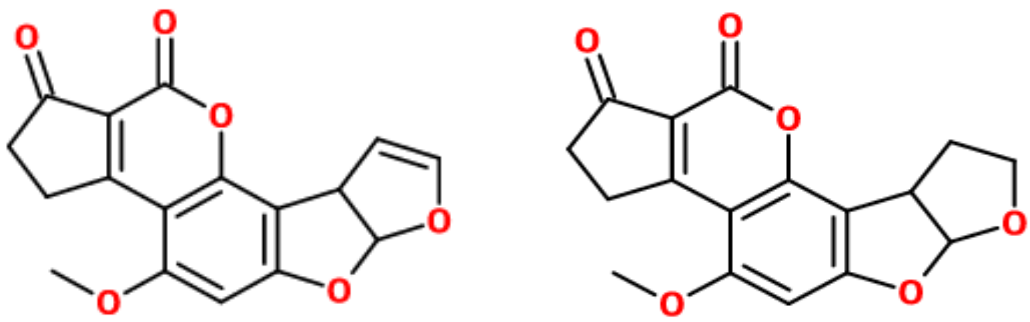
The fungus *A. flavus* is an opportunistic plant pathogen, affecting many agriculture crops such as maize (corn), cotton, and groundnuts (peanuts).[14] Because *A. flavus* lacks host specificity, it can attack seeds of monocots, dicots, and seeds produced both above ground (corn) and below the ground (peanuts).[14] Under weather conditions favorable for its growth, *A. flavus* can cause ear rot on maize, resulting in significant economic loss to farmers.[14] Pre-harvest and post-harvest contamination of these crops with aflatoxins is common.

To minimize exposure, maximum levels of aflatoxins in many commodities have been set at 20 ppb or lower by most countries.[15, 16] Regulatory guidelines of the U.S. Food and Drug Administration (FDA) specifically prevent the sale of commodities if contamination by aflatoxins exceeds 20 ppb total aflatoxins for interstate commerce of food and feedstuff and 0.5 ppb aflatoxin M1 in milk.[17] The Food and Agriculture Organization (of the United Nations) has estimated that 25% of the world's crop are affected by mycotoxins, of which the most severe are aflatoxins.[18] It has been estimated that in the US losses due to aflatoxins are a $225 million/year impact, which does not

include mitigation costs ($20-30 million/year just for testing).[18] A lower standard limit of 4 ppb has been adopted by the European Union. If adopted by the rest of the world, this 4 ppb aflatoxin level in peanuts would be estimated to cost US $450 million annually in lost exports.[18]

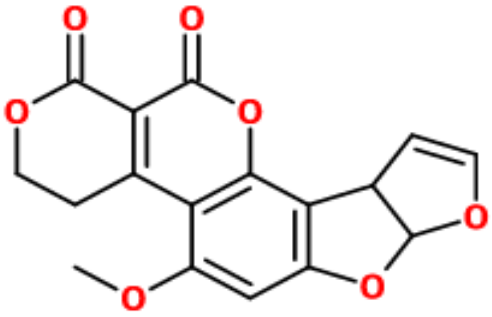
1.2 Aflatoxins and their biosynthesis

Aflatoxins were first identified in England about 60 years ago. They are believed to be the cause of Turkey X disease, which resulted in approximately 100,000 domestic turkey poult deaths in the 1960's.[19] Most *A. flavus* isolates produce aflatoxins B1 and B2, while *A. parasiticus* produces aflatoxins B1, B2, G1, and G2.[20] The structures for the four most prevalent aflatoxins can be found in Figure 1.1. Aflatoxin M1 can appear following consumption of contaminated feed by dairy cows, in milk, and milk products as the hydroxylated derivative of aflatoxin B1.[21] In addition to aflatoxin B1 and B2, *A. flavus* also produces many other mycotoxins such as cyclopiazonic acid, kojic acid, beta-nitropropionic acid, aspertoxin, aflatrem, and aspergillic acid.[22]

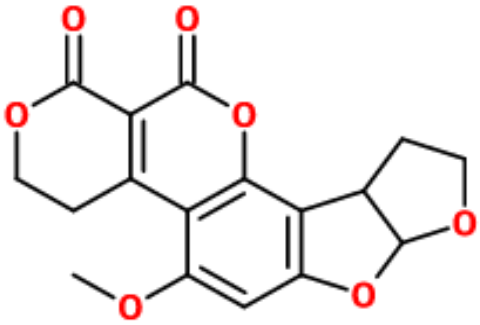


Aflatoxin B1

Aflatoxin B2



Aflatoxin G1



Aflatoxin G2

Figure 1.1 Chemical structures of aflatoxins B1, B2, G1, and G2.

Aflatoxin B1 is the most toxic of the four major aflatoxins. It is a potent carcinogen in humans[12] and animals including nonhuman primates,[23] birds,[24] fish,[25] and rodents.[26] Chronic exposure can result in suppressed immune response,[27] malnutrition,[28] fatty infiltration of the liver,[29] hepatic lesions,[30] and even hepatomas.[31] Because of a lack of detection, monitoring and regulating measures to safeguard the food supply in developing countries, food and feed contamination by aflatoxins is a significant world food safety issue.[14] For example, in western India in 1974, 108 people died from aflatoxin poisoning through the consumption of maize contaminated by *A. flavus*.[32] In April 2004, one of the largest aflatoxicosis outbreaks occurred in rural Kenya, resulting

in 317 cases of aflatoxin exposure and 125 deaths following the consumption of aflatoxin-contaminated homegrown maize.[33] Symptomatic aflatoxin poisoning is rarely observed in the U.S., but does occasionally occur in animals. The most notable recent case involved the reported death of over 100 dogs in 2006 following the consumption of tainted dog feed.[34]

Since the identification of aflatoxins, extensive efforts have been made and expenses incurred worldwide to monitor aflatoxin occurrence and to develop control strategies. The discovery of a colored mutant that accumulates the brick-red pigment, norsolorinic acid (NOR) (metabolite produced in *A. parasiticus),* marked a milestone in the understanding of the chemistry of aflatoxin biosynthesis.[35] Since NOR is the earliest and the first stable aflatoxin precursor in the aflatoxin biosynthetic pathway, this discovery led to the identification of other key aflatoxin intermediates and established the early step metabolites in the aflatoxin pathway.

This provided the opportunity to isolate the first aflatoxin pathway gene that encodes a reductase for the conversion from NOR to eventually aflatoxins. After the cloning of several important aflatoxin pathway genes, the aflatoxin pathway gene cluster was discovered in *A. parasiticus* and *A. flavus*.[36] The discovery of the cluster promoted the elucidation of the biosynthetic pathway, which includes biosynthetic proteins and the associates.[37-39] As many as 30 genes are potentially involved in aflatoxin biosynthesis. In *A. flavus* and *A. parasiticus* the aflatoxin pathway genes are clustered within a 75-kb region of the fungal genome on chromosome III roughly 80 kb away from a telomere.[40]

1.3 Microbial volatile organic compounds

Microbial volatile organic compounds (MVOCs) are produced during the primary and secondary metabolism of micro-organisms such as fungi and bacteria.[41] The primary metabolism of micro-organisms includes the synthesis of DNA, and amino and fatty acids, whereas secondary metabolism involves pathways and products that are not generally required for organism survival.[42] It is often stated that MVOCs are by-products of primary metabolism and secondary metabolism.

Fungi or bacteria are reported to produce a wide range of volatile organic compounds including alcohols, aldehydes, ketones, terpenes, esters, ethers, sesquiterpenes, and sulfur compounds.[43] Importantly, the production of MVOCs has been found to be strain specific; therefore, these compounds can be used to classify fungi at the species level. For example, Fischer *et al.*[44] monitored and screened thirteen airborne fungal species frequently isolated in compositing plants. 2-Methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol were found in high quantities for nearly all species tested whereas 2-methyl-butanoic acid methyl ester was only emitted from *Emericella nidulans*, and hexanoic acid ethyl ester was only released by *Aspergillus candidus*. Larson and Frisvad[45] have successfully classified *Penicillium* species containing 132 isolates of 25 different taxa using qualitative MVOC data. MVOC data have also been utilized to discriminate toxigenic and non-toxigenic strains of specific species. Jelen *et al.*[46] found some differences among the strains of same species where the pattern of volatile sesquiterpenes was characteristic and distinctive for both toxic and non-toxic strains.

MVOC production is also influenced by the media on which fungi or bacteria grow, and the environmental conditions. In the study by *Polizzi et al.,* a *Penicillium polonicum*, an *Aspergillus ustus,* and a *Periconia britannica* strain were isolated from water damaged environments. The production of microbial volatile organic compounds were investigated by means of headspace solid-phase microextraction followed by GC-MS analysis.[47] The influence of temperature and relative humidity on growth, metabolism, and resulting MVOC production was found to be significant within a single fungal species.

Volatile biomarkers have been utilized for fungal detection using an electronic nose. Schiffman *et al*.[48] studied the ability of an electronic nose which contained 15 metal oxide sensors for discriminating among the fungi. The electronic nose was able to quantify five volatile organic compounds emitted by fungi; these data were used for fungi discrimination. It is also possible to predict one type of mycotoxin, deoxynivalenol (DON), levels in barley samples naturally contaminated with *Fusarium* species, using the MVOC patterns detected and quantified by either GC-MS or an electronic nose.[49]

1.4 Aflatoxin detection and quantification techniques

Many separation, detection, and classification techniques including high-performance liquid chromatography (HPLC),[50, 51] gas chromatography mass spectrometry (GC-MS),[52] enzyme linked immune-sorbent assay (ELISA)[53, 54] and multiplex polymerase chain reaction (multiplex PCR) assays,[55, 56] have been developed for early detection of aflatoxins in order to reduce the economic loss of infected crops. Most of the protocols used for HPLC detection of aflatoxins are very similar. HPLC detection protocols involve extraction, concentration, and reverse phase separation. The most

widely used extraction solvents are chloroform-water, methanol-water, or acetonitrile-water.[57] For clean-up and concentration, immuno-adsorbent column[58] and solid phase extraction cartridges are frequently used.[59] The most common detection methods for analysis utilize UV, fluorescence, or mass spectrometry detectors.

ELISA has become very popular recently due to its relatively low cost and easy application.[60, 61] Commercially available ELISA kits for detection of aflatoxins are normally based on a competitive assay format that uses a primary antibody specific for the target molecule. They can be portable, rapid, and are highly specific as well as simple to use. The disadvantage of these kits lies in the fact that they are for single use, which can increase costs of bulk screening.

Multiplex PCR assay has also been developed to detect aflatoxin-producing fungi by amplifying the aflatoxin biosynthetic genes: norsolorinic acid reductase (*nor-1*), versicolorin A dehydrogenase (*ver-1*), and sterigmatocystin O-methyltransferase (*omt-A*).[62] This approach has been largely applied to detect different microbial species, to differentiate closely related species, and to recognize single species. This approach also allows the detection of species that are present at low levels.[63] However, cross-amplification reactions and false positive signals are becoming a major concern when this technique is used as a defining method for differentiating microorganisms in complex matrices.[63]

Table 1.1 Comparison of aflatoxin fast detection techniques. Revised (adapted) with permission from Sankaran *et al.*[64] Copyright (2015) Elsevier B.V.

Characteristics	Molecular techniques	Imaging and spectroscopic techniques	VOCs profiling-based techniques
Accuracy of the method	Most accurate Molecular detection kits are under development	Plant and disease specific The higher the visible symptoms the better the accuracy	This method is in the developmental stages Identification of fungus-specific biomarkers can improve the accuracy
Cost	Moderately expensive and labor intensive	Relatively expensive	Low cost
Applicability for field work/Ruggedness	Field kits are being developed. It is difficult to develop kits for all diseases. It is difficult to automate the process for rapid detection	Can be automated or can be performed through remote operation easily	Moderately rugged, depending on the detector used for sensing VOCs.
Speed of detection	May require 24-48 h for reliable results	May require minutes for disease detection; depends on the computational speed as well as speed of scanner	May require significantly less time Once established this method can be automated with a robotic vehicle for plant disease detection

1.5 Metabolomic approach for fungal discrimination and biomarker identification

Metabolomics is the endpoint of the "omics cascade" including genomics, transcriptomics, proteomics, and varies in a manner similar to phenotype expression. It is a valuable emerging tool to study phenotype and change in phenotype caused by environmental influences, disease, or changes in genotype.[65] The metabolome represents a large variety of chemicals with diverse physical and chemical properties such as amino acids, lipids, organic acids and nucleotides.[65] Metabolic profiling and metabolic fingerprinting approaches are usually used for metabolomics investigations (Figure 1.2).

Metabolic profiling focuses on quantitative analysis of metabolites in a selected biochemical pathway or analysis of a specific class of compounds (lipids or fatty acids methyl esters). The results of metabolic profiling can be used to build databases that can be integrated with pathway maps or other "omics" data, which will enhance our biological understanding. For example, the quantitative analysis of fatty acids as fatty acid methyl esters using GC-FID (flame ionization detection) allows for microorganism identification based on the specific fatty acid composition of the cell wall.[66] However, the disadvantage of metabolic profiling is a clear understanding of the identity of the metabolites and how these metabolites vary with changes in environmental conditions and nutrient. A clear understanding of what constitutes a biomarker and how they change remains an unsolved challenge for modern analytical methods.

Metabolic fingerprinting focuses on comparing patterns or "fingerprints" of metabolites that change in response to disease, toxin exposure, or environmental or

genetic alterations. Metabolic fingerprinting can be used as a diagnostic tool through the evaluation of MVOC profiles of healthy and unhealthy organisms.

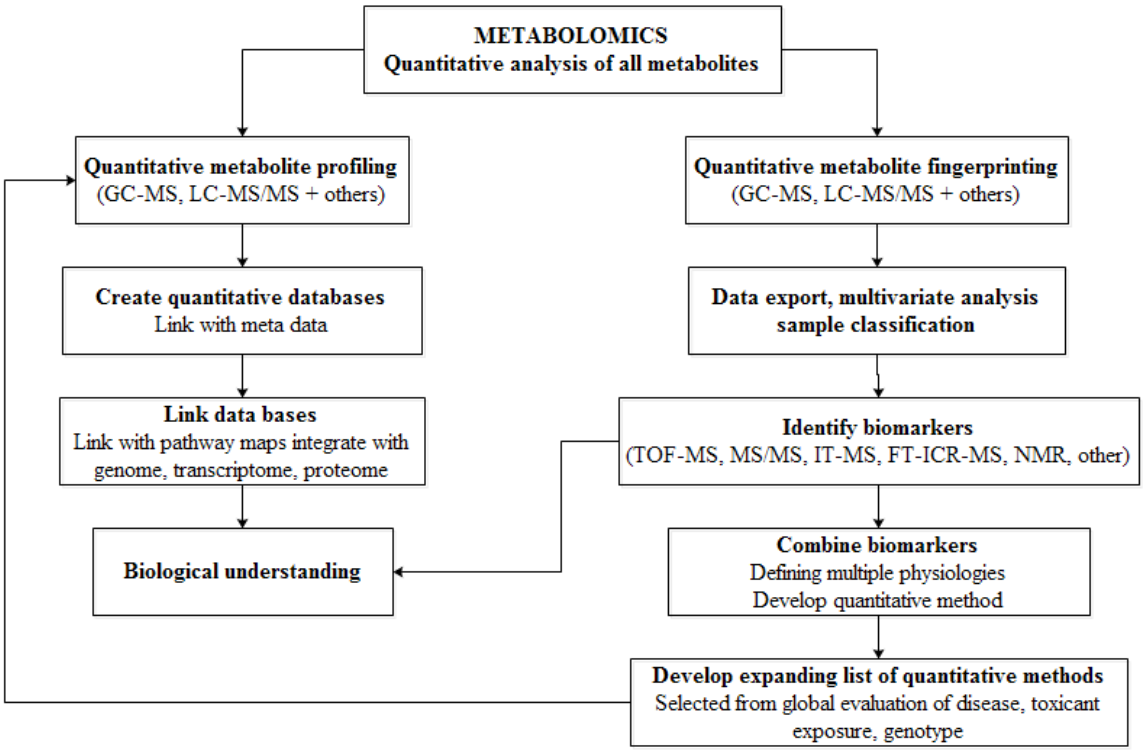


Figure 1.2 Approaches for metabolomic investigation. Revised (adapted) with permission from Dettmer *et al*. [67]

In our study, MVOC analysis has been used to determine the metabolic fingerprints of aflatoxigenic fungi. The MVOCs are collected by SPME fibers followed by analysis with GC-MS. Both the choice of analytical techniques and the experimental design for the metabolomics study are very important, and each requires careful consideration. In general, the GC-MS based metabolomics experiment needs to take the

following steps: 1) sample preparation and sampling, 2) sample analysis using GC-MS, 3) data export, and 4) data analysis.

1.6 Sample preparation and sampling

When conducting a preliminary search for biomarkers, one must reduce the influence of biological variability and obtain statistically validated data. At least five replicates of each sample and control were analyzed in each study. While processing fungi samples, special care must be taken to minimize the influence of growth conditions and contamination. For example, antibiotics should be added into the growth media to avoid the contamination of other bacteria and fungi before target fungus inoculations. The growth media should be fresh and cannot be used more than seven days after preparation. All growth media need to be identical in order to discriminate the *A. flavus* isolates because the number, quantities and even the identities of MVOCs can be significantly affected by the change of growth media.[68] The fungi isolates must be sub-cultured every two weeks to maintain the fresh culture and enough spores. The fungal cultures should be stored at a consistent temperature (an incubator at 30 ℃ was used for this study). Any glassware used for culturing must be sterilized to avoid contamination.

SPME was developed to address the need for rapid sample preparation both in the laboratory and on-site. In this technique, a small amount of absorbent dispersed on a solid support is exposed to the sample for a well-defined period of time. Quantification of target analytes can then be performed based on time accumulation of analytes in the coating. SPME includes three basic extraction modes: a direct extraction, a headspace extraction, and an extraction involving membrane protection. In this study, headspace extraction was used because the target analytes are volatile chemicals.

The performance of SPME is critically dependent on the selection of an appropriate coating. SPME fibers are commercially available with a wide range of sorbent coatings, and it is important to choose a fiber coatings that can extract the range of key analytes produced by the samples. To accomplish this, the types of SPME fiber must be compared before detailed metabolomics studies to obtain the desired quantitative and qualitative MVOCs data from the fungal samples. Types of commercially available SPME fiber coatings are shown in Table 1.2.

Table 1.2 Types of commercially available SPME fiber coatings and their target analytes[69]

Types of coating	Polarity	Target analyte
100 µm PDMS	Nonpolar	High affinity to nonpolar compounds, MW 50 ~ 310
85 µm Polyacrylate (PA)	Polar	Aromatic compounds and oxygenated analytes
60 µm PEG (Carbowax)	Polar	More selective toward polar analytes
65 µm DVB/PDMS	Bipolar	Semi-volatile analytes and large volatile analytes, MW 50 ~ 290
55 µm/30 µm DVB/CAR/PDMS	Bipolar	Polar and nonpolar compounds, MW 40 ~ 290
85 µm CAR/PDMS	Bipolar	Polar and nonpolar compounds, MW 30 ~ 160

DVB/PDMS is the cross-linked polydimethylsiloxane and divinylbenze coating.
CAR/PDMS is the cross-lined polydimethylsiloxane and carboxen coating.

Extraction parameters such as temperature, extraction time, pH, sample volume, and ionic strength of the solution are also essential to be optimized to achieve the goal of short analysis time, ideal sensitivity and reproducibility. However, the analytical optimization should not affect the chemical and physical properties of the biological

sample. For example, the change of sampling temperature can dramatically affect the growth cycle and MVOC production of fungi and bacteria. NaCl can increase solution ionic strength and thereby promote VOCs volatility; however, the effect of NaCl on the growth of fungi and bacteria are not well known. Hence, any MVOCs sampling strategy using SPME fiber must be closely followed to ensure reproducibility and reliability of the experiment data (Figure 1.4).

Figure 1.3 MVOCs sampling using SPME fiber in the incubator.

1.7 Multivariate analysis of VOCs data

A large amount of raw data are generated from GC-MS analysis of volatiles collected from biological samples. Multivariate analysis can be used for extracting important information from large datasets within a reasonable time. In chemistry and chemical engineering, the term "pattern recognition" is often used as a synonym for multivariate analysis. The "pattern" of a typical class (e.g. types of fungi) provides the

relations of observations within the class. If the "patterns" differ between classes, they can then be used to classify the observations on the basis of the similarity between their data and the "class pattern". Multivariate analysis can be applied to 1) give a brief overview of the data, 2) classify and discriminate among the groups of observations, 3) and build up a regression model between the variables **X** and response **Y**.

In order to provide a simple overview of the information in a data table, principal component analysis (PCA), one of the simplest multivariate analyses, is often used to understand the relationship of variables: which variables contribute the similar information to the PCA model, and which provide unique information about the observations.[70] For classification and discrimination, separate established class models are used for classifying new observations. The last stage of data analysis is regression modeling between **X**-variables (predictors) and **Y**-variables (responses). The responses are often laborious, time-consuming, and expensive to measure compared with predictors. In this case, partial least square (PLS) modelling is used to accomplish fast, accurate, and quantitative prediction of complex responses (e.g. quality of product, impurity of sample, concentration of toxins, or species identification). In this study, a special form of PLS was utilized - partial least square discriminant analysis (PLS-DA).

1.8 Pretreatment of data

In biological sample studies, data pretreatment needs to be considered because the variation of data is not only caused by biological variation but also caused by the properties of the data. For example, the average concentration of a single small biologically produced molecule can be much lower than the concentration of a highly abundant compound like ATP. However, from a biological point of view, metabolites

present in high concentration are not necessarily more important than those present at low concentration.[71] There are also possible large fluctuations in concentrations of many biologically produced chemicals under identical experimental conditions likely due to phenotype variations. Variations can also be introduced through sampling and analytical errors.[71] Data pretreatment is important in reducing the effect of a number of confounding factors that play no role in aiding isolate identification.

The selection of data pretreatment method not only depends on biological information for the analysis, but also on the data analysis method chosen such as PCA, PLS, and cluster analysis (CA). Table 1.3 lists the common data pretreatment methods and their goals, advantages and disadvantages.

Table 1.3 Overview of the pretreatment methods in this study[71]

Class	Method	Formula	Goal	Advantages	Disadvantages
I	Centering	$\tilde{x}_{ij} = x_{ij} - \bar{x}_i$	Focus on the differences and not the similarities in the data	Remove the offset from the data	When data is heteroscedastic, the effect of this pretreatment method is not always sufficient
II	Autoscaling	$\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{s_i}$	Compare metabolites based on corrections	All metabolites become equally important	Inflation of the measurement errors
	Pareto Scaling	$\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{\sqrt{s_i}}$	Reduce the relative importance of large values, but keep data structure partially intact	Stays closer to the original measurement than autoscaling	Sensitive to large fold changes
III	Log transformation	$\tilde{x}_{ij} = \log x_{ij}$ $\hat{x}_{ij} = \tilde{x}_{ij} - \bar{x}_i$	Correct for heteroscedasticity, pseudo scaling. Make multiplicative models additive	Reduce heteroscedasticity, multiplicative effects become additive	Difficulties with values with large relative standard deviation and zeros
	Power transformation	$\tilde{x}_{ij} = \sqrt{(x_{ij})}$ $\hat{x}_{ij} = \tilde{x}_{ij} - \bar{x}_i$	Correct for heteroscedasticity, pseudo scaling	Reduce heteroscedasticity, no problems with small values	Choice for square root is arbitrary

The mean is estimated as: $\overline{x_{IJ}} = \frac{1}{N}\sum_{N=1}^{N} x_{ij}$, and the standard deviation is estimated as $s_i = \sqrt{\frac{\sum_{N=1}^{N}(x_{ij}-\bar{x}_i)^2}{N}}$. $\tilde{x}_{ij}$ is the data after the pretreatment, and x_{ij} is the data before the pretreatment. i is the column which represents the relative concentration of each MVOC. j is the row which represents the samples (observations).

1.8.1 Multivariate data analysis methods

Multivariate data analysis is the expansion of univariate statistics used to study and solve multivariate theory and problems using mathematical statistical methods. It can be applied to determine the dependent relationship and statistical tendency among the multiple random variables. In complex medical studies, univariate analysis can ignore the correlation of the multiple variables, so that results do not accurately reflect circumstances objectively and comprehensively. Moreover, multivariate analysis not only can reveal the relationship of the intrinsic change of multiple variables, but can also serve to simplify a complex dataset.

One of the most commonly used multivariate analysis methods is PCA, which generate new variables to replace the original redundant variables using a mathematical dimension reduction method.[70] In PCA the original correlated variables are converted into linear uncorrelated variables called principal components. These new principal components represent the largest possible variance from the original variables. When selecting the first principal component, the so called F_1 variables, it must represent as much information of the original variables as possible. The "information" is measured using variance, and F_1 has the largest variance in all the linear combinations. If F_1 is not enough to represent the information from the original variables, the selection of the second principal component F_2 should be considered. The information included in F_1 will not be presented in F_2, so the mathematical expression is $Cov\ (F_1, F2) = 0$, the third, fourth…. and the pth component will be generated and so on.

PCA is widely used in food microbiology to identify bacterial contamination of foods. For example, Yun Xu et al. investigated the volatile metabolite profiles using gas chromatograph (GC) during the spoilage of pork and selected important regions in chromatograms to characterize pork contaminated with *Salmonella typhimurium*.[72] Yao Lu and Peter Harrington successfully classified 6 bacteria species using PCA based on fatty acid methyl esters profiles.[73] The metal-oxide based e-nose was able to detect ochratoxigenic fungal species using PCA for the contamination of wine and other wine grape products.[74]

In order to maximally achieve the goal of classification, another traditional multivariate data analysis method, discriminant analysis (DA) is commonly used. DA is a supervised classification model while PCA is an unsupervised classification model. DA

builds a predictive model for group membership composed of a discriminant function based on linear combinations of predictor variables. The predictor variables provide the best discrimination between groups. The purpose of DA is to 1) maximally separate the groups, 2) to determine the most parsimonious way to separate the groups, and 3) to discard variables which are little related to group distinctions.

DA is similar to regression analysis. A discriminant score can be calculated based on the weighted combination of the independent variables. One discriminant function is for 2-group discriminant analysis where the first function maximizes the difference between the values of the dependent variable and the second function maximizes the difference between the values of the dependent variable while controlling the first function. The first function is the most powerful discriminating dimension. The second and later functions may also represent additional significant dimensions of differentiation.

Sue *et al*.[75] investigated the possibility of early detection of microbial contamination to avoid process failure and costly delays in the fermentation industry. They obtained a 97% success rate using discriminant analysis in correctly classifying samples coming from contaminated or axenic cultures. Li *et al*.[76] performed linear discriminant analysis to classify onions inoculated with *Botrytis allii* and *Burkhdderia cepacia* with correct classification rates over 97.8%. They found sixteen compounds were present in *B. allii* and *B. cepacia* inoculated onion bulbs.

Partial least squares regression (PLS) is a statistical method that finds a linear regression model by projecting the predicted variables **X** and the observable variables to a new space. It is not originally designed for statistical discrimination. However, Barker

and Rayenes[77] proved that PLS is preferred over PCA when discrimination is the goal and dimension reduction is needed. Partial least squares discriminant analysis (PLS-DA) can be regarded as a linear two-class classifier.[78] This method aims to find a straight line that divides the space into two regions where algorithms are used to find the discriminator, or separator or decision function. A traditional disadvantage of Linear Discriminant Analysis (LDA) is that the number of variables needs to be less than the number of samples.[79] PLS-DA can overcome this problem by using PLS for dimension reduction.

PLS-DA consists of a classical PLS regression where the dependent variable **Y** is categorical and represents sample class membership.[80] By making use of class information, PLS-DA tends to improve the separation between the groups of samples. PLS-DA is commonly used for classification purposes and biomarker selection in metabolomics studies. For example, Jonsson *et al*.[81] developed a strategy using PLS-DA for identifying differences in large series of metabolomics samples analyzed by GC-MS. This method could detect the differences between the plant extract samples derived from the leaves of different development stages and plants subjected to small changes in day length. Kind *et al*.[82] developed a comprehensive urinary metabolomics approach for identifying kidney cancer where PLS-DA was used as one of the algorithms for statistical analysis. This linear method has the advantage that biomarkers are readily identified from the model using the loading values.

CHAPTER II

MONITORING MVOC PROFILES OVER TIME FROM ISOLATES OF *ASPERGILLUS FLAVUS* USING HS-SPME-GC-MS

2.1 Abstract

Fungi produce a variety of microbial volatile organic compounds (MVOCs) through primary and secondary metabolism. The fungus, *Aspergillus flavus*, is a human, animal, and plant pathogen which produces aflatoxin, a dangerous toxin and carcinogen. In this study, MVOCs were analyzed using solid phase microextraction (SPME) combined with GCMS from two genetically different *A. flavus* strains, an aflatoxigenic strain, NRRL 3357, and a non-aflatoxigenic strain, NRRL 21882. A PDMS/CAR SPME fiber was used over 30 days to observe variations in MVOCs over time. The relative percentage of individual chemicals in several chemical classes (alcohols, aldehydes, esters, furans, hydrocarbons, ketones, and organic acids) was shown to change considerably during the varied fungal growth stages. This changing chemical profile reduces the likelihood of finding a single chemical that can be used consistently as a biomarker for fungal strain identification. In our study, discriminant analysis techniques were successfully conducted using all identified and quantified MVOCs enabling discrimination of the two *A. flavus* strains over the entire 30 day period. This study underscores the potential of using SPME GCMS coupled with multivariate analysis for fungi strain identification.

2.2 Introduction

Aflatoxins are polyketide-derived, secondary fungal metabolites, and only three *Aspergillus* species, *A. flavus,*[83] *A. nominus,*[84] and *A. parasiticus*,[85] are known to produce these naturally carcinogenic compounds.[86] The economic impact is immense because fungal mycotoxin contamination is estimated to affect one quarter of the world's food crops including maize, cotton, and peanuts.[86] Crop losses are estimated to cost between $1 and $1.5 billion/year in the United States.[87] These value do not account for livestock losses or the impact on human health or healthcare costs from exposure to the fungi or to the toxins. In order to control exposures, maximum levels of aflatoxins for many commodities have been set at levels below 20 ppb by most countries.[88, 89] For example, the U.S Food and Drug Administration (FDA) has set limits of 20 ppb total aflatoxins for interstate commerce of food and 0.5 ppb for milk.[90] The European Commission has set the limits of 15 and 10 ppb for total aflatoxins on groundnuts and dried fruits, respectively.[89] Many methods have been proposed and are in development for the detection of aflatoxins or *A. flavus* including those that identify the presence of the toxins and those that identify the fungus.

Conventional analytical methods being used for aflatoxin detection are high-performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GC-MS), enzyme linked immune-sorbent assay (ELISA), and multiplex polymerase chain reaction (multiplex PCR) assay.[91] These methods can be sensitive, inexpensive, and give both qualitative and quantitative measurement of aflatoxins; however, initial enrichment or interference/inhibitor removal is generally required.

Common identification methods for fungi include fluorescence *in situ* hybridization, DNA array hybridization, and multiplex tandem PCR.[92] However, there are no published aflatoxigenic-specific PCR primers that are able to successfully differentiate aflatoxigenic and non-aflatoxigenic strains. This is an obvious inconvenience in many industrial applications, particularly in the field of maintaining food safety in crops destined for livestock and human consumption. Thus there is an urgent need for a practical, rapid, and cost-effective method to identify the presence of aflatoxin-producing fungi.

The method described here focuses on identification and quantification of fungal microbial volatile organic compounds (MVOCs) as a means of identifying the fungi presence. The major source of MVOCs produced by organisms such as fungi and bacteria are from primary (synthesis of DNA, amino and fatty acids) and secondary (oxidation of glucose) metabolism.[93] Some MVOCs, such as 3-methyl-1-butanol, 1-octen-3-ol, 3-octanone, and sesquiterpenes have been proposed as indicators for fungal species.[94-96] Nilsson, *et al.*[97] reported some unique biomarkers (1-octen-3-ol, 3-octanol and several sesquiterpenes) emitted from *Penicillium* spp. It has been reported that *A. flavus* produces strain-specific volatiles such as 3-methylbutanol, 2-methyl-1-propanol, hexanol, *trans*-caryophyllene, nonanal, and naphthalene.[98] Moreover, several studies have demonstrated that fungal species produce a unique pattern or grouping of MVOCs that can also be used for species identification.[99] Cluster analysis (CA), principle component analysis (PCA) in 2 or 3 dimensional space, and linear discriminant analysis (LDA) have utilized MVOC data to discriminate bacteria at the species level.[95, 96]

Techniques that involve solid phase vapor collection followed by thermal desorption are widely applied in MVOC analysis. Thermal desorption tubes have been used for field sample collection followed by transportation to a lab for analysis.[93, 97] Solid phase microextraction (SPME) has been used to collect and concentrate MVOCs from fungi and bacteria.[100] This technique has the potential to be part of an efficient method for field applications due to its portability and simplicity. The application of SPME in conjunction with GC-MS has been successfully applied to the detection of indoor mold[101, 102],[101, 102] fungal species identification,[103, 104] and the diagnosis of foodborne pathogen infection.[105, 106]

The focus of this study was 1) to monitor changes in fungal volatile profiles of two strains of *A. flavus* for 30 days and 2) to develop a method using multivariate analysis for discriminating aflatoxigenic and non-aflatoxigenic *A. flavus* that is viable over all 30 days of analysis. The general methods represented in this study can be applied to identify other strains and species of fungi.

2.3 Material and methods

2.3.1 Chemical standards

Twenty-six reference chemical standards were purchased from several suppliers: 2-heptanone (99%), 2-heptanol (98%), hexanal (≥ 97%), 2-methyl-1-butanol (≥ 99%), 3-methyl-1-butanol (98%), 2-nonanone (≥ 99%), 2-pentanol (98%), isovaleraldehyde (97%), 3-octanone (≥ 98%), 2-pentylfuran (≥ 97%), 2-undecanone (98%), 2-nonanol (99%), 1-octen-3-ol (98%), 2-methylbutyric acid (98%), methyl isobutyrate (99%), 1,2,4,5- tetramethylbenzene (98%), 2-octanone (98%), ethyl acetate (HPLC grade ≥99.7%), 2-heptanone (99%), octane (98%) and ethyl isobutyrate (99%) from Fluka

Analytical standards and ethyl isovalerate, ethyl butyrate and ethyl proionate were from Sigma-Aldrich (St. Louis, MO). Pentane (98%) was obtained from Alfa Aesar (Ward Hill, MA).

2.3.2 Fungal sample preparation

The aflatoxigenic strains, NRRL 3357 (L-strain; http://www.aspergillusflavus .org/) and NRRL 21882, were provided by the United States Department of Agriculture-Agricultural Research Service, Corn Host Plant Resistance Research Unit, Mississippi State University, Starkville, MS (USDA-ARS-CHPRRU).

Both fungal strains were cultured on potato dextrose agar (Difco, Sparks, MD), which was prepared by dissolving 39 g of the powdered agar in 1 L of purified water and autoclaving at 121 °C for 15 min. The fungal spores were then extracted using a 0.02% Tween20 solution and then diluted with distilled water to 2×10^6 spores/ml for inoculation using hemocytometer.

Corn media (2%) was prepared by mixing 0.6 g corn grit (Martha White Yellow Corn Meal, Jackson, Tennessee) with 28 ml distilled water. The mixture was then placed in sterile 40-ml glass headspace vials covered with a polypropylene screw cap and PTFE/silicone septum (Sigma-Aldrich, St. Louis, MO). This basal medium was chosen based on preliminary studies performed in this laboratory and studies performed by Demain.[107] The corn media were autoclaved for 1 hour to avoid contamination.

Inoculations were performed by adding 10 µl of each spore suspension to the cooled 2% corn media. Fungal growth took place in 30 ml of the 2% corn grit liquid media in the capped 40-ml glass vials. The aflatoxigenic and non-aflatoxigenic *A. flavus* cultures were prepared in five replicates each, and four replicates of non-inoculated corn

grit liquid media were used as control. Each vial was incubated in the absence of light at 30°C followed by MVOC analysis after 1, 3, 6, 10, 20, 24, and 30 d.

2.3.3 SPME fibers comparison and MVOCs analysis

A SPME fiber comparison study was done in order to optimize MVOC collection. Standard solutions of known fungal MVOCs (1-heptanol, 1-hexanol, 1-octen-3-ol, 2-heptanone, 2-methyl-1-butanol, 2-octanone, 3-methyl-1-butanol, 3-octanone, ethyl acetate, ethyl butyrate, ethyl isobutyrate, ethyl isovalerate, ethyl propionate, hexanal, methyl isobutyrate, and styrene) were mixed and diluted with dichloromethane to mixture concentrations between 300 ppm to 10,000 ppm. Final concentrations of hydrocarbons (5 ppb), alcohols (300 ppb), ketones (20 ppb), aldehydes (20 ppb), esters (20 ppb) and organic acids (20 ppb) were achieved when 1 µl of the standard solutions were injected with a 1 µl syringe into 30 ml of deionized water in 40 ml septa equipped vials. SPME fibers with the following materials and thickness were tested: 100 µm polydimethylsiloxane (PDMS), 85 µm carboxen/ PDMS (CAR/PDMS), 65 µm divinylbenzene/PDMS (DVB/PDMS), 85 µm polyacrylate (PA) and Carboxen/divinylbenzene/PDMS (CAR/DVB/PDMS) fibers (Supelco Inc., Bellefonte, PA, USA). The standard volatiles were extracted in triplicate for each type of SPME fiber for one hour at 30 ℃.

The CAR/PDMS fiber was selected for headspace extraction of the fungal isolates and non-inoculated corn control for one hour at 30 ℃. After 1 hour of exposure, the fiber was pulled into the needle sheath, and the SPME device was removed from the vial and then inserted into the injection port of the GC system for thermal desorption. In order to

monitor the changes in MVOC profiles from fungal species over time, the MVOCs were collected and analyzed after 1, 3, 6, 10, 20, 24 and 30 d.

2.3.4 GC-MS conditions

All GC-MS analysis was performed on an Agilent 5975C Inert XL MSD coupled with a 7890A Gas Chromatography system. SPME fibers were desorbed at 250 °C in a split/splitless injection port, equipped with a 78.5 mm × 6.5 mm × 0.75 mm SPME inlet liner (Supelco Inc., Bellefonte, PA, USA) while working in the splitless mode. The GC system was equipped with a DB-1 capillary column (60 m × 320 µm × 1 µm). Helium was used as a carrier gas with a flow velocity of 1.2 ml min^{-1}. The oven temperature program was as follows: 45 °C held for 9 min, 10 °C min^{-1} ramp to 85 °C followed by a 3 min hold; ramp to 120 °C at 3 °C min^{-1} followed by a 3 min hold, then a final ramp at 10 °C min^{-1} to 270 °C. The MS analysis was carried out in full scan mode (scan range from 35-350 amu) with ionization energy of 70 eV. Ion source and quadrupole temperatures were 230 °C and 150 ℃, respectively. Fungal metabolites were identified by comparing the retention time of chromatographic peaks with standards analyzed under the same conditions and by mass spectrum database searches using the NIST 08 spectral database.

2.3.5 Multivariate analysis

Discriminant analysis (DA) was employed to visualize resultant clustering of fungal culture samples based on MVOC profiles and to examine the relationship between toxigenic and non-toxigenic *A. flavus* isolates. Prior to analysis, peak area data were standardized by autoscaling. Discriminant analysis was performed using statistic software IBM SPSS statistics 21 (International Business Machines Corp.).

2.4 Results and Discussion

2.4.1 HS-SPME extraction method optimization

To investigate the extraction efficiency for the MVOCs, the following specific fibers were evaluated: 100 µm PDMS, 85 µm CAR/PDMS, 65 µm DVB/PDMS, 85 µm PA and 50/30 µm CAR/DVB/PDMS. Figure 2.1 shows the resulting TIC chromatograms for the 17 standard VOC mixture after one hour headspace extraction at 30 °C (best temperature for aflatoxin production). The data are displayed on the same scale to emphasize the difference in extraction efficiencies.

PDMS and PA fibers were determined to be not suitable because of relatively low collection amounts when compared to the other fiber types. CAR/PDMS, DVB/PDMS and CAR/DVB/PDMS fibers show similar TIC chromatograms. For further investigation, the peak areas of the 17 standard VOCs were compared as shown in Figure 2.2. The average relative standard deviations of the 17 standard VOCs for these fibers are 18.4% (CAR/PDMS), 13.1% (DVB/PDMS) and 14.9% (CAR/DVB/PDMS). Although DVB coated fibers extracted larger amount of high molecular weight alcohols and ketones (1-octen-3-ol, 2-octanone and 3-octanone), they have less affinity to esters (ethyl butyrate, ethyl isobutyrate and methyl isobutyrate) and low molecular weight alcohols (3-methyl-1-butanol and 2-methyl-1-butanol). The DVB/PDMS fiber also had insufficient absorption of the esters; therefore, the CAR/PDMS fiber was used in the subsequent fungus MVOC studies.

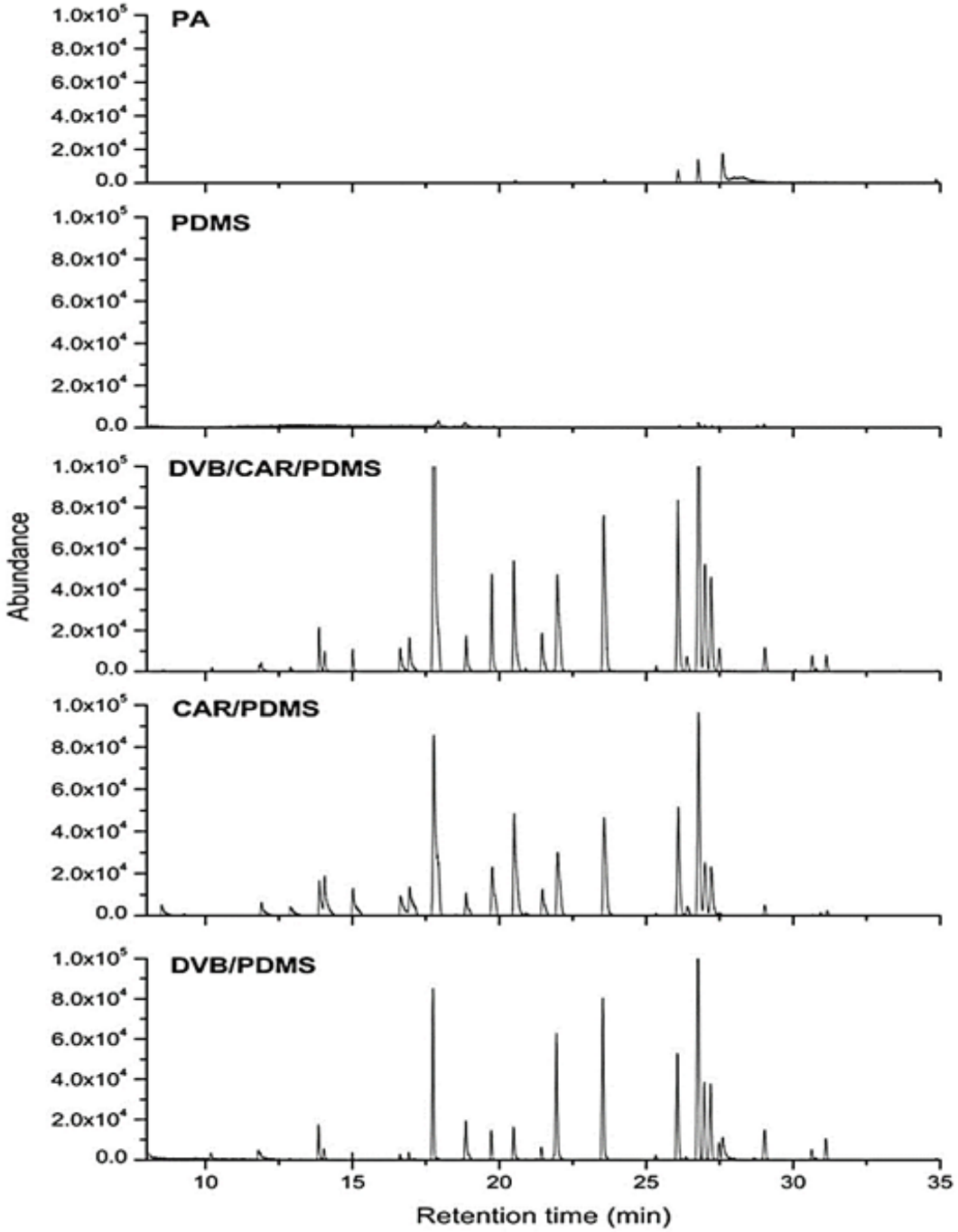


Figure 2.1 Comparison of TIC chromatograms from varied SPME extraction of 17 standard VOCs followed by GCMS analysis displayed on the same scale.

Best results were obtained using DVB/CAR/PDMS, DVB/PDMS, and CAR/PDMS.

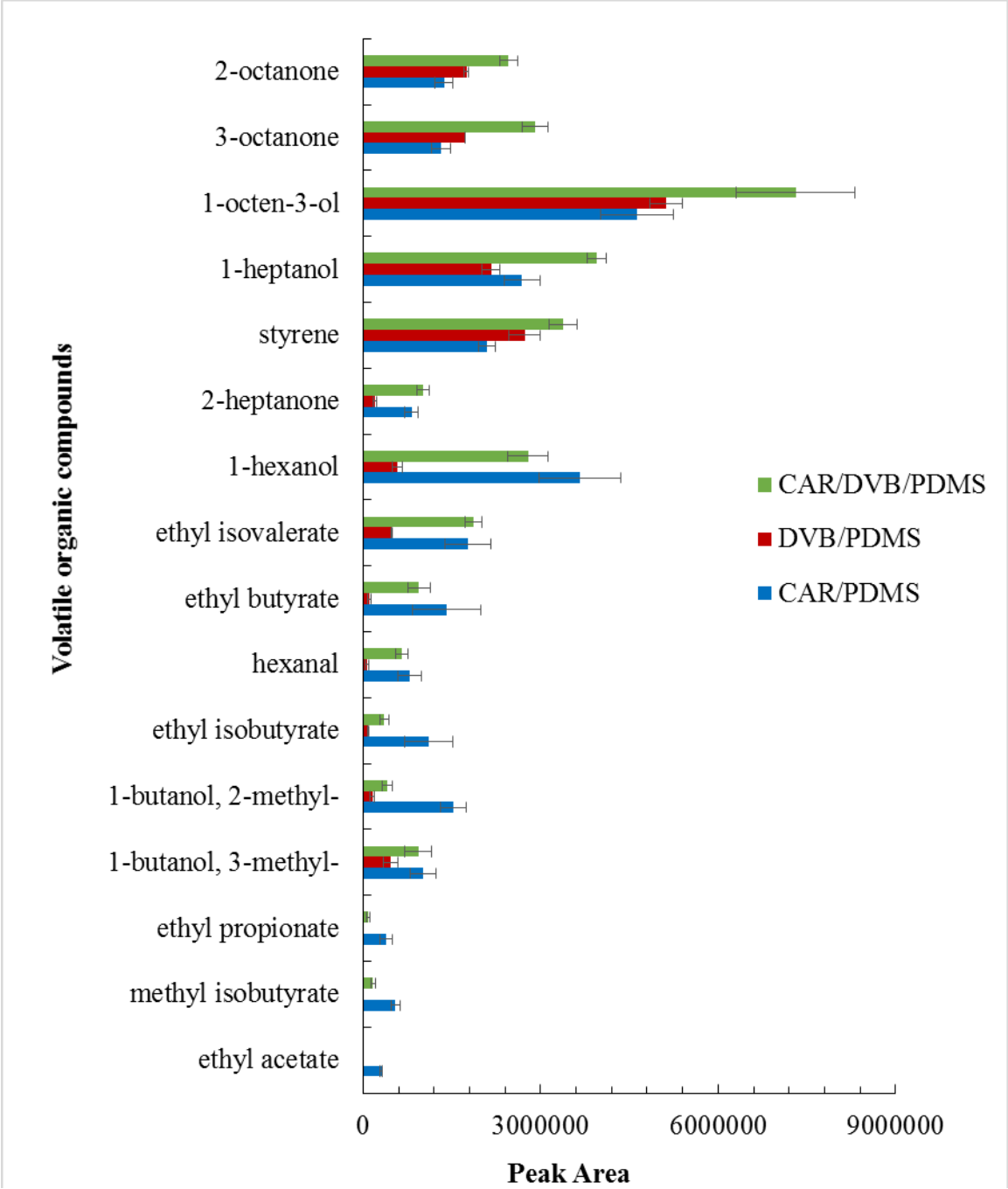


Figure 2.2 Comparison of peak areas and standard deviations showing 17 standard VOCs after HS-SPME-GCMS analysis using different SPME fibers, including CAR/DVB/PDMS, DVB/PDMS and CAR/PDMS.

Each fiber was tested in triplicate.

A culture media volume of 30 mL and 10 mL headspace volume provided sufficient amounts of VOCs during a 1 hour collection period at 30 °C. The choice of SPME fiber for MVOCs collection technique plays an important role in fungal species discriminations. For example, CAR/PDMS fibers are better for volatile analytes, while DVB/PDMS fibers are good for extracting semi-volatile analytes. The CAR/DVB/PDMS fiber contains two adsorbents and can extend the molecular weight range of analytes extracted with a single SPME fiber. However in this study, the CAR/PDMS fiber was selected because of our desire to focus on collection of the more abundant volatile organic compounds being emitted from the two fungal strains.

2.4.2 Identification of volatiles produced by *A. flavus*

The volatile MVOC profiles produced by aflatoxigenic and non-aflatoxigenic *A. flavus* were monitored over 30 d. The resulting chromatograms obtained from the headspace analysis of the emitted MVOCs after incubation for 6 days are shown in Figure 2.3 for the control (growth media only), toxic (aflatoxigenic *A. flavus*) and nontoxic (non-aflatoxigenic *A. flavus*) samples. A very clear difference in MVOCs abundance was observed, where the toxic strain produces significantly less MVOCs compared to the nontoxic strain. MVOCs produced by the fungal strains and control were identified by comparing with the standards and the NIST 08 library. Ethanol was produced in large amounts in all fungal cultures; we found that this chemical did not aid in discrimination and was therefore removed from consideration when looking for identifying MVOCs patterns. The most significant signals (detected in all replicates) with high abundance (TIC peak area > 1×10^4 units) are listed in Table 2.1 (excluding ethanol). This table contains the chemical retention time, standard deviation of this

retention, compound name, the days the specific chemical was detected in the samples and its relative composition.

Table 2.1 shows the average peak area percentage of each MVOC produced during the 30 days of incubation. The detected MVOCs were grouped by functional group including alcohols, aldehydes, esters, furans, hydrocarbons, ketones, and organic acids. In total, 56 different volatile compounds were identified in all samples (fungus and control). Forty-eight compounds were detected in the non-aflatoxigenic strain, and forty compounds were detected in the aflatoxigenic strain. The predominant MVOCs were alcohols (ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol), aldehydes (3-methylbutanal, 2-methylbutanal), esters (ethyl isobutyrate, methyl isovalerate), hydrocarbons (toluene, α-pinene, and styrene), ketones (2, 3-butanedione, 3-octanone) and organic acids (acetic acid, 2-methylpropanoic acid).

Alcohols and esters were found in the fungal culture samples and were not detected in the corn control media. In addition, most of the hydrocarbons were produced by the corn control; however some hydrocarbons (toluene, styrene and α-pinene) were only emitted by the non-aflatoxigenic strain. A relatively high percentage of 2-heptanol (2.23%) consistently appeared in volatiles produced by the aflatoxigenic strain; however this compound was not found in the non-aflatoxigenic strain. Moreover, a low percentage of furans (2-methylfuran, 2-ethylfuran, and 2,4-dimethylfuran) were detected at day 6 and dimethyl sulfide was detected at day 3 only in the aflatoxigenic strain.

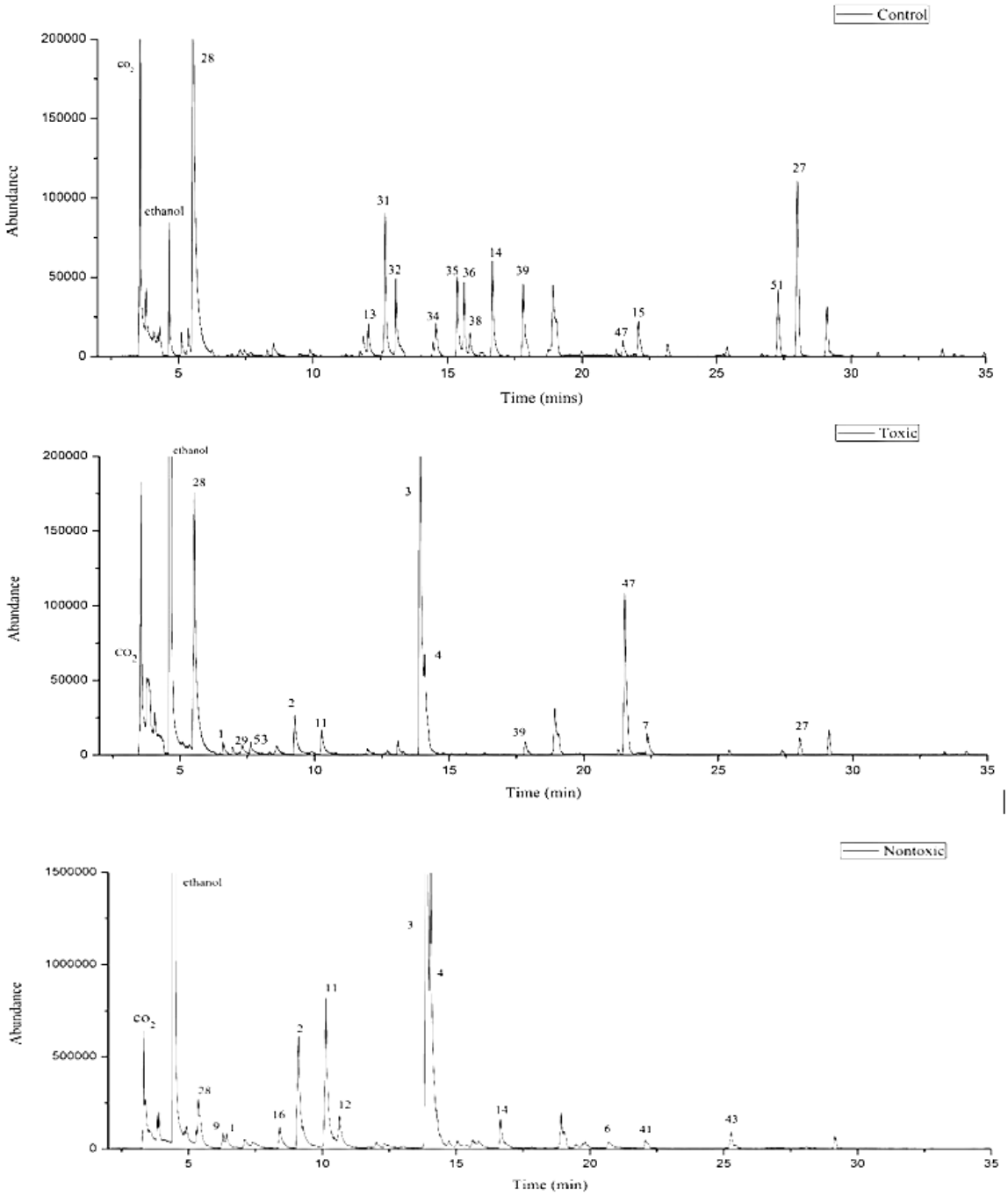


Figure 2.3 HS-SPME-GCMS total ion current (TIC) chromatogram showing MVOCs identified from the fungal strains and non-inoculated media at day 6 for the control (upper), toxigenic *A. flavus* (center), and non-toxigenic *A. flavus* (lower).

Peak numbers refer to the volatiles listed in Table 2.1 (ethanol and carbon dioxide were detected in all samples).

Table 2.1 Headspace SPME-GCMS analysis of 56 microbial volatile metabolites from both aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus*.

No.	Ret time (min)	STD[d]	Compound Name[e]	Days detected			Average Peak Area % [a]		
				Nontoxic	Toxic	Control	Nontoxic	Toxic	Control
Alcohols									
1	6.560	0.035	1-propanol	3,6,10,20,24, 30	6,10,20,24, 30	n.d[b].	0.91	0.66	n.d.
2	9.300	0.016	2-methyl-1-propanol	A.D[c].	3,6,10,20,24 ,30	n.d.	7.89	2.77	n.d.
3	13.917	0.009	3-methyl-1-butanol	A.D.	A.D.	n.d.	39.50	38.24	n.d.
4	14.105	0.006	2-methyl-1-butanol	A.D.	A.D.	n.d.	15.54	10.42	n.d.
5	15.468	0.015	1-pentanol	10	A.D.	n.d.	0.04	n.d.	n.d.
6	20.712	0.024	1-hexanol	6,10	A.D.	n.d.	0.21	n.d.	n.d.
7*	22.310	0.009	2-heptanol	n.d.	A.D.	n.d.	n.d.	2.23	n.d.
8	27.004	0.028	1-octen-3-ol	6	A.D.	n.d.	0.01	n.d.	n.d.
Aldehyde									
9	6.464	0.015	2-methyl-propanal	1,3,6	n.d.	n.d.	0.86	n.d.	n.d.
10	7.492	0.032	butanal	n.d.	n.d.	A.D.	n.d.	n.d.	0.45
11	10.247	0.015	3-methylbutanal	A.D.	6,10,20,24,3 0	n.d.	6.93	1.22	n.d.
12	10.774	0.018	2-methylbutanal	A.D.	10,24,30	n.d.	1.16	0.10	n.d.
13	12.039	0.080	pentanal	6	n.d.	A.D.	0.07	n.d.	2.06
14	16.684	0.015	hexanal	6,10,20	n.d.	A.D.	0.39	n.d.	4.71
15	25.082	0.014	2-heptenal	6	n.d.	A.D.	0.01	n.d.	3.37
Esters									
16	8.557	0.013	ethyl acetate	A.D.	6,10,20,24,3 0	n.d.	1.68	3.69	n.d.
17	12.964	0.023	propanoic acid, ethyl ester	20,24,30	n.d.	n.d.	0.09	n.d.	n.d.
18	15.178	0.283	ethyl isobutyrate	6,10,20,24,30	20,24,30	n.d.	0.56	0.22	n.d.

Table 2.1 (Continued)

19	15.946	0.010	ethyl butyrate	6,10,20,24,30	20,24,30	n.d.	0.02	0.27	n.d.
20	16.029	0.390	methyl isovalerate	6,10,20,24,30	20,24	n.d.	0.38	0.13	n.d.
21	19.736	0.041	ethyl 2-methylbutyrate	6,10,20,24,30	20,24,30	n.d.	0.19	0.20	n.d.
22	19.840	0.037	ethyl 3-methylbutyrate	6,10,20,24,30	20,24,30	n.d.	0.42	0.24	n.d.
Furan-related compounds									
23	5.374	0.013	furan	1,20,24,30	1,3,6,10,24,30	A.D.	0.48	0.73	1.29
24*	8.329	0.022	2-methylfuran	n.d.	6	n.d.	n.d.	0.04	n.d.
25*	12.729	0.010	2-ethylfuran	n.d.	6	n.d.	n.d.	0.08	n.d.
26*	13.287	0.009	2,4-dimethylfuran	n.d.	6	n.d.	n.d.	0.06	n.d.
27	28.025	0.012	2-pentylfuran	1,3,6,10	6,10,20,24,30	A.D.	0.29	0.43	10.25
Hydrocarbons									
28	5.535	0.011	pentane	A.D.	A.D.	A.D.	6.94	11.45	36.51
29	7.321	0.015	2-methylpentane	n.d.	6,10	n.d.	n.d.	0.26	0.52
30	8.544	0.015	hexane	n.d.	n.d.	A.D.	n.d.	n.d.	1.05
31	12.684	0.007	2,2,3,3-tetramethylbutane	1,3	1,3	A.D.	1.78	0.41	8.61
32	13.061	0.060	heptane	6,24	6	A.D.	0.08	0.12	3.87
33	14.463	0.010	2,5-dimethylhexane	1,3	n.d.	A.D.	0.19	n.d.	0.73
34	14.583	0.012	2,4-dimethylhexane	1,3	n.d.	A.D.	0.61	n.d.	2.49
35	15.371	0.007	2,3,4-trimethylPentane	1,3	1,3	A.D.	1.48	0.43	5.06
36	15.609	0.008	2,3,3-trimethylPentane	1,3	1,3	A.D.	1.80	0.68	4.01
37	15.681	0.138	toluene	6,10,20,24	n.d.	n.d.	0.33	n.d.	n.d.
38	15.843	0.005	2,3-dimethylhexane	1,3	3	A.D.	0.42	0.09	1.62
39	17.826	0.009	octane	6,30	6,24	A.D.	0.06	0.22	4.64
40	21.284	0.024	2,3,4-trimethylhexane	1	6	A.D.	0.05	0.05	0.48

Table 2.1 (Continued)

41	22.085	0.027	styrene	6,10	n.d.	n.d.	0.14	n.d.	n.d.
42	22.367	0.007	p-xylene	3,20,24	n.d.	n.d.	0.09	n.d.	n.d.
43	25.295	0.016	α-pinene	6,10,20,24,30	n.d.	n.d.	0.25	n.d.	n.d.
44	35.812	0.007	decane	3	n.d.	n.d.	0.20	n.d.	0.20
Ketones									
45	5.126	0.017	acetone	A.D.	A.D.	A.D.	1.19	1.24	1.22
46	7.263	0.041	2,3-butanedione	3,6,10,14,20,24	20,24,30	n.d.	0.58	0.11	n.d.
47	11.758	0.012	2-pentanone	1,3,10,20,24,30	1,3,24,30	n.d.	0.39	0.45	n.d.
48	12.375	0.022	3-hydroxy-2-butanone	3,6,10,20,24,30	n.d.	n.d.	0.37	n.d.	n.d.
49	21.486	0.006	2-heptanone	1,3	A.D.	A.D.	2.36	17.67	1.21
50	27.147	0.005	3-octanone	6	1,20,24,30	n.d.	0.01	0.16	n.d.
51	27.341	0.014	2-octanone	1	1,6,10,20,24,30	A.D.	0.13	0.36	0.24
52	34.108	0.005	2-nonanone	1	1,3,6	n.d.	0.30	3.28	n.d.
Organic acids									
53	7.463	0.179	acetic acid	10,20,24,30	6,10,20,24,30	n.d.	1.52	1.08	n.d.
54	14.828	0.172	2-methylpropanoic acid	6,10,20,24,30	30	n.d.	0.72	0.16	n.d.
55	19.575	0.169	2-methylbutanoic acid	6,10,20,24,30	n.d.	n.d.	0.36	n.d.	n.d.
Sulfur containing compounds									
56*	5.740	0.004	dimethyl sulfide	n.d.	3	n.d.	n.d.	0.07	n.d.

[a] Average peak area % is the average peak area percentage of each compound collected on the 7 sampled days (1,3,6,10,20,24,30), and the peak area of each compound for days not detected was counted as zero.
[b] n.d. : not detected in the culture samples which were analyzed by GC-MS
[c] A.D.: detected in all days sampled (1,3,6,10,20,24,30)
[d] STD: standard deviation of each compound retention time in five replicates
[e] Identification based on the comparison of retention time and mass spectra with standards under the same conditions
* : VOCs detected in aflatoxigenic *A. flavus* only
Note: Ethanol and carbon dioxide were detected in all samples; they are not listed due to large amount of VOC production interference the other peak area% result.

Significant amounts of ethanol were formed from the metabolic oxidation of glucose during the primary and the secondary metabolism of non-aflatoxigenic and aflatoxigenic *A. flavus* cultures. Ethanol was also observed by Jurjevic, *et al.*[98] in the headspace gases produced by the aflatoxigenic and non-aflatoxigenic strains grown on the corn substrate for 25 days incubation. Several MVOCs were found in our study on most days in both the non-aflatoxigenic and aflatoxigenic strains including 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanol and 3-octanone.

Certain MVOCs produced by *A. flavus* have been reported to be biomarkers that can be used for identifying pathogenic fungal strains. For example, $C_{15}H_{24}$ volatile compounds (alpha-gurjunene, *trans*-caryophyllene, and cadinene) were detected using a purge and trap technique and were considered to be unique "fingerprints" for aflatoxigenic strains of *A. flavus*.[108] These markers were not found in this study. A possible explanation for this is the lack of enough nutrients (0.2 g of corn grit) for significant sesquiterpene biosynthesis.

Dimethyl disulfide and nonanal were reported to be associated only with the aflatoxigenic *A. flavus*, while hexanal, 1-hexanol, 1-octen-3-ol, and 2-pentyl furan were only associated with non-toxigenic *A. flavus*.[98] Our study also identified several compounds that were found in only one isolate. However, these compounds cannot be used for discrimination because this trend did not hold up over time during each lifecycle stage. For example, in agreement with the literature, we found dimethyl disulfide only in our toxic sample, but this chemical was present only on the 3rd day, while 1-hexanol, propyl ethyl ester, and 2-methylbutanoic acid where only found in the nontoxic *A. flavus,* but again these chemicals were not present on each day. One exception was 1-heptanol,

this chemical was present in all toxic samples, but was not found in any of the non-toxic samples.

2.4.3 Investigation of the fungal VOC profile over time

Variations in MVOCs patterns over time were determined using peak area percentage. The total peak ion current from aflatoxigenic and non-aflatoxigenic strains during 30 days of incubation are shown in Figure 2.4. Total peak areas for each day were calculated by summing the peak areas of all detected MVOCs in a sample (excluding ethanol). The results show that the amount of MVOCs significantly increases by day 6 for the nontoxic isolate relative to the toxic isolate due primarily to increasing amounts of alcohols and esters being produced. After 10 days the quantity of MVOCs begins to decrease, possibly because a lack of nutrients remaining in the media retards continued biosynthetic processes of fungi.

The results found in Figure 2.4 show that non-aflatoxigenic and aflatoxigenic *A. flavus* produce different amount of MVOCs over 30 days. We hypothesize that the difference in amount of MVOCs production are caused by the following reasons: 1) Aflatoxin biosynthesis is induced by simple carbohydrates, such as glucose and sucrose, [109] therefore aflatoxin production reduces nutrients available for fungi growth. 2) The non-toxigenic isolate has a characteristic gene for rapid growth compared to toxigenic isolate. 3) The presence of aflatoxin inhibits some biological pathways that produce MVOCs.

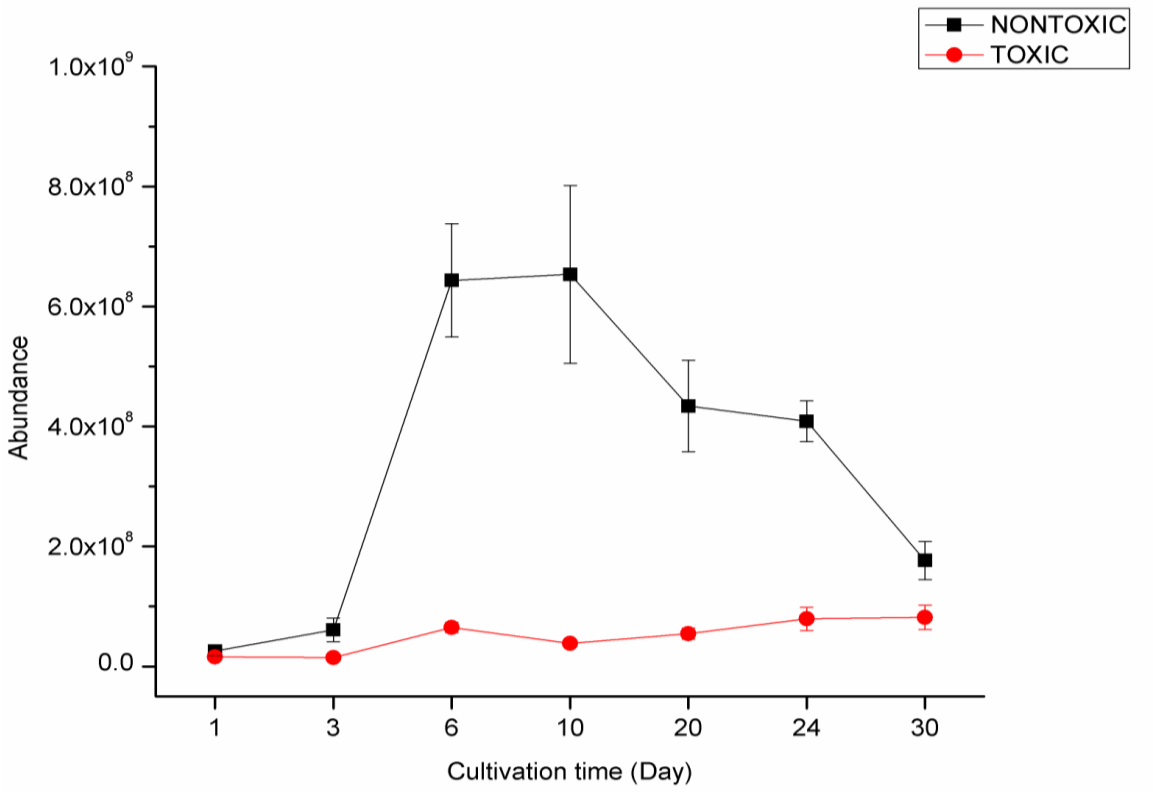


Figure 2.4 Comparison of the total ion current for identified MVOCs between aflatoxigenic and non-aflatoxigenic *A. flavus* during a cultivation period of 30 days.

The abundance is the summed total peak area of all compounds detected from both aflatoxigenic and non-toxigenic *A. flavus*.

Figure 2.5 shows time-dependent expression patterns of six chemical classes. The (non-ethanol) alcohol production (mainly 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-methyl-1-propanol) significantly increased during incubation and reached maximum at 20-24 days (Fig. 2.5A). In the late period of incubation (20-30 days), the relative percentages of esters are much higher in the aflatoxigenic strain compared to non-aflatoxigenic strain (Fig. 2.5C). The production of esters (ethyl isobutyrate, methyl isovalerate, and ethyl 3-methylbutyrate) and organic acids (acetic acid and 2-

methylpropanoic acid) increased significantly starting on day 6 with another significant increase for the ester in the toxic isolate beginning on day 20. The relative percentage of aldehydes and ketones decreased during the 30 days of fungal culture incubations. The large percentage of ketones produced by the aflatoxigenic strain in the early stage of incubation (Fig. 2.5E) is primarily from 2-heptanone production. Thus, we can report that even though some trends are observed we see significant variations in MVOCs production over time.

Our results demonstrate that there are numerous qualitative and quantitative fluctuations in MVOCs profiles during different days as shown in Table 2.1 and Figure 2.5 consistent with the findings of Borjesson, *et al.*[110] and Jurjevic, *et al.*[98]. A significant distinction in the relative amounts of MVOCs production from aflatoxigenic and non-aflatoxigenic *A. flavus* provides a possible direction for discriminating these fungal isolates. However, developing a method that discriminates on a specific growth day is not applicable for field analysis, since the growth stage of fungal species cannot be ascertained when collecting MVOCs in the field. Korpi *et al.*[93] also emphasized that an individual MVOC cannot be related to a certain microbial species because the same MVOC may be produced by different microorganisms. In order to reduce the reliance on any one specific MVOC we have applied chemotaxonomy techniques to reveal potential species-specific MVOC patterns by comparing entire qualitative and quantitative datasets of MVOCs. More importantly, this method enables the discrimination of fungal strains during any growth stage during the first 30 days.

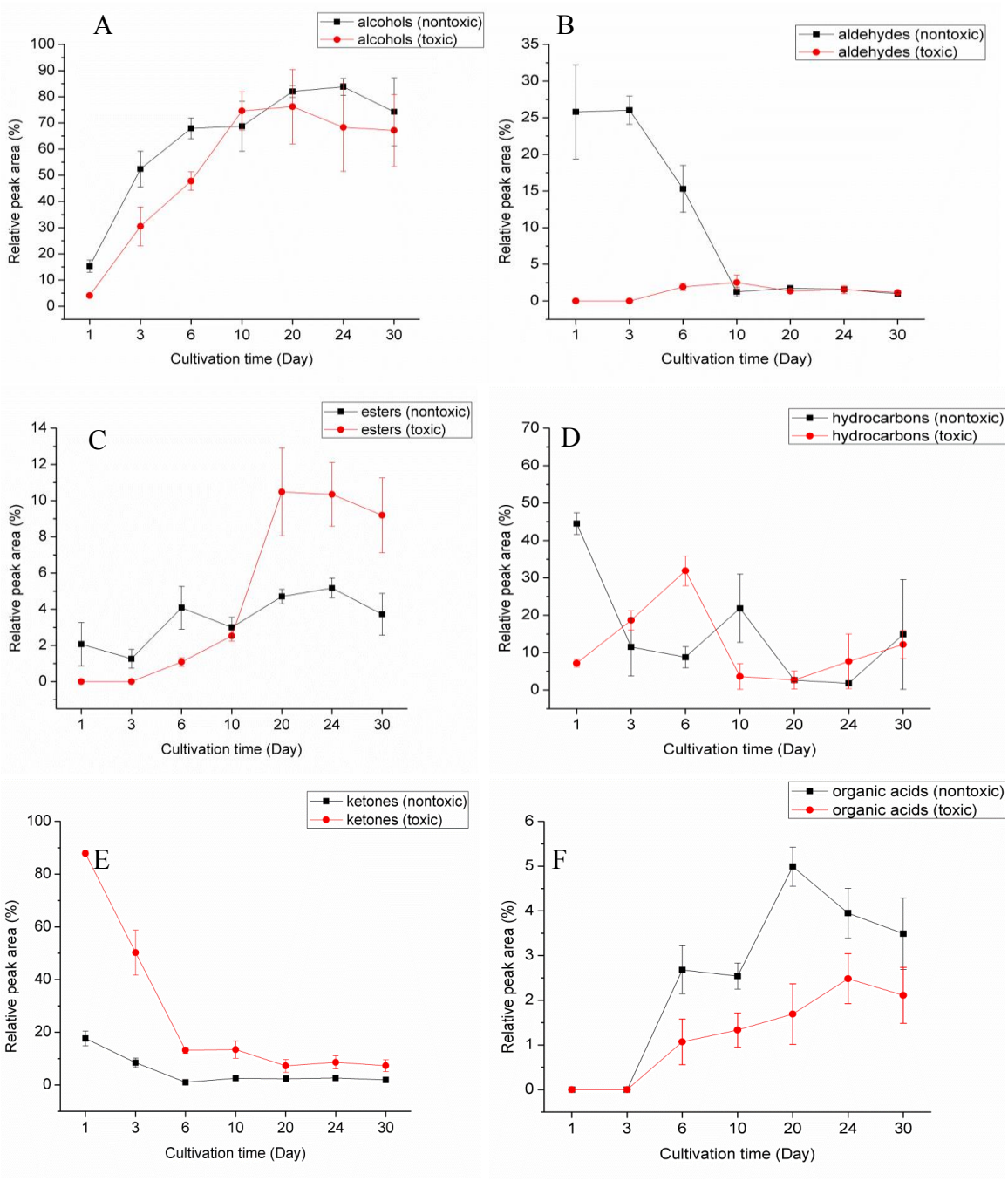


Figure 2.5 Variation of MVOCs expression patterns of aflatoxigenic and non-aflatoxigenic *A. flavus* during a cultivation period of 30 days for selected volatiles from classified compounds of A) alcohols, B) aldehydes, C) esters, D) hydrocarbons, E) ketones, and F) organic acids.

2.4.4 Multivariate analysis of MVOC profile

Due to the large number and varying concentrations of MVOCs produced, multivariate analysis is required to recognize patterns in the data leading to discrimination of the different fungal strains. To evaluate the capability of this HS-SPME-GCMS method for distinguishing aflatoxigenic and non-aflatoxigenic *A. flavus*, the GCMS data (day 1, 3, 6, 10, 20, 24, 30) from fungi and control samples were collected and analyzed using discriminant analysis (DA) models. DA builds up a predictive model which is composed of a discriminant function based on linear combinations of predictor variables. It can be used to discard variables that are little related to group distinctions and to maximally separate the groups. Using this approach, 13 MVOCs (Table 2.2) were identified with 2-methyl-1-propanol, 2-heptanol, propanoic acid ethyl ester, ethyl isobutyrate, ethyl 3-methylbutyrate, furan, 2-pentylfuran, 2,3-butanedione, 2-heptanone, 2-octanone, and 2-methylpropanoic being the most significant compounds for group classification.

Fig. 2.6 shows the plot of discriminant scores of the analyzed samples. The three classified groups (toxic, nontoxic, control) were satisfactorily separated, showing that this method can be used to discriminate these strains of aflatoxigenic and non-aflatoxigenic *A. flavus* during the fungi growing process in a laboratory environment. 93.5% of cross-validated group cases were correctly classified by the discriminant functions built by the model, thus achieving perfect discrimination (Table 2.3). However, it is important to note that MVOC profiles will vary with growth conditions and with specific isolate.

Table 2.2 Standardized canonical discriminant function coefficients for HS-SPME-GC-MS data from samples analyzed during 30 days culture incubation

Standardized Canonical Discriminant Function Coefficients[a]		
Variable	Discriminant Function[b]	
	1	2
2-methyl-1-propanol	-1.016	0.338
2-heptanol	-3.056	-3.086
propanoic acid, ethyl ester	1.051	0.240
ethyl isobutyrate	-0.109	-1.219
2-pentylfuran	1.889	1.535
ethyl, 3-methylbutyrate	-1.066	-0.723
furan	0.954	1.664
2,3-dimethylhexane	1.787	1.672
styrene	-0.552	-1.071
2-octanone	1.010	0.634
2-heptanone	1.479	0.531
2,3-butanedione	1.450	2.008
2-methylpropanoic acid	-0.709	1.066

[a] Discriminant analysis was performed using standardized GC-MS data from aflatoxigenic, non-aflatoxignic *A. flavus* and control samples analyzed in day 1, 3, 6, 10, 20, 24, 30.

[b] Discriminant function 1 and 2 were used as linear combinations of independent variables for the three groups.

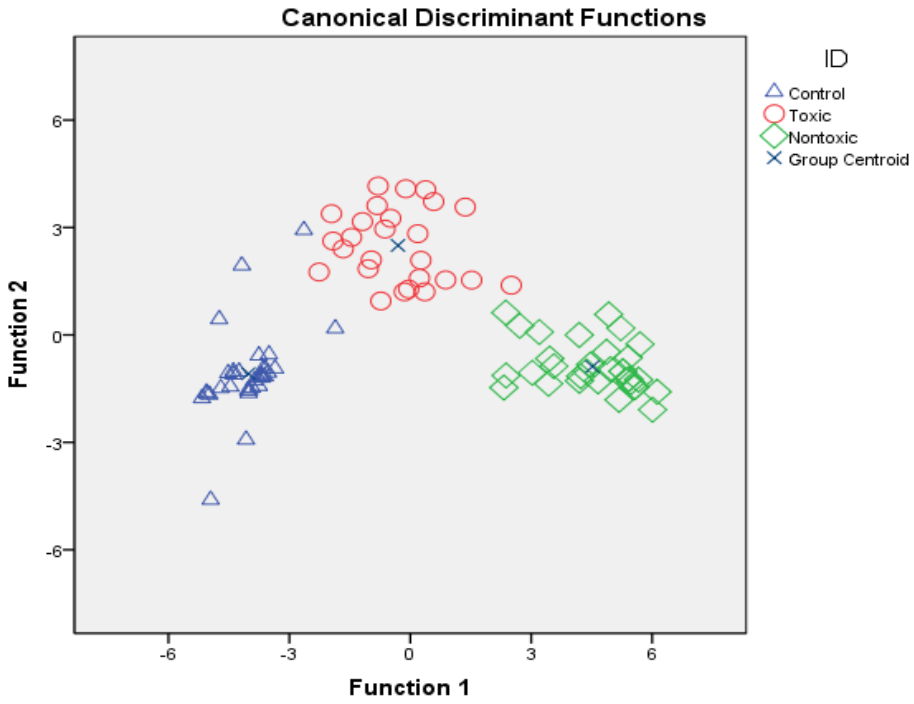


Figure 2.6 Discriminant score plot of the MVOCs analyzed by HS-SPME-GCMS grouped by chemical classes of toxigenic and non-toxigenic isolates and non-inoculated control during 30 days incubation.

Table 2.3 Classification and cross-validation results using HS-SPME-GC-MS data from samples analyzed during 30 days culture incubation

		Classification Results [a,c]			
		Predicted Group Membership[d]			Total
	ID	0	1	2	
Original	Control	97.1%	2.9%	0	100%
	Toxic	0	100%	0	100%
	Nontoxic	0	0	100%	100%
Cross-validated[b]	Control	91.2%	8.8	0	100%
	Toxic	7.7	88.5%	3.8	100%
	Nontoxic	0	0	100%	100%

[a] 98.9% of original grouped cases correctly classified.
[b] Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all other cases.
[c] 93.5% of cross-validated grouped cases correctly classified.
[d] Predicted group membership includes non-inoculated control, non-aflatoxigenic strain culture and aflatoxigenic strain culture.

Multivariate analysis was performed using data for each identified compound produced by control and fungal strains to discriminate aflatoxigenic and non-aflatoxigenic strains. Multivariate analysis is a powerful technique for this sort of complex data because it can reveal hidden patterns and reduce the information to a more comprehensive format. In this study, discriminate analysis was used, unlike principle component analysis and cluster analysis, object groups are known in discriminate analysis and the goal is to determine the best fit parameters of the model to separate the objectives base on independent variables of samples. In this case, the categorical groups are aflatoxigenic and non-aflatoxigenic strains, and the corn control. The independent variables used for discrimination are qualitative (compound name) and quantitative (peak area).

DA was applied to calculate the discrimination functions for classification of aflatoxigenic, non-aflatoxigenic *A. flavus* and control in clusters, which minimizes the variance within the classes and maximizes the variance among the classes. DA provides a number of discriminant functions equal to the number of categories of grouping variables minus one. Since three categories were considered including toxic, nontoxic and control, two discriminant functions were obtained in which the first function maximizes the difference between the values of the dependent variables, and the second function maximizes the difference between the values of the dependent variable while controlling the first function. Two discriminant functions were calculated, with the first accounting for 84.1% of the variance.

In summary, the standardized discriminant function coefficients indicate the relative importance of the independent variables in predicting the dependence, where

coefficients with large absolute values (Table 2.2) correspond to variables with greater discriminating ability. A stepwise method was performed by automatically selecting the best MVOCs to use in this model. The "leave-one-out" cross-validation method was performed in order to determine the accuracy of the predictive model, where each identity tested is removed one-at-a-time from the initial matrix of data; then the classification model is rebuilt and the case removed is classified in this new model. The discriminant analysis model based on MVOCs of inoculated samples correctly classified 93.5% of the observations based on cross-validation. The result obtained from DA can be considered very satisfactory for the detection of aflatoxin producing *A. flavus* growing in corn media.

2.5 Conclusions

Our results clearly show that the production of MVOCs is significantly affected by microbial species and growth cycles, and we know from the literature that growth conditions such as media, pH, humidity and temperature also affect MVOC production.[111-113] More than 200 volatile compounds have been reported as fungi MVOCs in the literature. The combination of large number and variable MVOC composition requires multivariate analysis for specific fungal isolate identification.

Based on standard VOCs absorption data, the CAR/PDMS SPME fiber was considered to be the best fiber for *A. flavus* VOCs profiling. The time course experiments (carried out over 30 days) revealed that MVOCs production is time-dependent and that aflatoxigenic and non-aflatoxigenic strains had significantly different MVOCs expression patterns. HS-SPME-GCMS was applied successfully to detect and differentiate two *A. flavus* strains (aflatoxigenic and non-aflatoxigenic strains). A discriminate analysis plot

achieved satisfactory performance in classifying *A. flavus* strains and control based on quantitative MVOCs data even though different isolates produce similar MVOCs.

Results indicate that it is possible to build a database for chemotaxonomic application by performing MVOC monitoring at controlled growth conditions (temperature, humidity and substrate). Our sample size is small but clearly shows that specific MVOCs are unlikely to be useful for the confident identification of different *A. flavus* isolates. Future studies will be done to expand the number of fungal strains that can be discriminated using patterns of MVOCs instead of individual MVOCs that have been identified with HS-SPME-GCMS using multivariate analysis in order to build up a fungi screening database.

CHAPTER III

EFFECTS OF EXPERIMENTAL PARAMETERS ON FUNGI VOLATILE METABOLITES

3.1 Abstract

Aspergillus flavus produces dangerous metabolites known as aflatoxins. These compounds are toxic and carcinogenic, and their contamination of agricultural products results in health issues and economic hardships in the US and around the world. Early identification of aflatoxigenic isolates of *A. flavus* is key in the management of these fungi. An emerging method for specific isolate identification involves the analysis of volatile metabolites of the fungus. Complicating this approach is the understanding that many factors influence metabolic production including growth parameters such as growth media, temperature and spore counts. In addition, analytical methods can influence results. In this chapter we evaluate several growth and analysis methods in order to better understand the requirements of an analytical method that will elucidate metabolomic chemical signatures of these fungi.

3.2 Introduction

Aflatoxins are secondary metabolic products produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*.[86] Aflatoxin contamination of corn, peanut, and other agricultural commodities have a significant impact on health and the agricultural

economy, especially in the southeastern United States where aflatoxin contamination cost farmers, buyers, and sellers an annual average of $ 22.7 million.[114] The production of aflatoxins is always associated with the production of other metabolites, some of which are volatile. These volatile metabolites are produced during both primary and secondary metabolism and are often collectively referred to as microbial volatile organic compounds (MVOC).[115] MVOC are widely investigated as the indicator of fungal growth,[116-119] mycotoxins production[120-122] and for fungal taxonomy.[123-125]

In recent years, metabolomics approaches have been widely used for the investigation of metabolites of biological samples for identifying biomarkers that correlate to a disease,[126, 127] drug toxicity,[128, 129] or genetic or environmental variation.[130] Metabolites can belong to a wide variety of compound classes, such as amino acids, lipids, organic acids, nucleotides, alcohols, esters, and hydrocarbons.[67] These compounds are very diverse in their physical and chemical properties and occur in a wide concentration range. Some of these metabolites are volatile enough for headspace sampling. Metabolic profiling and fingerprinting methods are used to elucidate a microorganism's life processes.[67] Metabolic profiling is a determination of the chemicals and their concentrations produced by specific biosynthesis pathway of organisms. Metabolic fingerprinting is the screening approach to classify samples based on metabolite patterns or "fingerprints". The metabolomics study process often includes sample preparation, sample collection, instrumental analysis, data pretreatment, and data analysis.

Relative humidity, temperature, substrate (growth medium), and number of fungal spores inoculated are the main factors influencing fungal growth, metabolism and

MVOCs production in a laboratory setting.[68, 131] For example, Lopez-Malo *et al.*[132] studied the effect of incubation temperature (10-30 ℃), pH (3.0-4.0), and vanillin concentration (350-1200pm) on the growth of *A. flavus*. They concluded that the germination time and radial growth rate were significantly affected by the three studied variables. Joffe and Lisker studied the effects of light, temperature, and pH value for aflatoxins production.[133] They indicated that 24 ℃ was the optimal temperature for aflatoxin production. Polizzi *et al.*[68] studied the influence of various growth parameters on fungal growth and volatile metabolite production by indoor molds. They proved that the range of MVOCs and the quantities were larger on malt extract agar than on wallpaper and plasterboard. Clearly fungal growth conditions are an important consideration when conducting a metabolomic fingerprinting study involving the production of MVOCs.

Sampling methods such as thermal desorption tube (Tenax TA),[95, 134, 135] purge and trapping of headspace gases,[136, 137] headspace sorptive extraction,[124, 138] and solid phase microextraction (SPME)[139-142] have been used for the collection of MVOCs. SPME is a popular technique because it has the advantages of low cost per analysis and portability. Volatile chemicals can be selectively enriched on SPME fibers depending on fiber coating selection. Therefore the SPME fiber coating selection is important and should be tailored for specific applications. Optimization of fiber selection for 15 volatile and semivolatile analytes representing 13 organic classes were performed, and extraction efficiencies of the fibers for each of the analytes were compared.[143] This study illustrated key considerations involved in the selection of a SPME fiber including: (a) the polarity

and functionality of the polymer absorbent and (b) the volatility and functional groups of the target analyte.

The separation of MVOCs is efficiently accomplished with the use of gas chromatography (GC).[141, 144] Detection methods include flame ionization detection (FID) [142, 145, 146] and mass spectrometry (MS)[147-152] for the quantification and qualification of metabolic profiles. Among the MS techniques, single quadrupole mass detection is most widely used; however, more advanced techniques such as triple quadrupole (MS/MS),[153, 154] time of flight (TOF),[155] and ion mobility mass spectrometry (IMS-MS)[156, 157] are utilized depending on the purpose of the analysis.

In metabolomics research, different data pretreatment methods are applied in order to generate 'clean' data in the form of normalized peak areas that reflect metabolite concentrations. These clean data can then be used as the input for data analysis. Data pretreatment aids to enhance relevant (biological) information and to reduce the influence of confounding factors from random error and spurious chemicals from column and absorbent bleeding.[71] Three classes of data pretreatment methods are normally utilized including centering, scaling and transformations. Centering converts all the concentrations to fluctuations around zero.[158] Scaling enable the adjustment of fold differences between the metabolites, increasing the importance of low abundant metabolites. Transformations including log and power transformations are generally applied to correct for heteroscedasticity.[159]

Statistical data analysis methods including multivariate data analysis (MVDA) can be used for extracting important features from large or small data sets containing a number of variables and observations. MVDA includes multivariate ANOVA

(MANOVA), linear discriminant analysis (LDA), cluster analysis (CA), principal component analysis (PCA), partial least square analysis (PLS). These methods are widely used in fungal detection and classification.[123, 160-162]

In this study, the effect of sample collection strategy using SPME fibers and sample preparation (fungal growth parameters) on the MVOCs production from *A. flavus* was investigated. For SPME fiber evaluation, the extraction efficiency of three commercial available SPME fibers coated with Carboxen/Polydimethylsiloxane (CAR/PDMS), Divinylbenzene /Polydimethylsiloxane (DVB/PDMS) and Carboxen/Divinylbenzene /PDMS were compared. A single *A. flavus* isolate was selected for this study in an attempt to reduce variations due to phenotype differences associated with different isolates.[140] For the growth parameters' effect study, the *A. flavus* isolate was grown under varied conditions using different temperatures and number of spores inoculated and on different substrates to evaluate the influence of these factors on MVOCs production. One aim of this study was to optimize fungal growth conditions for large MVOCs production and to determine MVOC variability within a single isolate. Data pretreatment methods including scaling, centering and transformations were applied to MVOCs data sets from *A. flavus* grown on five different substrates.

3.3 Chemicals and Equipment

3.3.1 Chemicals

An alkane mixture standard, methanol (≥99.5%), sucrose, $FeSO_4 \cdot 7H_2O$ (99+%), and L-asparagine monohydrate were purchased from Sigma-Aldrich (St. Louis, MO). $(NH_4)_2SO_4$ (99.7%), KH_2PO_4 (99.7%), $MgSO_4$ Anhydrous, $CaCl_2 \cdot 2H_2O$, $ZnSO_4 \cdot 7H_2O$,

$MnCl_2$ (97%), and Tween 20 solution was bought from Thermo Fisher Scientific (Pittsburgh, PA).

3.3.2 Growth medium

Malt Extract Agar (MEA), Czapek Solution Agar (CSA), and Corn Meal Agar (CMA) were purchased from Becton, Dickinson and Company (Franklin Lakes, New Jersey). The ingredients of chemical defined agar (CDA) was mixed based on the literature.[163] The ingredients of the growth medium are listed in Table 3.1.

Table 3.1 Growth substrates and their ingredients used in the study

Growth substrate name	Abbreviation	pH	Media conc.	Ingredients
Corn meal agar	CMA	6.0±0.2	17 g/L	2 g corn meal, infusion from solid, 15 g agar
Czapek solution agar	CSA	7.3±0.2	49 g/L	30 g saccharose, 2.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01g $FeSO_4$, and 15 g agar
Chemical defined agar	CDA	-	60.28 g/L	30 g sucrose, 10 g Asparagine, 3.5 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 500 mg $MgSO_4$, 200 mg $CaCl_2$, 10 mg $ZnSO_4 \cdot 7H_2O$, 5 mg $MnCl_2$, 2 mg $FeSO_4$, and 15 g agar
Chemical defined liquid	CDL	-	45.28 g/L	30g sucrose, 10 g Asparagine, 3.5g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 500 mg $MgSO_4$, 200mg $CaCl_2$, 10mg $ZnSO_4 \cdot 7H_2O$, 5 mg $MnCl_2$, 2 mg $FeSO_4$
Malt extract agar	MEA	4.7±0.2	33.6 g/L	12.75 g maltose, 2.75 g Dextrin, 2.35 g Glycerol, 0.78 g Peptone, and 15.0 g agar

3.3.3 Fungi growth apparatus

The 100 mm diameter petri dish for fungi growth was purchased from Fisher Scientific Inc. (Pittsburgh, PA). The hemocytometer was obtained from (C. A. Hausser

and Sons, Philadelphia, USA). The incubator and the 50 mL Erlenmeyer flasks were purchased from Fisher Scientific Inc. (Pittsburgh, PA).

3.3.4 SPME fibers

Carboxen/Polydimethylsiloxane (CAR/PDMS), Divinylbenzene/ Polydimethylsiloxane (DVB/PDMS), and DVB/CAR/PDMS SPME fibers were purchased from Sigma-Aldrich (St. Louis, MO).

3.3.5 Analytical equipment

The 7890 GC coupled with 5975c MSD was obtained from Agilent Technologies (Santa Clara, CA). The SPME inlet liner was purchased from Supelco Inc. (Bellefonte, PA, USA). Ultra pure helium was purchased from Airgas, Inc. (Columbus, MS). The 60-m J&W DB-1 capillary column was obtained from Agilent Technologies (Santa Clara, CA).

3.3.6 Fungal Isolates

The aflatoxigenic isolate K73 was collected from corn sampled in Sunflower County, MS. This isolate was used for SPME fiber comparison study. Two aflatoxigenic isolates 5-3B and 4-3A were isolated from pig feed in Maben, MS. These isolates were used for the growth parameters (growth substrate, concentration of spore suspension, and temperature) effects study.

3.4 Methods

3.4.1 Fungal growth method

In a typical experiment, the fungal isolate was cultured in a Petri dish containing MEA medium at 30 ℃ in the incubator for 7 d and was subcultured every two weeks to

maintain fresh spores. Fresh spores were extracted using 0.02 % Tween20 solution and diluted to the desired concentration using sterile distilled water while the number of spores was counted using the hemocytometer. A spore suspension (10 ul) was injected into a 50 ml Erlenmeyer flask containing 30 ml of the sterile growth medium. The Erlenmeyer flasks were then covered with aluminum foil and sealed with parafilm.

3.4.2 Selection of SPME fibers

The aflatoxigenic isolate K73 was used for the SPME fiber comparison study. The growth medium used was MEA and spores concentration was maintained at 1×10^6 spores/mL. Fungal cultures were incubated in the absence of light at 30 ℃ for 7 days. Fungal cultures were prepared in 6 replicates for each type of SPME fiber for a total of 18 experiments.

After 7 days of culture incubation, SPME fibers were exposed to the headspace of the Erlenmeyer flasks containing the fungus at 30 ℃ for 5 h. After the sampling period, the fiber was pulled into the needle sheath, the SPME device was removed from the flask and then inserted into the hot injection port of the GC-MS for thermal desorption.

3.4.3 Effects of growth parameters on the MVOCs production

The aflatoxigenic isolate, 5-3B, was used for the growth parameters (growth substrate, concentration of spore suspension, and temperature) effects study. The growth parameters evaluated are listed in Table 3.2. CAR/PDMS fibers were used for the effects of growth parameters study.

Table 3.2 Growth parameters evaluated for the effects on MVOCs production

Effect factors	Growth parameters
Media	CMA, CSA, CDA, CDL, and MEA
Concentration of spore suspension	See Table 3.3 (next section)
Temperature (ºC)	15, 30, 37, 45

3.4.4 Effects of Different Media on MVOCs production

The fungal growth media, their ingredients and amount used are listed in Table 3.1. The growth substrates were prepared by dissolving the agar in 1 L of deionized water followed by autoclaving at 121 ℃ for 15 min. The chemical defined liquid medium (CDL) was prepared using the same ingredients as in the chemical defined agar (CDA) medium without adding 15 g agar. Fungal cultures were prepared with 6 replicates for each growth medium for a total of 30 experiments. The fungi was grown in the absence of light at 30℃ for 7 days. The MVOCs were extracted using the PDMS/CAR fiber for 5 h at 30 ℃.

3.4.5 Effects of spore concentration on MVOCs production

The aflatoxigenic isolate 4-3A was used in this study. The concentration of spore suspension used is listed in Table 3.3. The fungal growth method was identical to the method described in section 3.3.1, except that four different concentrations of the spore suspension were injected on the MEA medium instead of the only 1×10^6 spores/ml. The toothpick method involves taking a small piece of spores and mycelium of fungi using a sterile toothpick and directly inserting it on the surface of agar medium. The fungi was grown in the absent of light at 30 ℃ for 7 days. The fungal culture were prepared in 6 replicates for each concentration inoculum for a total of 24 experiments. The MVOCs were extracted using PDMS/CAR fiber for 5 hours at 30 ℃.

Table 3.3 The concentration of spores suspension of *A. flavus* 4-3A used in the study

Treatment	Spores concentration (spores/mL)
High concentration inoculation	1.2×10^7
Medium concentration inoculation	2.4×10^6
Low concentration inoculation	4.8×10^5
Toothpick inoculation	--

3.4.6 Effects of growth temperature on MVOCs production

In the temperature effect study, each flask was incubated in the absence of light at four temperatures (15 ℃, 30 ℃, 37 ℃ and 45 ℃) for 7 days. The concentration of spores was 1×10^6 spores/mL and the fungi was grown on the MEA media. The fungal growth method was identical to the method described in section 3.3.1 except for the use of 4 different temperatures. The fungal isolates were prepared in 6 replicates at each temperature level for a total of 24 experiments. CAR/PDMS fibers were used to extract MVOCs for 5 hours at 30 ℃.

3.4.7 GC-MS analysis

The analysis of collected MVOCs was performed with a GC-MS. Extracted volatiles were thermally desorbed from the SPME fiber in the injection port (at 270℃), equipped with a 78.5 mm × 6.5 mm × 0.75 mm SPME inlet liner. Thermal desorption was setup for 5 min and the SPME fiber was conditioned for 1 h at 270 ℃ following manufacture instructions before the next usage. The gas chromatography capillary column used for separation was a 60-m DB-1 capillary column with an internal diameter of 320 µm and a film thickness of 1 µm. Helium was used as a carrier gas was with a flow velocity of 1.2 ml min^{-1}. The following GC oven temperature program was applied: 45 ℃ for 9 min, 10 ℃ min^{-1} to 85 ℃, hold for 3 min, 3 ℃ min^{-1} to 110℃, hold for 3 min,

3 ℃ min^{-1} to 120℃, hold for 3 min, and 10 ℃ min^{-1} to 270 ℃, hold for 5 min. The MS analysis was carried out in full scan mode (scan range from 35-350 amu) with ionization energy of 70 eV. Ion source and quadrupole temperatures were 230 ℃ and 150 ℃, respectively.

3.4.8 GC-MS MVOC data manipulation

3.4.8.1 Data processing

Tentative chromatographic peak identification was made by library matching using the NIST 08 MS Library. Compounds were considered positively identified when both mass spectra and retention index (RI) led to the same identification. Quantitative data for each analyte was determined using peak area. Peak alignment adjustments were required due to instrument drift and experimental error. Peak alignment procedures for samples from GC-MS measurements play an important role in biomarker detection and metabolomic studies in general.[81] The peak alignment procedures are illustrated in Appendix B.

Additional data processing required that peak areas of zero were replaced with values equal to 1 count to allow for log transformation.[71] The lowest peak areas in the rest of the data are on the order of 10^5. Any MVOCs detected less than three times in the 6 replication experiments were removed from further data treatment. Silicon containing peaks with m/z of 73, 207 and 281 are believed to have originated from the column stationary phase and were also removed from the processed data.

3.4.8.2 Data pretreatment

Systematic data pretreatment can be used to enhance the results of follow-on classification methods including PCA and PLS. The data pretreatment methods listed in Table 3.4 were compared using PCA to evaluate the classification results of five media types with six replications. In the SPME fiber selection study, log transformation (Table 3.4) were applied to achieve better group separations. Each MVOC detected represents the dependent variable in PCA and each replication is the observation.

Table 3.4 Overview of the pretreatment methods in this study[71]

Class	Method	Formula	Goal
I	Centering	$\tilde{x}_{ij} = x_{ij} - \bar{x}_i$	Focus on the differences and not the similarities in the data
II	Autoscaling	$\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{s_i}$	Compare metabolites based on corrections
	Pareto Scaling	$\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{\sqrt{s_i}}$	Reduce the relative importance of large values, but keep data structure partially intact
III	Log transformation	$\tilde{x}_{ij} = \log x_{ij}$	Correct for heteroscedasticity, pseudo scaling. Make multiplicative models additive
	Power transformation	$\tilde{x}_{ij} = \sqrt{(x_{ij})}$	Correct for heteroscedasticity, pseudo scaling
	Area normalization	$\tilde{x}_{ij} = \frac{x_{ij}}{\sum x_j} \times 100$	Relative quantity of analyte

The mean is estimated as: $\overline{x_{ij}} = \frac{1}{N}\sum_{N=1}^{N} x_{ij}$, and standard deviation is estimated as $s_i = \sqrt{\frac{\sum_{N=1}^{N}(x_{ij}-\bar{x}_i)^2}{N}}$. $\tilde{x}_{ij}$ is the data after the pretreatment and x_{ij} is the data before the pretreatment. i is the column and represents the relative concentration of each MVOC. j is the row and represents the samples (observations).

3.4.8.3 Data analysis

MANOVA was performed to examine whether there is significant difference in the quantities of MVOCs emitted by the fungal culture inoculated with different spore doses. 15 MVOCs, commonly emitted by the fungi, were selected to compare the

quantitative variation caused by the change in spore dose. These 15 compounds are ethanol, 1,4-pentadiene, 2-methylfuran, 2-methyl-1-propanol, 3-methylbutanol, 2-methylbutanol, toluene, (-)-aristolene, β-elemene, α-farnesene, cubenene, δ-cadinene, β-germacrene, β-panasinsene, and β-cadinene. The data was treated by log transformation and then MANOVA was performed using SAS 9.3 software (SAS Institute Inc.). PCA was performed using software program SIMCA-P+ 11.0 (Umetrics, Umea, Sweden). PCA classification results were evaluated using score plots.

3.5 Results and Discussion

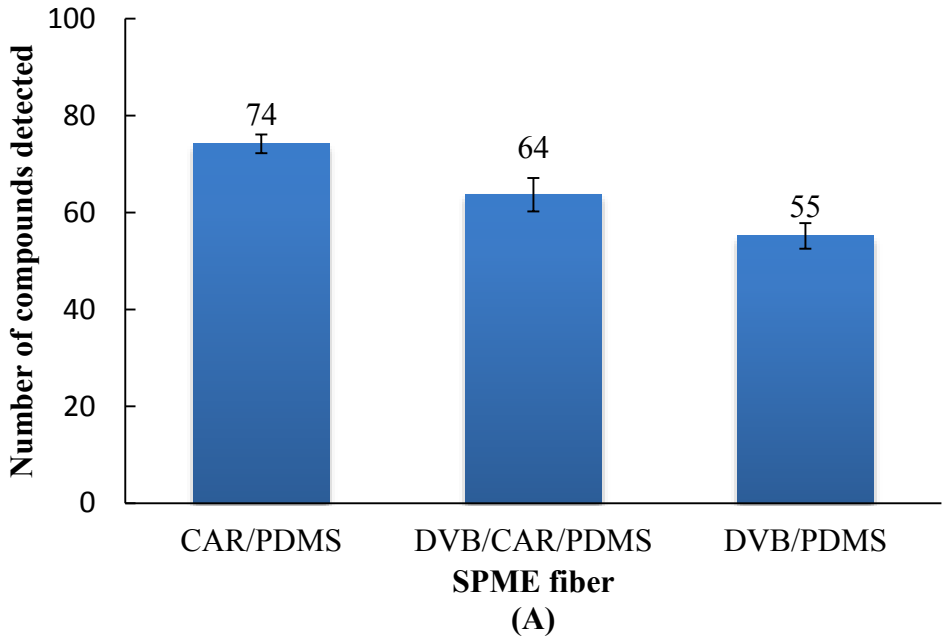
3.5.1 Evaluation of SPME fiber on metabolic profiling

Extracted MVOC profiles and quantities were determined and the information was used to select the best SPME fiber for metabolic fingerprinting. Experiment precision (repeatability) was evaluated based on relative standard deviation (RSD%) of the six replicates of three SPME fiber types: CAR/PDMS, DVB/PDMS and DVB/CAR/PDMS. These fibers were evaluated in terms of their efficiency in extracting volatile metabolites emitted by *A. flavus* K73 growth on a MEA substrate. The fungal culture was incubated for 7 d at 30 ℃ with initial inoculation spores concentration of 1×10^6 spores/mL. The SPME extraction was maintained at 30℃ for 5 hours. The extraction efficiency evaluation included two aspects, MVOC selectivity and quantity (peak area).

Three evaluated fibers showed different abilities to extract volatile metabolites of *A. flavus* as shown in Figure 3.1 A and B. The CAR/PDMS fiber not only extracted the largest number of MVOCs (Figure 3.1A), but also extracted the largest amount of MVOCs based on the total peak area of all the volatile metabolites (Figure 3.1B). A

closer look at the data revealed MVOC functional group selectivity. Identified MVOCs were divided into 9 chemical classes including alcohols, aldehydes, furans, hydrocarbons, ketones, organic acids, organosulfur compounds, sesquiterpenes, and other compounds. Among the chemical classes, hydrocarbons were divided into hydrocarbon1 (fewer than ten carbons) and hydrocarbon2 (ten or more carbons). Other compounds include seven unknown compounds, one ether and one ester.

The CAR/PDMS fiber extracted greater amount of alcohols, furans, hydrocarbons1, hydrocarbon2 and ketones, while DVB/PDMS extracted larger amount of high molecular weight compounds containing the organosulfur compounds, sesquiterpenes and other compounds (Figure 3.2). These results agree with the literature which describes the CAR/PDMS as likely to extract low molecular weight compounds while DVB/PDMS is better at extracting high molecular weight compounds.[164]



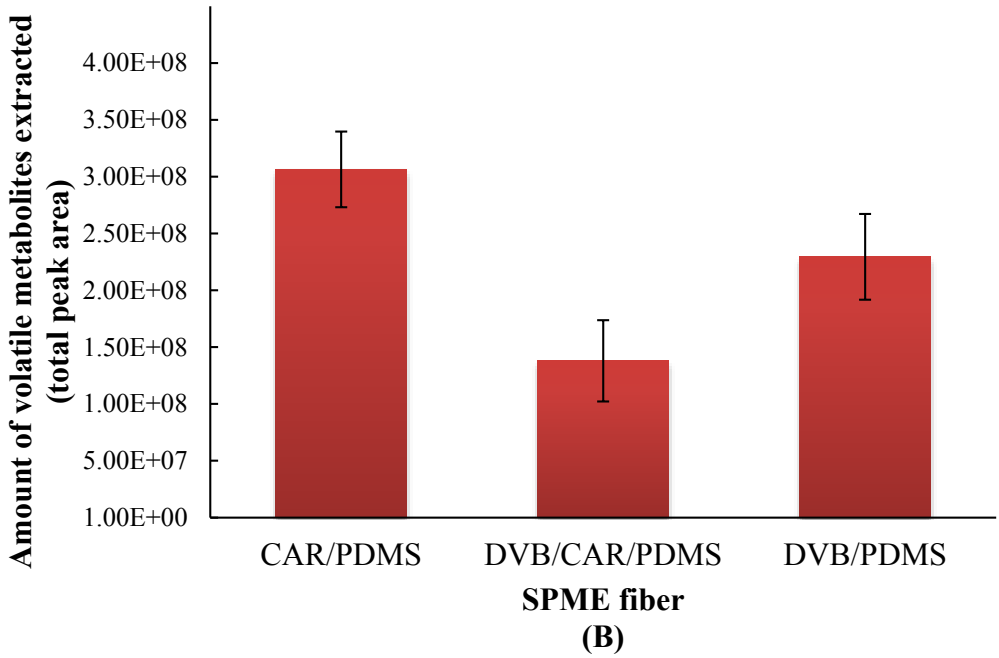


Figure 3.1 SPME fibers comparison through the number of (A) and amount of (B) volatile metabolites extracted from *A. flavus* culture using three types of SPME fibers CAR/PDMS, DVB/CAR/PDMS and DVB/PDMS in six replications.

The error bars indicate the standard deviation of six replicates.

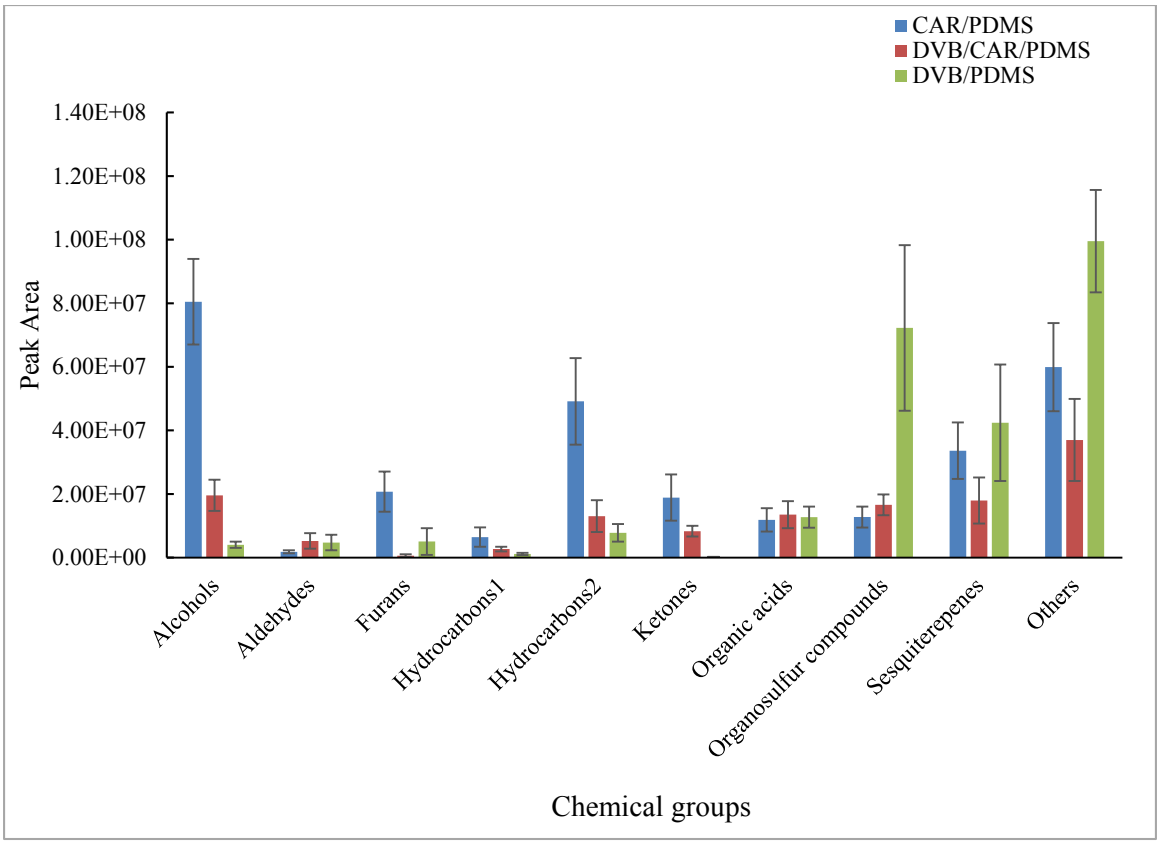


Figure 3.2 SPME fibers comparison though the amount of volatiles in chemical groups extracted from *A. flavus* culture using three types of SPME fibers CAR/PDMS, DVB/CAR/PDMS and DVB/PDMS in six replications.

The error bars indicate the standard deviation of peak areas of six replicates. Among the chemical classes, hydrocarbons were divided into hydrocarbon1 (fewer than ten carbons) and hydrocarbon2 (ten or more carbons).

Since the CAR/PDMS fiber has difficulty adsorbing higher molecular weight analytes and DVB/PDMS has difficulty extracting analytes with low molecular weights, a DVB/CAR/PDMS fiber was developed by the manufacturer.[165] The advertised extraction advantage of extended molecular weight range of VOCs was not observed based on our results (Figure 3.1A and B). CAR/PDMS was determined to be an excellent SPME fiber

coating choice for fungal MVOC profiling based on its ability to collect the largest number and greatest quantity of MVOCs.

The precision or repreatibility of this method was examined using the relative standard deviation percentage (RSD%) of each extracted and identified fungal MVOC. RSD% of each metabolite was calculated using both peak area and peak area percentage data. Table 3.5 lists 15 common MVOCs detected and their RSD% for each SPME fiber (the entire list of identified compounds can be found in Appendix A Table A.1). The peak area percentage was obtained by dividing peak area ion currents of each compound by chromatograms total ion current (TIC) times one hundred. This is referred to as the area normalization method (Table 3.4). This method is the earliest and most straightforward of the data pretreatment methods and requires no reference standards or calibration to be prepared. The average RSD% for each SPME fiber type is listed in Table 3.5.

Table 3.5 15 selected MVOCs and their RSD% (using both peak area and peak area percentage) obtained using three types of SPME fibers CAR/PDMS, DVB/PDMS, and DVB/CAR/ PDMS with 6 replications each.

No.	Compound name	R.T. (min)	Peak Area RSD%			Peak Area% RSD%		
			CAR	DVB CAR	DVB	CAR	DVB CAR	DVB
4	1,4-Pentadiene	5.543	48.8	61.9	-	40.4	40.0	-
9	Propanoic acid, 2-methyl-, anhydride	8.313	68.0	31.1	-	64.1	65.4	-
10	Furan, 2-methyl-	8.568	38.4	20.3	-	30.1	22.4	-
11	1-Propanol, 2-methyl-	9.046	30.4	32.6	-	29.7	30.8	-
15	1-Butanol, 3-methyl-	13.825	52.6	51.3	-	57.7	74.6	-
16	1-Butanol, 2-methyl-	13.943	37.8	34.3	-	41.8	32.3	-
18	Toluene	15.501	13.8	15.9	-	25.1	26.2	-
22	Styrene	21.901	23.8	17.3	19.3	35.7	24.5	19.7
36	Undecane, 2,6-dimethyl-	38.682	73.5	37.2	43.5	48.0	37.0	20.0
52	2,4,4,6,6,8,8-Heptamethyl-2-nonene	43.784	26.0	27.0	31.5	15.3	40.0	11.7
56	β-Elemene	44.256	26.8	24.7	30.2	27.1	23.8	7.8
64	α-Selinene	45.591	34.7	46.5	44.5	39.6	0.6	42.7
68	Cedrene	46.060	29.5	37.6	28.4	14.6	20.4	29.0
70	Calamenene	46.338	27.3	52.4	48.8	23.7	45.1	29.0
72	π-Calacorene	46.627	17.0	32.6	36.5	22.8	21.7	23.1
	Average RSD%[a]	**--**	**56.7**	**40.9**	**49.8**	**56.7**	**52.08**	**39.5**

[a] Total RSD% is the average RSD% of 133 MVOCs listed in Appendix A Table A.1 (see Appendix A Table A.1 for complete data)

The average peak area RSD% for CAR/PDMS, DVB/CAR/PDMS, DVB/PDMS are 56.7%, 40.9%, and 49.8%, respectively. Using peak area %, the RSD% of CAR/PDMS, DVB/CAR/PDMS, DVB/PDMS are 56.7%, 52.1%, and 39.5%, respectively. Previous studies show that RSD% determined using analytical standards calculated by peak area were 18.4%, 14.9% and 13.1%, for CAR/PDMS, DVB/CAR/PDMS and DVB/PDMS, respectively.[140] Several volatile metabolites show large fluctuations in concentration under identical experimental condition – this is due in

part to uninduced biological variation.[71] In this study, the uninduced biological variation added between 26% and 38% to our experimental variability.

PCA was performed to aid in the evaluation of the extraction efficiency of the SPME fiber types by exploring the correlation between the specific volatile metabolites and SPME fiber types. The raw peak area data (Appendix A Table A.2) was treated using log transformation to reduce the heteroscedasticity (uninduced biological variation), to convert the non-normal MVOC distribution into a normal one, and to make skewed distributions more symmetric.[71] This also helps to add emphasis to MVOCs present in trace quantities that may play a role in fungi identification. PCA was performed using the log transformed MVOCs peak area data. A subset of this data, 15 selected MVOCs, can be found in Table 3.6 (see appendix A Table A.3 for the complete data set).

Table 3.6 15 Selected MVOCs profile and log transformed data obtained using three types of SPME fibers CAR/PDMS, DVB/PDMS, and DVB/CAR/ PDMS with 6 replications each.

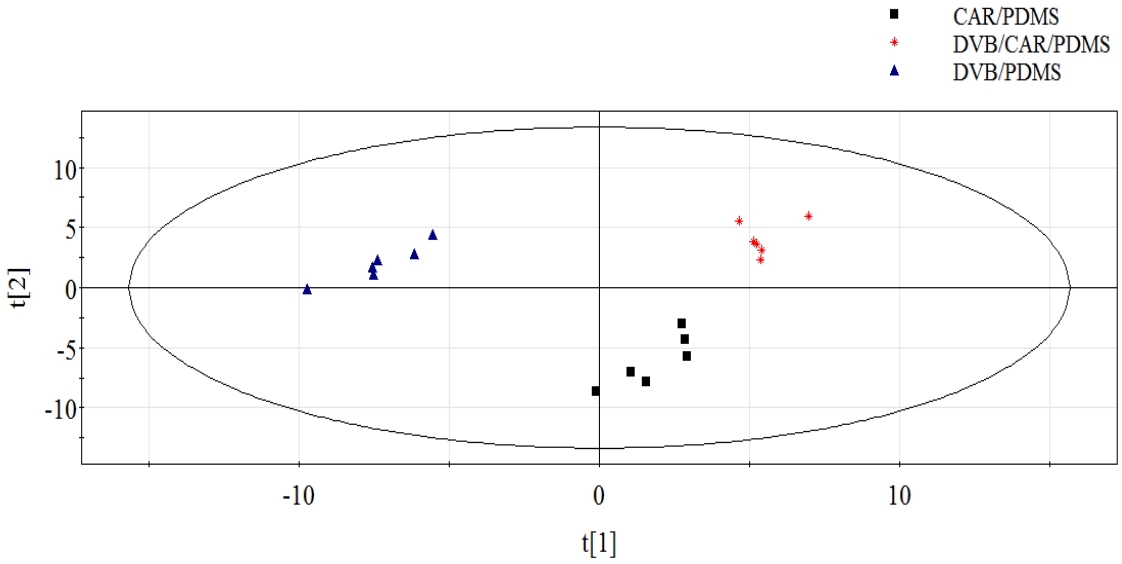
No.*	Library/ID	Log transformed data																	
		CAR/PDMS						DVB/CAR/PDMS						DVB/PDMS					
		REP 1	REP 2	REP 3	REP 4	REP 5	REP 6	REP 1	REP 2	REP 3	REP 4	REP 5	REP 6	REP 1	REP 2	REP 3	REP 4	REP 5	REP 6
1	Ethanol	6.4 7	6.4 4	6.29	6.6 8	6.9 7	6.9 3	6.5 8	6.3 1	6.3 0	6.5 4	6.3 0	6.3 2	-**	-	-	-	-	-
4	1,4-Pentadiene	7.0 5	7.2 2	7.42	7.2 2	7.2 1	7.6 0	7.0 3	6.5 2	7.1 5	6.6 5	6.7 8	6.5 6	-	-	-	-	-	-
5	Acetic acid, methyl ester	6.6 6	6.7 9	6.50	6.4 0	6.3 2	6.8 6	6.2 0	-	5.8 0	5.6 6	5.4 4	5.3 3	-	-	-	-	-	-
7	Butanal	5.4 6	6.6 6	5.90	6.1 0	5.9 9	5.3 6	-	-	-	-	-	-	-	-	-	-	-	-
8	Propanal, 2-methyl-	6.1 7	6.8 1	6.09	6.0 6	5.9 4	5.9 1	-	-	-	-	-	-	-	-	-	-	-	-
9	Propanoic acid, 2-methyl-, anhydride	5.8 3	6.4 6	6.02	5.8 3	5.9 0	6.3 4	5.1 1	-	5.2 6	5.0 7	5.0 3	5.3 4	-	-	-	-	-	-
10	Furan, 2-methyl-	6.9 8	7.1 3	7.21	7.1 7	7.3 2	7.4 5	6.9 1	6.7 2	7.0 1	6.9 3	6.9 5	6.9 1	-	-	-	-	-	-
11	1-Propanol, 2-methyl-	7.6 6	7.6 0	7.28	7.6 4	7.7 5	7.7 2	7.0 6	6.6 1	6.8 1	6.9 6	6.8 6	6.9 2	-	-	-	-	-	-
21	Ethylbenzene	-	-	-	-	-	-	5.0 4	5.2 3	5.4 4	5.4 7	5.4 7	5.3 2	-	-	-	-	-	-
55	(-)-Aristolene	6.6 0	6.8 9	6.31	6.5 5	6.5 4	6.3 9	6.1 9	6.2 1	6.2 1	5.9 1	6.2 1	6.2 2	6.5 9	6.5 6	6.5 5	6.5 3	6.5 6	6.16
56	β-Elemene	7.2 7	7.2 0	7.07	7.3 1	6.9 7	7.2 3	6.9 7	6.9 0	7.0 2	6.6 7	6.8 9	6.9 8	7.1 4	7.2 3	7.3 0	7.2 9	7.4 4	7.06
67	Copaene	-	-	-	-	-	-	-	-	-	-	-	-	7.5 4	7.7 2	7.5 5	7.7 8	7.3 9	7.65
69	γ-Cadinene	5.8 9	5.8 0	5.56	5.8 1	5.8 0	5.9 5	-	-	-	-	-	-	5.7 9	5.8 1	6.0 1	6.1 9	5.9 8	5.92
72	π-Calacorene	6.3 5	6.2 6	6.29	6.4 5	6.2 9	6.3 7	6.5 0	6.0 6	6.3 6	6.3 4	6.2 4	6.2 9	5.9 8	6.0 8	6.3 2	6.2 6	6.4 0	6.10
84	Octadecanal	-	-	-	-	-	-	-	-	-	-	-	-	6.4 6	6.5 0	6.4 9	6.1 9	6.8 3	6.18

* Compound number and names are identical to those in Appendix A Table A.1. The full table can be found in appendix (Table A.3).

** Below detection threshold.

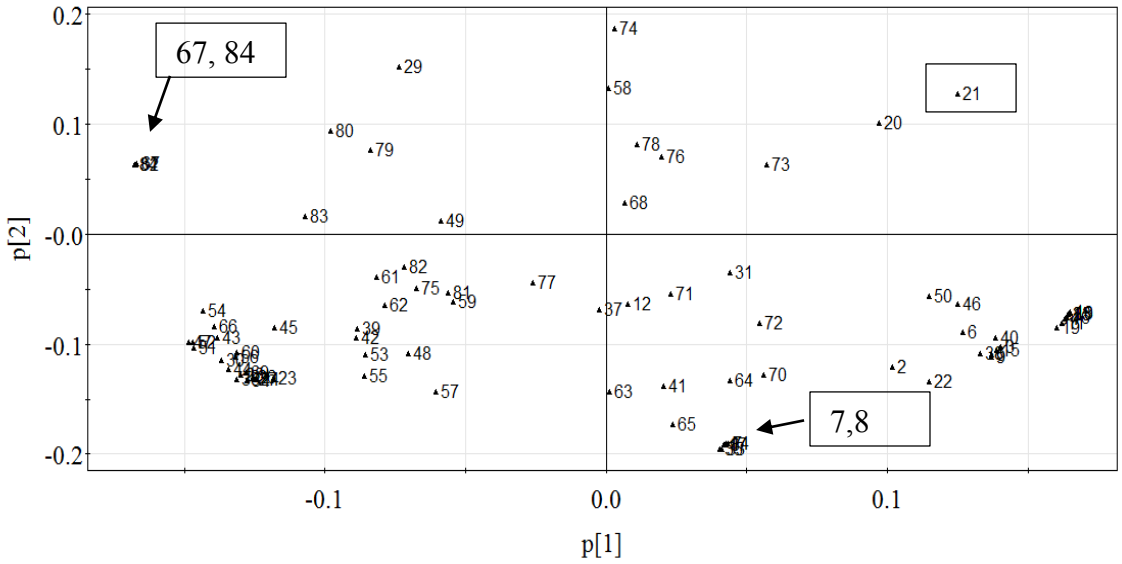
Figure 3.3 shows the score plot (A) and loading plot (B) from PCA analysis using the log transformed peak area MVOC profiles (dependent variables) and the SPME fiber coating types (independent variables). The score plot shows the classification results of the observations (data from different types of SPME fibers) and the loading plot describes the relationships among the variables (MVOCs). Even though the uninduced biological variation is large, the three types of SPME fiber can be classified successfully using the score plot. This is an indication that the SPME fibers do absorb differently emphasizing the importance of choosing the proper fiber for specific groups of target analytes.

A SPME fibers relative extraction selectivity can be seen in the loading plot. The chemicals absorbing most differently on the three SPME fibers are located on the periphery of the loading plot (farthest away from the plots center point). For example, octadecanal (**84**) and copaene (**67**) were only extracted by the DVB/PDMS fiber. These two compounds are located in the same region (upper left quadrant) of the loading plot. Butanal (**7**) and 2-methyl-propanal (**8**) are located in the lower right quadrant of the loading plot because they were only absorbed by the CAR/PDMS fiber. Ethylbenzene (**21**) was only extracted by the DVB/CAR/PDMS fiber located at upper right region of the loading plot. PCA is a fast and convenient method to identify the MVOC absorption patterns of the types of SPME fibers.



R2X[1] = 0.38 R2X[2] = 0.28 Ellipse: Hotelling T2 (0.95)

(A)



R2X[1] = 0.38 R2X[2] = 0.28

(B)

Figure 3.3 PCA score plot (A) and loading plot (B) by comparing the SPME fibers CAR/PDMS, DVB/CAR/PDMS and DVB/PDMS using volatile metabolites profiles.

The number in loading plot represents the volatile metabolites number listed in Appendix A Table A.1.

3.5.2 Effect of the growth substrates on MVOCs production

Growth factors affecting aflatoxin production by *Aspergillus paraciticus* has been reported by Reddy *et al.*[163] They found the amino acid asparagine to be essential for aflatoxin production. A number of authors have reported how substrate composition influences volatile production by fungi. For example, Larsen and Frisvad[45] found that while volatile profiles from *Penicillium* isolates were generally similar when the isolates were grown on either yeast extract sucrose agar or malt extract agar, fewer VOCs were produced by the same isolates when grown on Czapek yeast autolysate agar. Kahlos *et al.*[166] found that VOCs produced by the brown rot fungus Gloeophyllum odoratum varied, depending on the presence in the media of different growth elicitors, indicating the importance of media in studies of this nature. Hence, precedence exists for expecting MVOC variability due to growth media. The relationship between growth medium and *A. flavus* MVOC profiles are explored in the section below.

Five growth media substrates (Table 3.1) were evaluated. CMA contains the least amount of organic nutrients (around 2 grams), MEA is most commonly used in the MVOC studies[138, 167, 168] while CSA is typically used for *A. flavus* cultures.[84, 169] CDA and CDL are chemically defined medium that have previously been used for an aflatoxin production study.[163] The fungal culture was incubated for 7 days at 30℃ with initial inoculation spores concentration of 1×10^6 spores/mL. CAR/PDMS fiber was used for extracting the MVOCs for 5 hours at 30℃. Table 3.7 lists a subset of the identified MVOCs and their relative quantity (expressed in peak area) produced by *A. flavus* isolate 5-3B on the five different incubation medium (CMA, CSA, CDA, CDL and MEA).

Table 3.7 41[c] selected common MVOCs and their relative quantities (expressed in peak area) produced by *A. flavus* isolate 5-3B on the five different incubation medium (CMA, CSA, CDA, CDL and MEA).

No.	MVOC[a]	Chemical Classes	R.T. (min)	Media (Peak area × 10^5)				
				CDA	CSA	CDL	MEA	CMA
1	Ethanol	alcohol	4.595	15.9±7.29[b]	590±239	2441±2797	50.6±31.8	188±92
2	Acetone	ketone	5.022	-	282±97	142±155	266±151	71.1±24.7
4	Isopropyl Alcohol	alcohol	5.149	4471±601	312±66	-	78.4±46.3	57.03±7.79
7	1,4-Pentadiene	alkene	5.547	-	700±153	466±76	211±103	12.7±2.91
18	Furan, 2-methyl-	furan	8.567	131.7±41.4	360±45	1136±317	172±66.2	11.71±3.08
29	Toluene	aromatic hydrocarbons	15.506	12.9±4.35	24.7±7.95	101±27.2	24.3±3.35	8.63±1.71
36	Styrene	aromatic hydrocarbons	21.984	-	37.7±8.33	91.6±15.7	42.3±10.1	23.7±10.6
50	D-Limonene	aromatic hydrocarbons	30.733	-	34.9±3.24	334±548	3.76±2.38	8.23±2.14
54	Dodecane	alkane	33.053	14.1±2.11	46.4±16	46.5±14.8	96.6±99.6	30.9±5.98
63	2,2-Dimethylheptane -3,5-dione,	ketone	35.810	-	10.1±2.82	40.6±25.8	10.4±13.1	5.38±0.64
67	Decanal	aldehyde	39.186	6.65±0.864	14.9±8.36	238±178	11.1±8.25	8.58±2.82
69	1,3,7-Octatriene, 3,7-dimethyl-	alkene	40.716	12.4±2.45	-	13.5±11.9	13.5±7.87	-
73	Heptacosane	alkane	42.160	19.6±6.76	-	11.2±10.1	53.5±22.2	9.73±4.97
74	δ-Cadinene	sesquiterpene	42.380	-	38.1±17.2	29.7±32.2	11±4.47	20.7±10.8
81	(Z)-2-Hexadecene	alkene	43.313	84.9±22.9	19.7±5.31	-	33.7±8.69	15±7
82	trans-α-Bergamotene	sesquiterpene	43.379	282±77	38.7±25.8	76.7±37.3	13.7±5.65	16.1±8.52

Table 3.7 (Continued)

83	α-Cubebene	sesquiterpene	43.489	100±27	81.0±18.3	542±441	188±46.2	-
84	3-Hexadecene, (Z)-	alkene	43.619	61.3±13.8	155±56.7	68.6±35.1	91.6±23.9	75.7±34.3
85	trans-7-Hexadecene	alkene	43.781	-	83.7±30.9	44.2±28.7	14.4±5.98	37.7±16.7
87	Ylangene	sesquiterpene	43.988	10572±1341	96.2±32.1	813±345	39±20.6	-
88	(-)-Aristolene	sesquiterpene	44.106	15240±2280	944±251	13881±6678	154±41.4	27.3±8.56
89	β-Elemene	sesquiterpene	44.253	266±64.2	2770±1203	18305±8626	15.3±5.53	46.5±20.4
90	Isoledene	sesquiterpene	44.383	-	74±10.1	422±318	8.37±6.73	6.03±2.1
92	β-Humulene	sesquiterpene	44.502	-	89.9±34.4	366±194	8.16±2.46	-
95	α-Farnesene	sesquiterpene	44.690	4354±846	63.1±26.8	317±208	-	7.95±3.88
96	α-Gurjunene	sesquiterpene	44.778	647±138	187±79.1	5032±2941	18.3±8.73	-
97	β-Cubebene	sesquiterpene	44.871	2067±470	61.2±18.4	942±514	-	5.61±3.25
101	Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	sesquiterpene	45.110	3410±679	-	441±269	16.7±3.74	3.42±1.65
104	Valencene	sesquiterpene	45.511	1694±458	425±215	2675±2334	52.3±18.1	1.74±0.71
105	α-Selinene	sesquiterpene	45.590	10481±2747	536±115	3143±2099	30±20.2	-
106	α-Farnesene	sesquiterpene	45.742	1662±320	1079±553	13975±9284	-	3.58±1.06
109	Cubenene	sesquiterpene	46.030	2869±817	504±235	9414±6261	-	4.51±1.6
111	δ-Guaiene	sesquiterpene	46.181	9186±5597	100±39.7	1255±890	-	1.46±0.44
113	δ-Cadinene	sesquiterpene	46.329	1503±177	3196±1441	11706±8150	-	6.42±1.85
114	β-Germacrene	sesquiterpene	46.421	606±126	335±117	2428±1666	15.2±5.42	-
115	β-Panasinsene	sesquiterpene	46.489	1508±382	161±65.9	1218±853	21.8±3.71	-
116	β-Cadinene	sesquiterpene	46.616	164±39.9	307±178	4222±3500	9.23±3.28	-
122	Cadina-1(10),6,8-triene	alkene	47.141	60.7±16.9	34.9±14	299±145	12.3±8.27	-

Table 3.7 (Continued)

130	Germacrene D	sesquiterpene	47.993	52.8±19.9	37.8±15.4	438±298	10.7±4.56	-
131	α-Cadinol	alcohol	48.145	272±67	23.3±6.58	370±256	-	2.86±2.72
132	Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	aromatic hydrocarbons	48.345	-	68.7±22.8	603±65	-	13.85±5

[a] MVOCs selected are the compounds appeared in 4 out of 5 growth media

[b] Mean and standard deviation of peak area (6 replicates)

[c] Forty-one of the MVOCs were produced by the fungus in at least 4 out of 5 growth media substrates. The entire list of MVOC detected can be found in Appendix A. Table A.4

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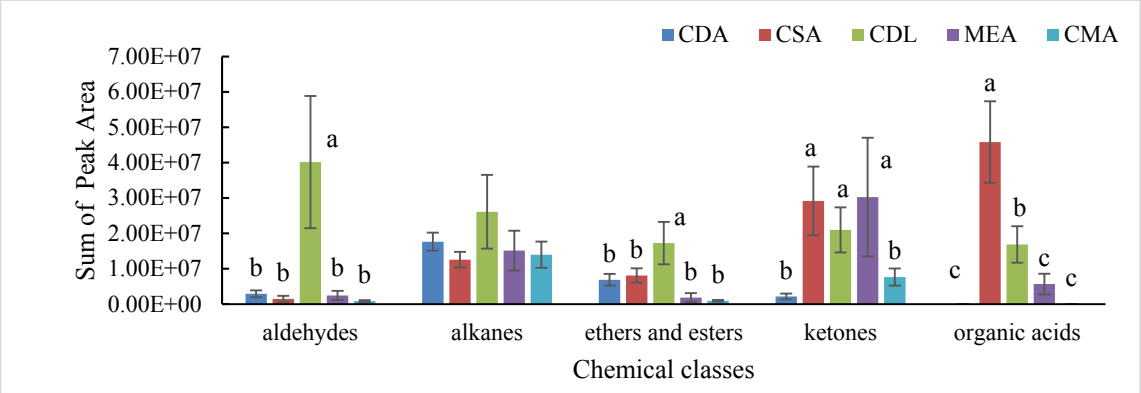
The entire list of compounds detected in any replicate in any media can be found in Appendix A Table A.4. A total of 132 MVOCs are detected in fungal cultures grown on all substrates. Forty-one of the MVOCs were produced by the fungus in at least 4 out of 5 growth media substrates. Ten MVOCs were produced on all growth media including ethanol (**1**), 2-methyl-furan (**18**), toluene (**10**), dodecane (**54**), decanal (**67**), (Z)-3-Hexadecene (**84**), and some sesquiterepenes (trans-α-bergamotene (**82**), (-)-aristolene (**88**), β-elemene (**89**), and valencene (**104**). These general fungal MVOCs have the potential to be considered as a group indicators of fungal growth.

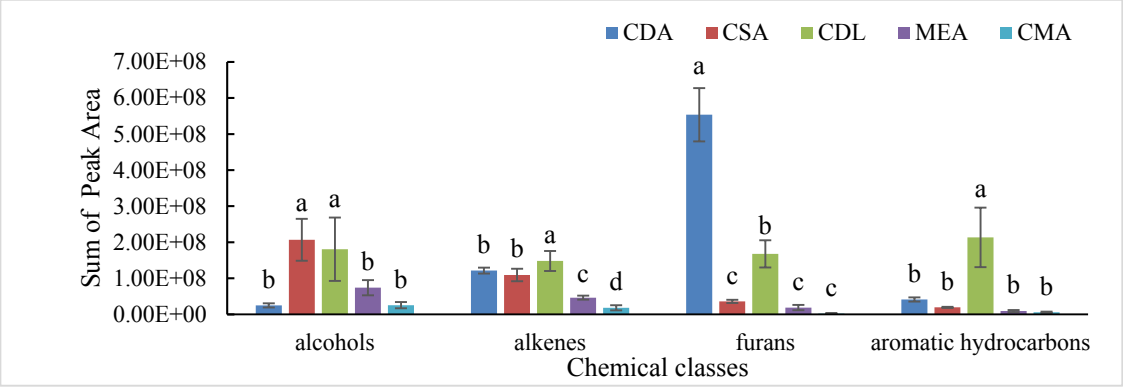
Different MVOC profiles are produced when the fungus is grown on different media. This difference can be seen when MVOC are divided into different chemical classes (alcohols, aldehydes, alkanes, alkenes, esters, ethers, furans, hydrocarbons, aromatic hydrocarbons, ketones, organic acids and sesquiterpenes) as shown in Figure 3.4. The 132 MVOCs were grouped by chemical class and their raw peak areas summed for Figure 3.4. MANOVA was performed to test whether there are significant differences among the total quantities of MVOCs in each chemicals class across the five growth media.

Figure 3.4B illustrates that the MVOCs production in CDA media has larger amount of furans than the other media. Among these growth medium, the fungi growth on the CDL media produces the largest amount of aldehydes, combination of ether and esters, aromatic hydrocarbons and alkenes. CDL and CDA have an identical list of ingredients except for the addition of 15 g of agar to the CDA media which helps to produce a gel like media. The quantities of MVOCs produced by the fungi growth on the liquid media (CDL) are much greater than those growth on the agar medium (CDA) for

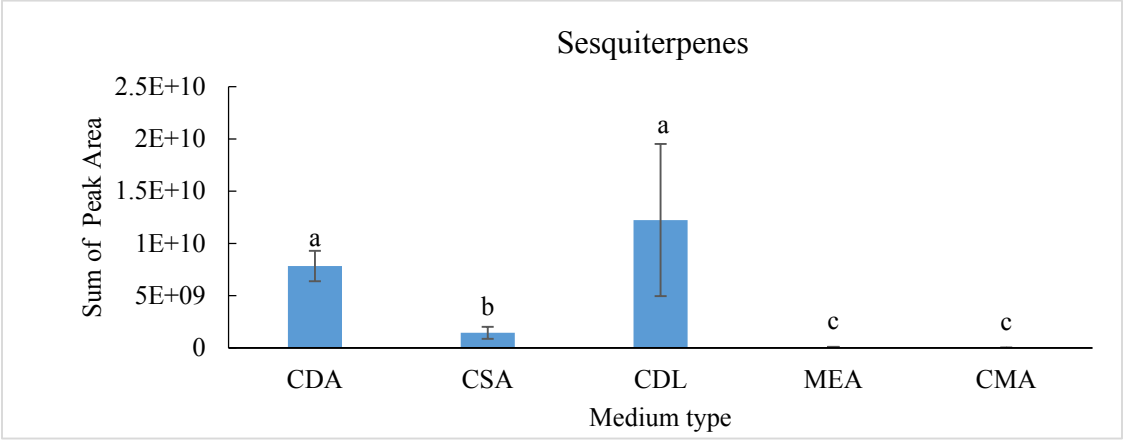
all chemical classes expect the furans. Interestingly, both CDL and CDA produced significantly more sesquiterepenes than the other growth media (Figure 3.4C). One possible explanation is the addition of L-asparagine which has been shown to enhance the production of sesquiterpenes and aflatoxins.[163] Hence, MVOCs production is clearly affected by growth media and growth media, must be considered when developing methods for classification of fungal species.



(A)



(B)



(C)

Figure 3.4 Comparison of amount of volatile metabolites (sum of peak area with SD (6 replicates)) emitted by *A. flavus* 5-3B on growth medium CDA, CSA, CDL, MEA and CMA.

Different letters above the bars indicate significant differences (MANOVA, $P<0.05$). The data with the highest total TIC for each chemical class starts with label a. The TIC of each medium shows no significant difference in the alkane group.

3.5.3 Effect of the concentration of spores suspension on MVOCs production

Traditional fungal inoculation and subculture methods are quite simple. A small piece of fungal culture is removed with sterile blade and then transferred to the surface of the growth medium. For *A. flavus*, it typically takes 7 days for the fungi to cover the entire agar surface of the 100 mm diameter petri dish. In this study differing spore suspensions were precisely prepared. Using these suspensions a controlled spore dose could be added to the growth media in order to elucidate the relationship between dose and MVOC production.

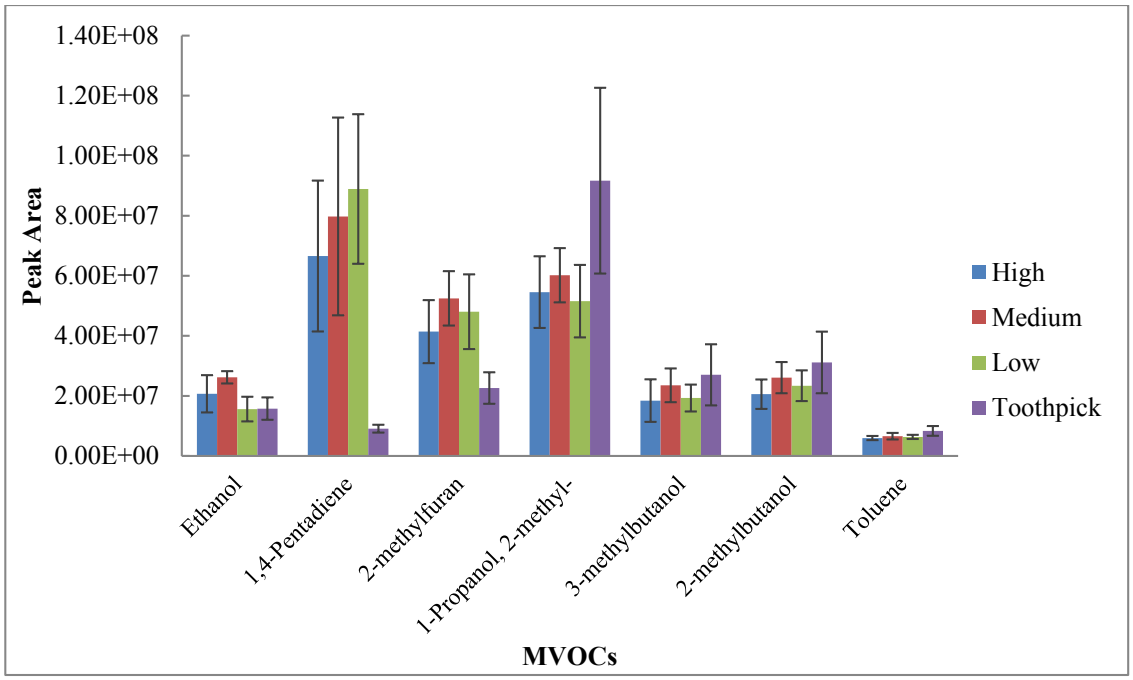
Spores of *A. flavus* were collected with a Tween 20 solution, diluted as needed with water and counted using a hemocytometer. Then 10 uL of a specific concentration of the spore suspension was injected on the malt extract agar (MEA) media surface before the standard 7 days of incubation at 30 ºC. CAR/PDMS fiber was used to extract MVOCs for 5 hours at 30ºC. Four concentration of spores' suspension were used including a high concentration (1.2×10^7 spores/mL), medium concentration (2.4×10^6 spores/mL), and low concentration (4.8×10^5 spores/mL). A toothpick inoculation method was also used for comparison.

The peak areas of 15 commonly detected MVOCs emitted by *A. flavus* 5-3B were selected to compare the different spore doses (Table 3.8). No consistent trends were observed that correlate spore count with specific MVOC peak ion counts. The variation of 15 MVOCs expressed in peak areas caused by different spores inoculum concentrations are shown in Figure 3.5.

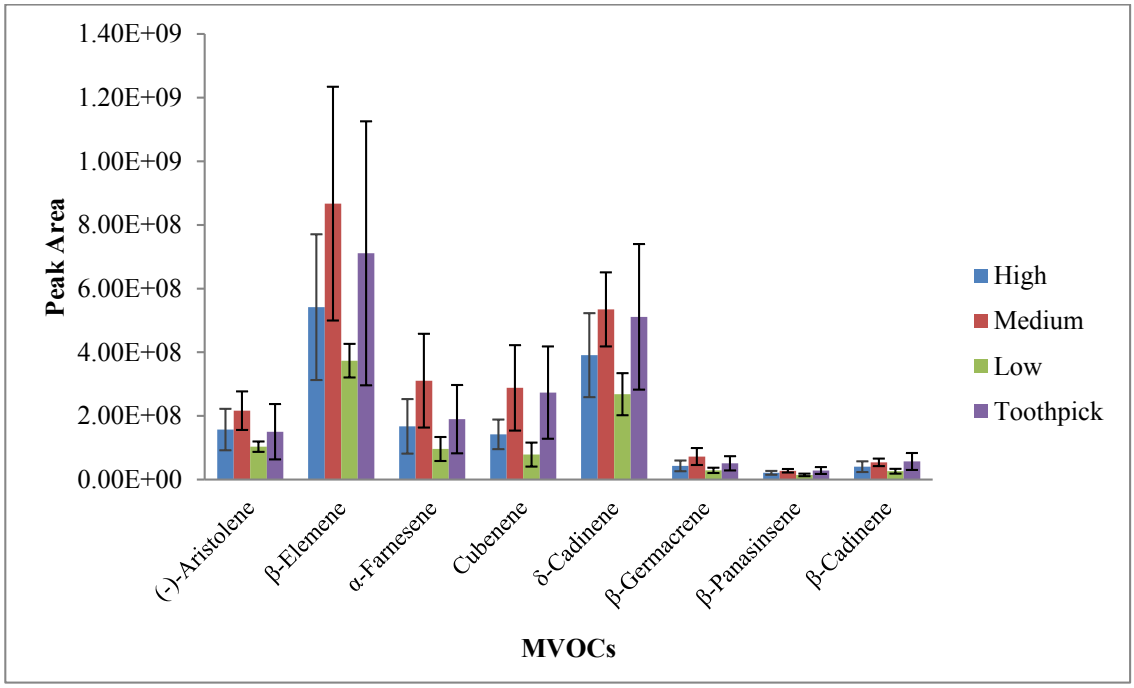
Table 3.8 Quantities of 15 selected common MVOCs of fungi culture inoculated with four different spores' concentrations

No.	Compound name	Peak Area ($\times 10^7$)[a]				P value[b]
		High	Medium	Low	Toothpick	
1	Ethanol	2.36±0.9	2.62±0.2	1.56±0.41	1.58±0.37	0.791
2	1,4-Pentadiene	6.99±2.39	7.98±3.29	8.89±2.49	0.91±0.13	0.010
3	2-methylfuran	4.4±1.13	5.25±0.9	4.8±1.24	2.26±0.52	0.009
4	1-Propanol, 2-methyl-	5.38±1.09	6.02±0.9	5.16±1.21	9.17±3.09	0.045
5	3-methylbutanol	1.83±0.63	2.35±0.56	1.93±0.45	2.7±1.02	0.061
6	2-methylbutanol	2.07±0.44	2.61±0.52	2.34±0.51	3.12±1.03	0.012
7	Toluene	0.66±0.16	0.66±0.11	0.63±0.07	0.84±0.16	0.005
8	(-)-Aristolene	19.27±10.45	21.66±6.07	10.33±1.63	15.04±8.68	0.597
9	β-Elemene	66.06±35.61	86.67±36.71	37.35±5.26	71.08±41.45	0.381
10	α-Farnesene	23.52±18.37	31.07±14.74	9.61±3.76	18.99±10.72	0.499
11	Cubenene	19.48±13.56	28.81±13.4	7.86±3.78	27.33±14.48	0.363
12	δ-Cadinene	50.58±30.48	53.46±11.63	26.83±6.6	51.12±22.88	0.712
13	β-Germacrene	6.22±4.88	7.24±2.67	2.92±0.83	5.12±2.25	0.174
14	β-Panasinsene	3.45±3.22	2.77±0.55	1.46±0.4	2.85±1.06	0.402
15	β-Cadinene	5.94±4.84	5.44±1.18	2.65±0.75	5.7±2.66	0.613

[a] data expressed as means and standard deviations of peak area
[b] comparison of high, medium, low concentrations and toothpick method.
Note: The mean difference is significant with 95% confident interval.



(A)



(B)

Figure 3.5 Amount of MVOCs expressed in peak area (SD for 6 replicates) of 15 selected MVOCs from *A. flavus* 5-3B grown on MEA medium inoculated with different concentrations of spore suspensions.

High, Medium, Low and Toothpick indicate spore dose (see Table 3.3).

3.5.4 Effect of temperature on MVOCs production

Temperature has been proven to affect *A. flavus* growth and aflatoxin production. MVOC production from *A. flavus* was investigated using four different temperatures: 15 ℃, 30 ℃, 37 ℃ and 45 ℃. Again spores were grown on MEA media for 7 days, inoculated spore count was 10 µL of a 10^6 spores/mL mixture and the MVOCs were collected with a CAR/PDMS SPME fiber for 5 h at 30℃. The TIC chromatograms obtained from analyzing the MVOCs emitted by *A. flavus* 5-3B grown in different temperatures were compared to select the preferred temperature (Figure 3.6). The fungi grown at 15 ℃ and 30 ℃ produced more amounts of sesquiterpenes compared to the fungi grown at 37 ℃ and 45 ℃ (Figure 3.6 B). The morphology of the fungi grown in 15 ℃, 30 ℃, and 37 ℃ are similar after 7 days; however, the fungi grows slowly at 45 ℃. The compound 1-butanol can be used as the indicator of fungi growth condition because 1-butanol is produced largely from MEA medium. The present of large amount of 1-butanol (Figure 3.6A) in the fungi culture indicated the slow growth rate of fungi 45 ℃.

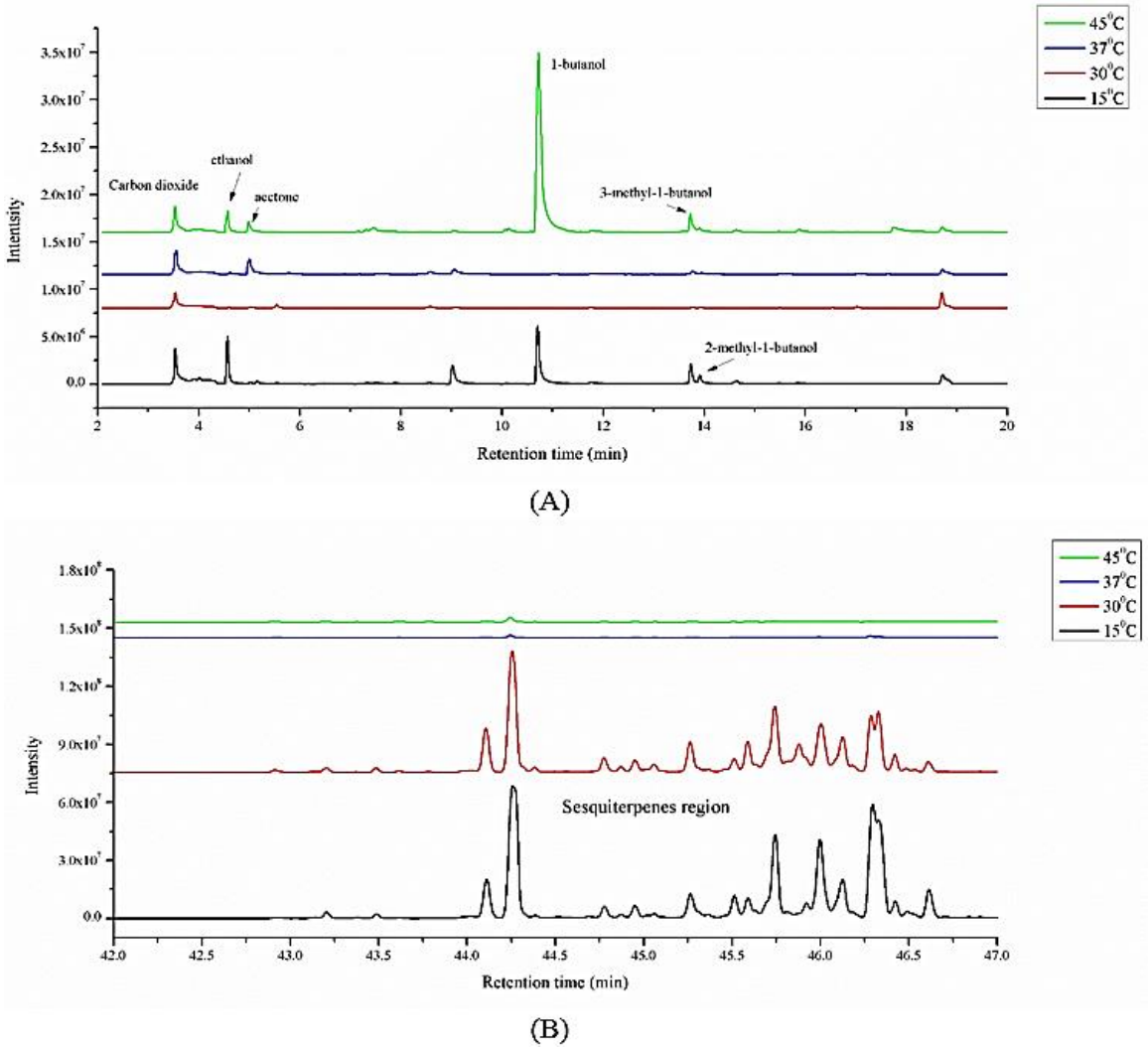


Figure 3.6 TIC chromatogram comparison of MVOCs profiles obtained from *A. flavus* 5-3B grown in different temperatures (15℃, 30℃, 37℃, and 45℃).

Schindler et al. [169] reported the optimum temperature for aflatoxin production occurred from at 24 ℃, and the maximal growth of *A. flavus* isolates occurred at 29 ℃ and 35 ℃. However, the optimum growth and aflatoxin production temperature may vary when growing on the different substrates. Karunaratne and Bullerman[170] reported the mycelial growth and sporulation of *A. flavus* occurred faster at 35 ℃ at all spore levels than 28 ℃ on rice. At 28 ℃, high amounts of aflatoxin B1 were produced, while the

lower and the higher spore levels produced comparatively lower levels of aflatoxin. A suitable temperature for fungal growth and aflatoxins production should be used when selecting the optimum temperature for a MVOCs study. A growth temperature of approximately 30 ℃ was determined to be the best temperature for this MVOC profiling study.

3.5.5 Effect of data pretreatment methods

Data pretreatment methods can be utilized to convert raw data to a different scale (for instance, logarithmic scale or relative scale) which reduces unwanted biases to more clearly depict important biological signals. The effect of data pretreatment have been illustrated through the application of six data pretreatment methods on MVOCs data of *A. flavus* grown on the five different media substrates (data from section 3.4.2). Results of these methods are shown in Figure 3.7 where A) is the raw data. The other graphs represent: B) centering, C) autoscaling, D) pareto scaling, E) log transformation, F) power transformation, and G) area normalization. The pretreatment methods were performed according to the equations listed in Table 3.4.

The MVOCs raw data obtained from the five growth media study was used for data pretreatment methods evaluation. The raw peak area data for the 132 identified MVOC (Appendix A. Table A.4) are shown in Figure 3.7A (MVOC profile from CDL media replication 1). Mean centering was applied to obtain a mean value of zero in order to improve the interpretability of the model (Figure 3.7B). Autoscaling is a combination of mean centering and scaling to unit variance where the scaling weight employed is $1/s$, and s represents the the standard deviation of the variable (peak area of a specific MVOC). After autoscaling, “long” variables are “shrunk” and “short” variables are

"stretched" (Figure 3.6C). In pareto scaling, the scaling weight is $1/\sqrt{s}$, and it is intermediate between the extremes of no scaling and autoscaling (Figure 3.6D). The data does not become dimensionless as after autoscaling, so this method stays closer to the original measurement than autoscaling.

Another objective of data pre-treatment is converting a non-normal distribution of the specific variable into a normal one. One way to accomplish this is through log transformation (Figure 3.6E). The benefits of this sort of transformation includes 1) simplifying the response function by linearizing a non-linear response-factor relationship, 2) stabilizing the variance of the residuals, and 3) making the distribution of the residuals more normal, which can serve to eliminate outliers. Power transformation plots the square root of the data (Figure 3.6F) and is similar to the pareto scaling method. Finally, the area normalization method (Figure 3.6G) showed similar results when compared to the original data (Figure 3.6A). The area normalization method is a semi-quantitative approach using the relative percentage of each compound of the total MVOCs extracted.

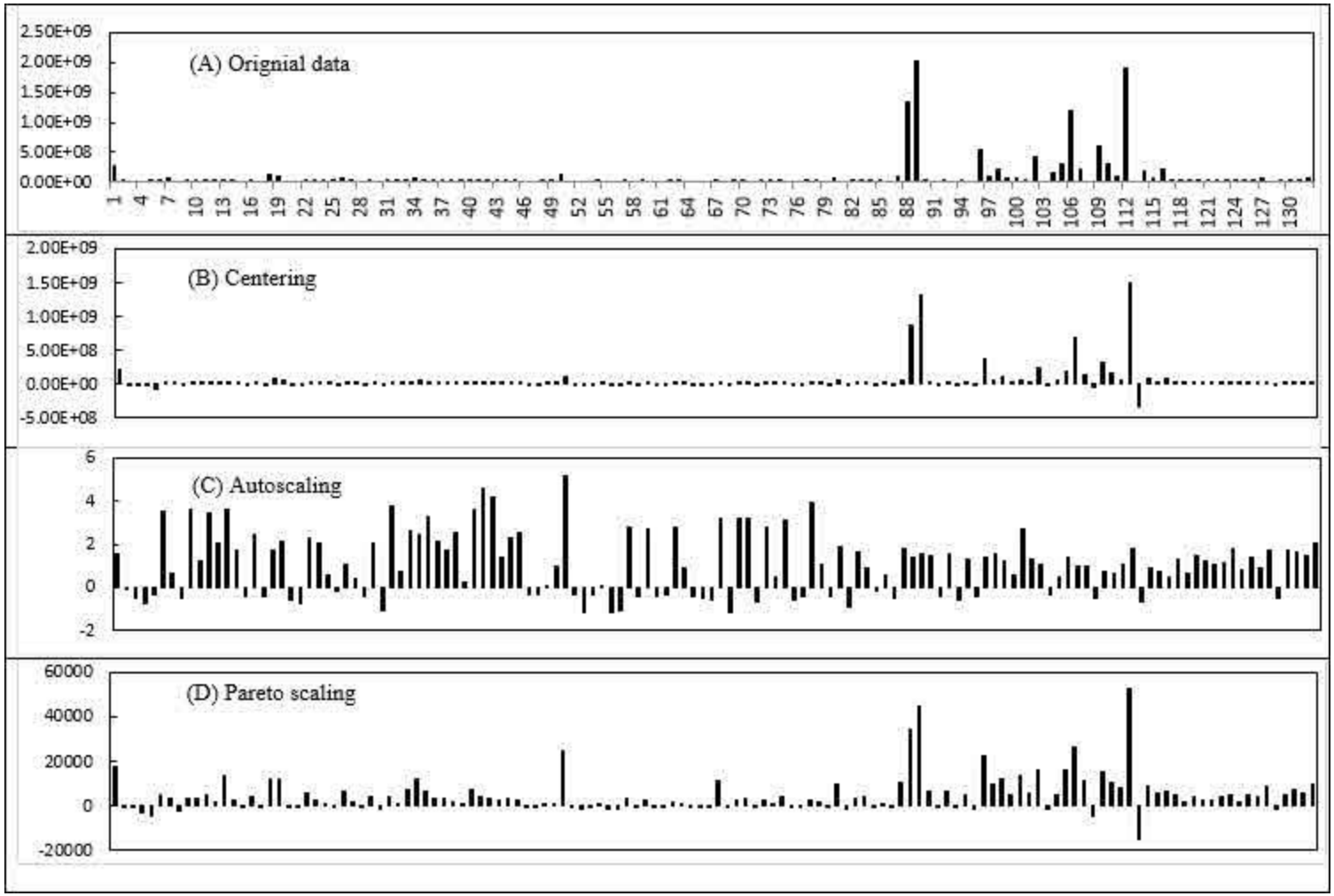


Figure 3.7 Effect of data pretreatment on the original data.

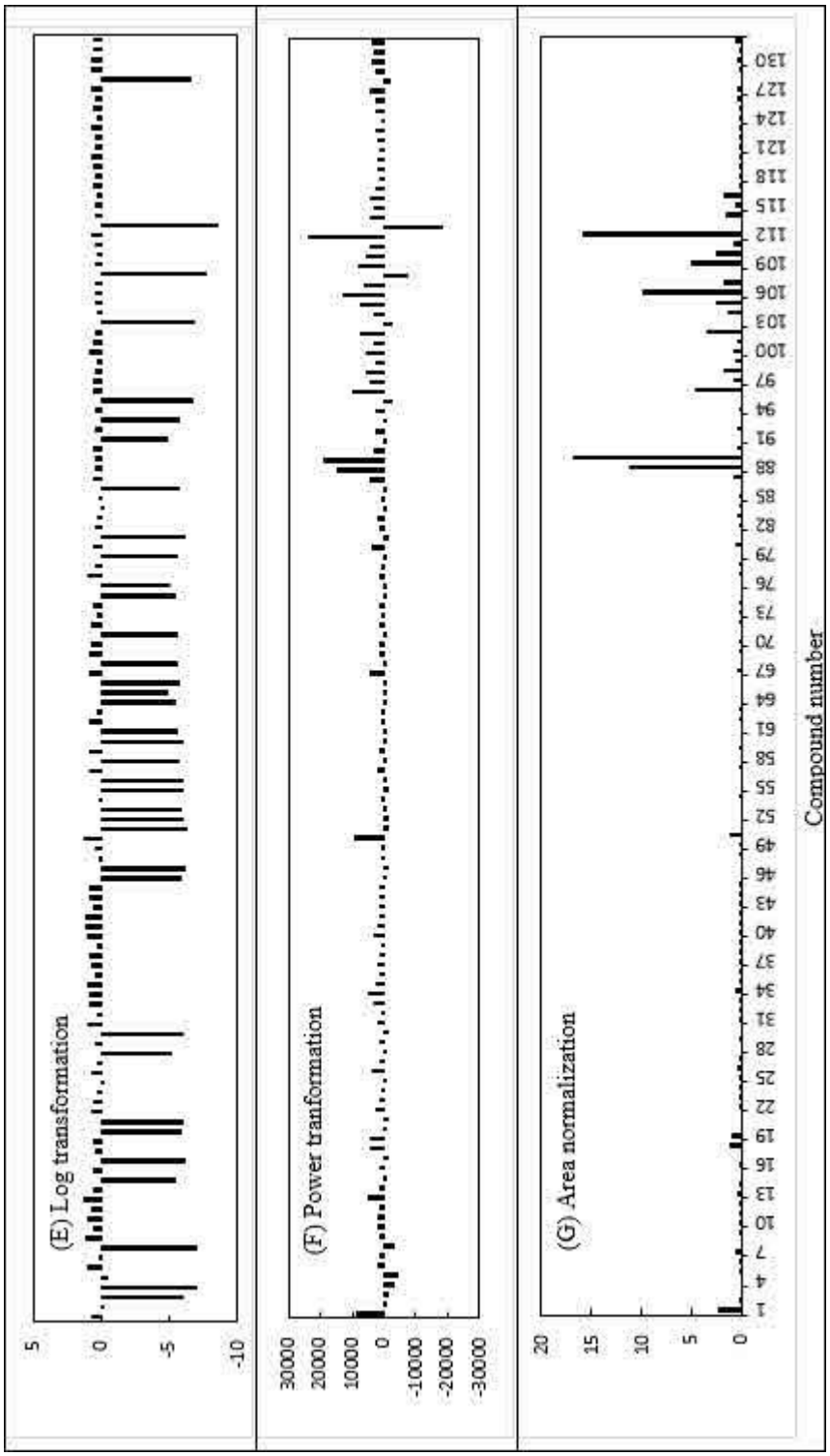


Figure 3.7 (Continued)

Each of the 6 data pretreatment methods were applied to the entire data set. PCA was used to analyze the effect of each method (Figure 3.8). PCA can also identify important MVOCs contributing to classification by analysis of the loadings. Suitable data pretreatment methods help provide good cluster separation where the distance within the cluster of a specific category (Media type) and the distance between the clusters of the categories are favorable. The application of log transformation (Figure 3.7E) provided the best clustering results in the score plots. PCA analysis of the centering (Figure 3.7B), pareto scaling (Figure 3.7D) and the original data (Figure 3.7A) provided poor clustering results compared to the other pretreatment methods. Power transformed data showed intermediate cluster separation. Tight clusters were produced with the area normalization method (Figure 3.7G), however, the CDA, CSA and CDL clusters were not well separated.

In the original (3.8A) and centering data (3.8B), MEA, CMA and CSA clusters "squeeze" together because CDA and CDL have much larger variances in the data caused by higher concentration of MVOCs. Large variances play an important role for classification of different categories (media) in PCA analysis. Poor cluster separation resulted from data pretreatment without a "hard" scaling method such as autoscaling. Pareto scaling is the intermediate between no scaling and autoscaling, which also showed unfavorable classification.

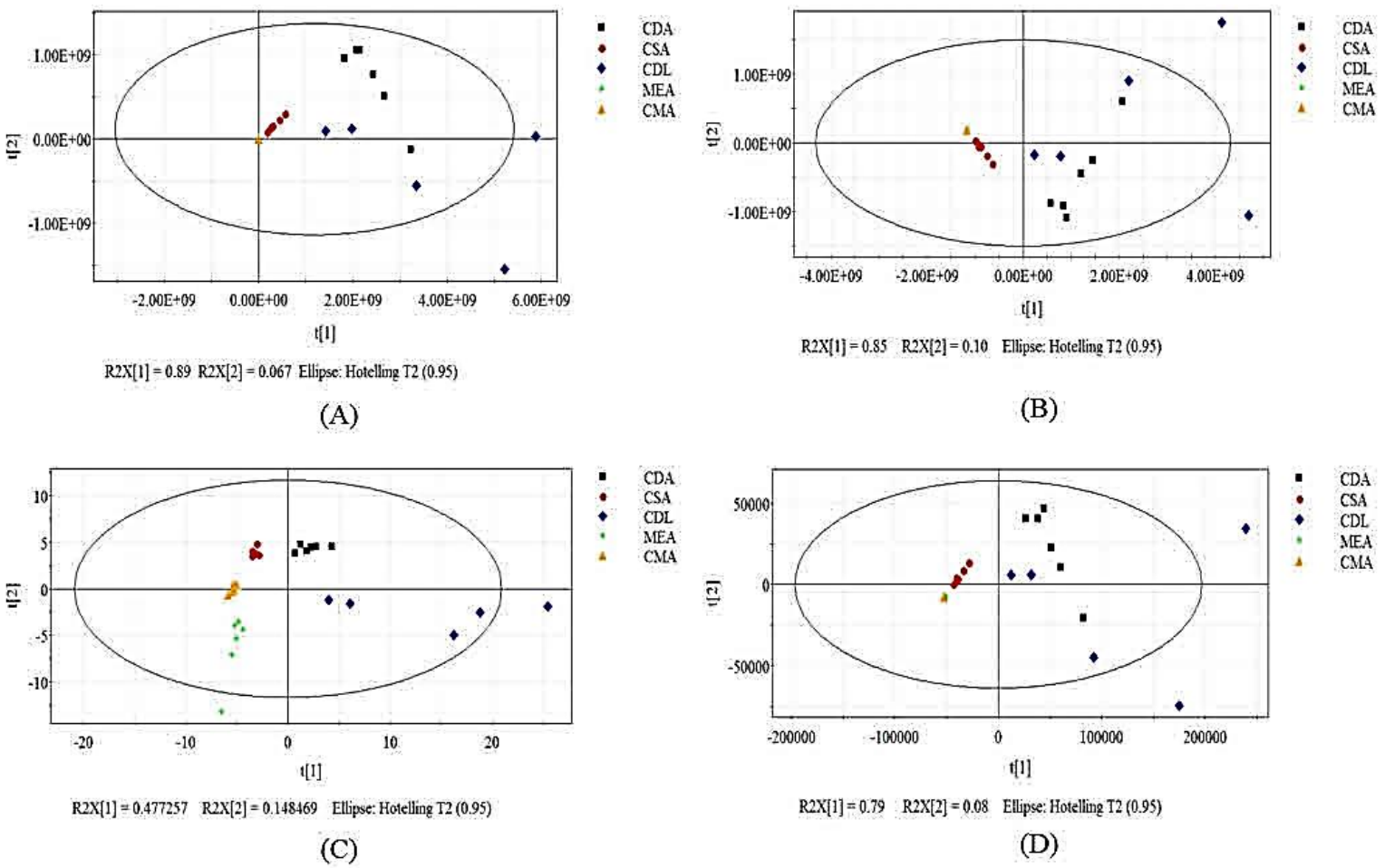
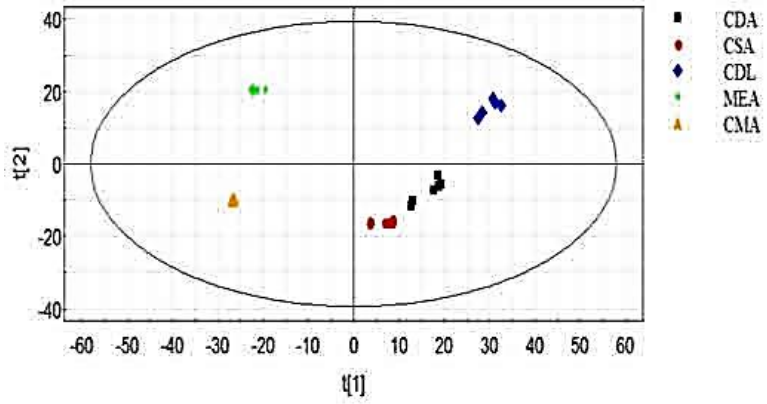
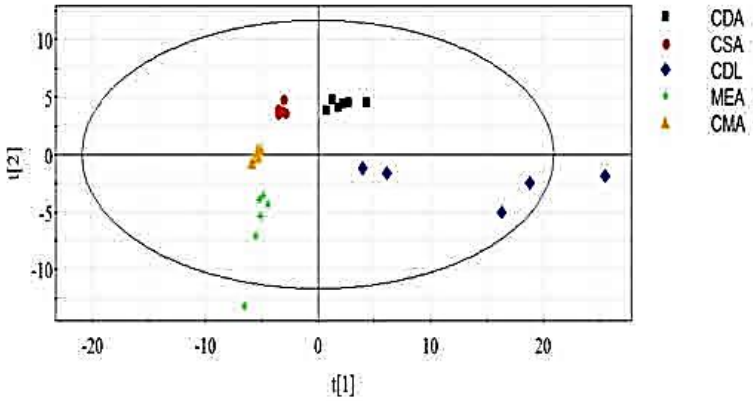


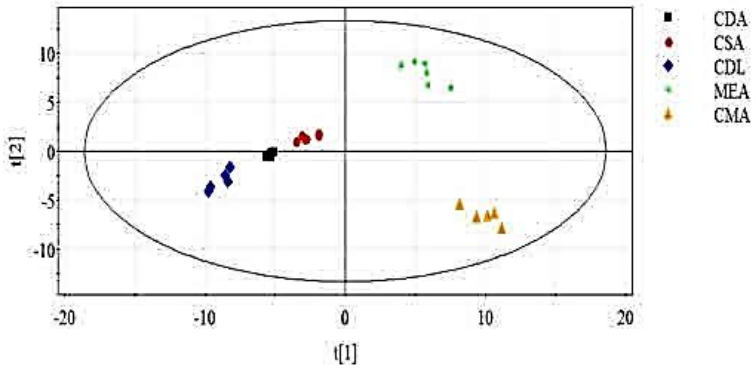
Figure 3.8 Effect of data pretreatment on the PCA results. PCA results of original data (A), centered data (B), autoscaled data (C), pareto scaled data (D), log transformed data (E), power transformed data (F), and area normalized data (G).



(E)



(F)



(G)

Figure 3.8 (Continued)

3.6 Conclusion

This study demonstrates that the experimental parameters used for MVOCs fingerprinting are crucial to the outcomes of MVOCs profiles and the data analysis. The identity and quantity of MVOCs extracted can be affected by many factors. MVOC profile trends were observed for: 1) the selection of SPME fiber, 2) fungal growth medium, and 3) growth temperature. Original spore dose also changes MVOC profiles; however, no clear trends were observed. The CAR/PDMS fiber seem to perform better than the other SPME fibers by collecting a larger variety and quantity of MVOC. Fungi grown on the CDL media produced much larger quantities of MVOCs compared to CSA, CDA, CMA and MEA medium. The highest MVOC production were found at 30 ℃ (this is known to be the optimal temperature for aflatoxin production).

Data pretreatment method is a key component of data analysis. The proper pretreatment methods will lead to better cluster separation which will aid in the discovery of relevant biomarkers. d

Many of the results presented here highlight the difficulties associated with chemotaxanomy of species from MVOC profiles even in a controlled laboratory environment. The goal of development of an aflatoxigenic *A. flavus* monitoring system for food storage is extremely challenging. Our results indicate that changes in growth media and conditions will result in significantly different profiles than those produced in a laboratory environment. However, the methods used, both for MVOC profiling and analysis, along aflatoxin analysis can be applied to generate new isolate profiles. These new profiles can then be used to develop monitoring strategies for the early identification of aflatoxigenic *A. flavus* contamination in an industrial setting.

CHAPTER IV

METABOLIC FINGERPRINTING OF AFLATOXIN-PRODUCING *ASPERGILLUS FLAVUS* USING HS-SPME-GCMS AND MULTIVARIATE ANALYSIS

4.1 Abstract

The identification and classification of *Aspergillus flavus (A. flavus)* from an examination of the microbial volatile organic compounds (MVOCs) emitted by the fungus has the potential to be the part of an early warning system for aflatoxigenic fungi isolate contamination. MVOCs profiles of different *A. flavus* isolates have been identified using a headspace solid phase microextraction gas chromatography mass spectrometry (HS-SPME-GCMS) strategy. Multiple statistical analysis approaches were used to discriminate the aflatoxigenic and non-aflatoxigenic *A. flavus* isolates using their MVOC profiles. Significant variations were found when comparing both individual MVOCs and groups of MVOCs by chemical classes (with the same functional group) using Multivariate ANOVA (MANOVA) analysis. Partial least-squares discriminant analysis (PLS-DA) models were used for discriminating isolates using 78 individual key MVOCs. The PLS-DA model has excellent classification specificity, where (-)-aristolene, calarene, β-germacrene, and γ-muurolene were discovered as possible volatile biomarkers for identifying aflatoxigenic isolates. This study strongly supports the concept that MVOC profiling can be used for identification of toxigenic fungal isolates and that HS-SPME-

GCMS combined with PLS-DA is a powerful method for fungal contamination identification and potential biomarkers discovery.

4.2 Introduction

Aspergillus flavus (*A. flavus*), one of the most abundant soil-borne fungi on earth, has a severe economic impact on both the agriculture and food industry because it can cause ear rot on maize and produce aflatoxins.[171] These aflatoxins are considered to be among the world's most potent naturally occurring carcinogens and a powerful toxin.[172] *A. flavus* has the ability to survive on many organic nutrient sources including corn, cotton, stored grains, dead insects, and plant debris.[14] It can survive in extremely harsh environments by forming sclerotia, which germinate to produce new colonies or conidiophores when conditions become favorable, resulting in significant losses to farmers.[173] Extensive efforts have been made worldwide to detect and quantify aflatoxins,[174-178] to develop control strategies for aflatoxigenic *A. flavus*,[179-182] and to study the biosynthetic mechanism of aflatoxins.[36, 183]

Many techniques including high-performance liquid chromatography mass spectrometry (HPLC-MS),[185, 186] enzyme linked immune-sorbent assay (ELISA),[53, 187] and multiplex polymerase chain reactions (multiplex PCR) assay[184] have been developed for detection and quantification of aflatoxins in order to reduce the economic loss of infected crops. Several studies have suggested that volatile fungal metabolites, also called microbial volatile organic compounds (MVOCs), vary from species to species and can enable chemotaxonomy of the fungal species.[44, 124] These MVOCs are produced during the primary and secondary metabolism of fungi and represent a small portion of the metabolome with molecular weights generally less than 250 Da. For example, a total of

132 isolates of 25 different terverticillate *Penicillium* fungi have been successfully classified using MVOC data and cluster analysis (CA).[188] The utilization of MVOC profiles obtained from Aspergillus species have allowed the identification of species-specific patterns for *Aspergillus versicolor*, *Aspergillus ustus* and *A. flavus*.[185]

Substantial efforts have been exerted on the development of electronic nose technology for fast detection or identification of fungal contamination on food[141, 189-191] or in an indoor environment.[119, 162] However, a detailed understanding of how volatile metabolites integrate with other metabolic processes like mycotoxin formation is still not known. The situation is complicated because MVOC profiles from fungi are significantly affected not only by the fungal genus and species, but also by growth phase,[192] temperature, humidity,[193] and media.[194]

Solid phase microextraction (SPME) has been successfully employed as part of a MVOC analysis (profiling) strategy. SPME is widely used for MVOCs sampling because it is a portable, non-invasive, and solvent free absorption technique. [115] When coupled with GCMS analysis it has been shown to provide accurate results, producing calibration curves with good fits over relevant concentrations.[195] This strategy has been well established for collecting MVOCs from species of *Aspergillus*,[153] *Penicillium*,[97] and *Fusarium*.[196] Hundreds of volatile metabolites are typically detected by this technique, complicating the identification of a unique pattern of specific chemicals associated with a specific fungal species. To overcome this problem, multivariate data analysis (MVDA) including principle component analysis (PCA),[190, 197-199] linear discriminant analysis (LDA),[203, 204] partial least squares projections to latent structures (PLS) analysis,[200] and CA[185, 188] have been widely utilized in classification and discrimination of fungal genus

and species based on MVOC profiles. Qualitative and quantitative information from detected MVOCs are treated as dependent variables and fungal isolates are set as independent variables to perform multivariate analysis. In this way large data sets can be analyzed in seconds.

The aim of this study was to 1) determine the MVOC profiles of aflatoxigenic and non-aflatoxigenic isolates of *A. flavus* grown on malt extract agar (MEA) medium, 2) identify MVOC patterns for each isolate by performing multivariate chemometric analysis, 3) identify volatile biomarkers specific to the group of aflatoxigenic and non-aflatoxigenic isolates. To our knowledge, this is the first attempt to classify the *A. flavus* species at the isolate level from MVOC profiles.

4.3 Material and methods

4.3.1 Chemicals and materials

An alkane mixture standard (for retention index value determination) and methanol (≥99.5%) were purchased from Sigma-Aldrich (St. Louis, MO). MEA was purchased from Becton, Dickinson, and Company (Franklin Lakes, New Jersey). Tween20 solution was purchased from Thermo Fisher Scientific (Pittsburgh, PA).

4.3.2 Fungal species

The *A. flavus* isolates used in this study are listed in Table 4.1. The aflatoxigenic isolates NRRL 3357 and a non-aflatoxigenic isolate NRRL 21882 were provided by the United States Department of Agriculture-Agricultural Research Service, Corn Host Plant Resistance Research Unit, Mississippi State University, Starkville, MS (USDA-ARS-CHPRRU). The aflatoxigenic isolate 5-3B was isolated from pig feed in Maben, MS.

Aflatoxigenic isolate K73 and non-aflatoxigenic isolates K35 and K32 were collected from corn sampled in Sunflower County, MS.

Table 4.1 *A. flavus* isolates used in the study.

Aflatoxigenic isolates	Non-aflatoxigenic isolates
NRRL 3357	NRRL 21882
5-3B	K35
K73	K32

4.3.3 Fungal growth

Fungal growth media was prepared by dissolving 33.6 g of the MEA powder in 1 L of purified water followed by autoclaving at 121 ℃ for 15 min. All the fungal isolates were cultured in a Petri dish (Fisher Scientific Inc.) containing malt extract agar at 30℃ in an incubator (Fisher Scientific Inc.) for 7 days. Fungal spores were then extracted using a 0.02% Tween 20 solution and diluted to 1×10^6 spores/ml with distilled water for spores inoculation. The concentration of the spore suspension was determined using a hemocytometer (C. A. Hausser and Sons, Philadelphia, USA). A 10-µL spore suspension was injected into a sterile 50 ml Erlenmeyer flask containing 30 mL sterile MEA media. The Erlenmeyer flasks were then covered with aluminum foil and sealed with parafilm (Bemis Manufacturing Company). Three aflatoxigenic (NRRL 3357, 5-3B and K73) *A. flavus* isolates, three non-aflatoxigenic (NRRL 21882, K32, K35) *A. flavus* isolates and a control (MEA medium) were prepared in 12 replicates each. Each flask was incubated in the absence of light at 30 ℃ for 7 days. A limited number of samples could be tested in one day (6 samples per day); therefore, 12 replicate samples were prepared over two

different days (6 each day). However, the incubation time and growth conditions of each replication were identical. Experiment and data analysis steps are shown in Figure 4.1.

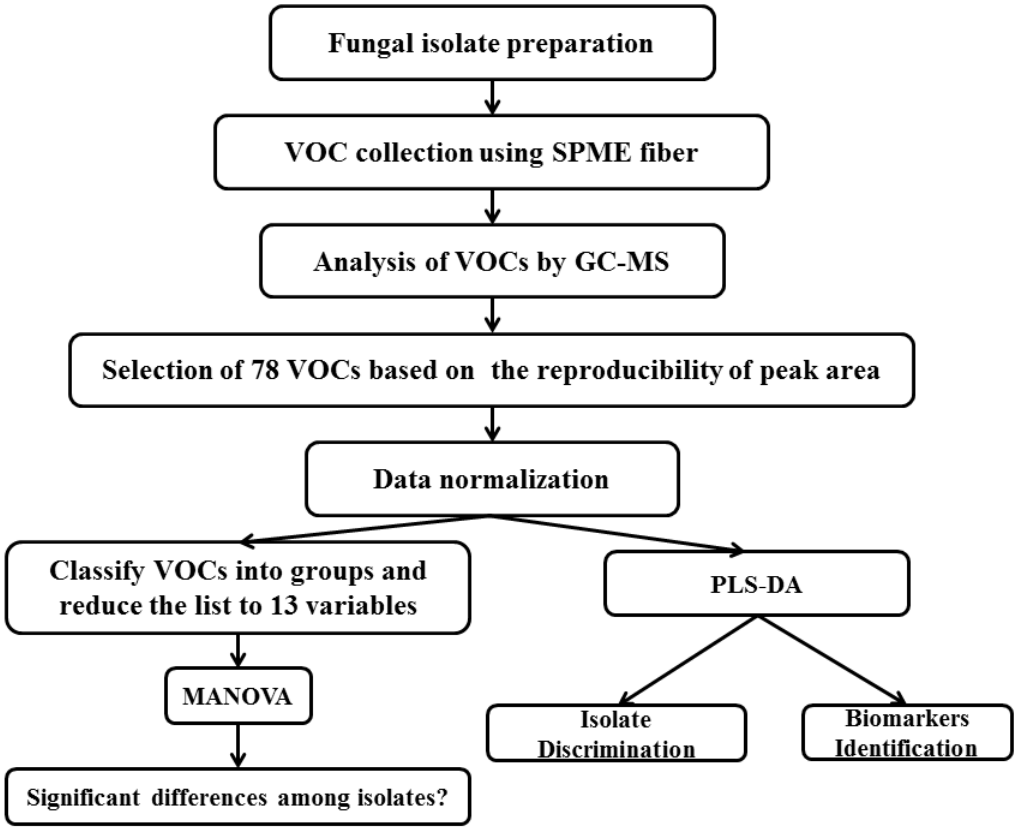


Figure 4.1 Summary of the experiment and data analysis procedures

4.3.4 Sampling of MVOCs

MVOCs were collected using an 85 µm Carboxen/Divinylbenzene/ Polydimethylsiloxane (CAR/DVB/PDMS) SPME fiber which has excellent VOCs absorption characteristics,[40] particularly for the selective absorption of sequiterpenes. After 7 days of culture incubation, SPME fibers were used to collect VOCs from the headspace of the Erlenmeyer flasks containing the fungal cultures for 5 hours. After the

sampling period, the fiber was pulled into the needle sheath, the SPME device was removed from the flask and was then inserted into the hot injection port of GC-MS for thermal desorption (270 ℃) within five hours of sample collection.

4.3.5 Aflatoxin production confirmation

Aflatoxin production was confirmed by VICAM Aflacheck, which has a 10 ppb limit of detection (Appendix C Figure C.1). In this method, 10 mL of 70% methanol was poured into the Erlenmeyer flask containing the fungal culture followed by shaking the flask for 1 minute using a vortex mixer (Scientific Industry, Inc.). A 250 µL sample extract was transferred to the strip test dilution tube, and 250 µL of distilled water was added with a micropipette. The solution was mixed by capping the strip test tube and shaking by hand. The test strip was then inserted into the dilution tube. A negative result could be determined if both a test line and a control emerged after 3 min. A positive result could be confirmed if no test line appeared after 5 minutes. Three replications of the Alfacheck test were done for each isolate sample (3 samples were randomly selected from 12 replications) collected on day 7 from the growth media. Results show that each of the aflatoxigenic isolates (K73, NRRL 3357 and ASP 5-3B) tested positive and each of the non-aflatoxigenic isolates (NRRL 21882, K32, K35) tested negative for aflatoxins production on all 18 tests.

4.3.6 Analysis of MVOCs by GC-MS

The analysis of collected volatile metabolites was performed on a 7890 gas chromatography (Agilent technologies) coupled with 5975C inert XL MSD. Extracted volatiles were thermally desorbed from the CAR/DVB/PDMS fiber in the injection port

(at 270℃), equipped with a 78.5 mm × 6.5 mm × 0.75 mm SPME inlet liner (Supelco Inc., Bellefonte, PA, USA). Thermal desorption was set up for 5 min, and the SPME fiber was conditioned for 1 h at 270℃ following manufacture instructions before the next usage. Analyte separation was done on a 60-m DB-1 capillary column with an internal diameter of 320 µm and a film thickness of 1 µm. The carrier gas was helium with a flow velocity of 1.2 ml min^{-1}. The following GC oven temperature program was applied: 45 ℃ for 9 min hold, 10 ℃ min^{-1} ramp to 85 ℃, hold for 3 min, 3 ℃ min^{-1} to 110 ℃, hold for 3 min, 3 ℃ min^{-1} to 120℃, hold for 3 min, and 10 ℃ min^{-1} to 270 ℃, hold for 5 min for a total analysis time of 50.6 min. MS analysis was carried out in full scan mode (scan range from 35-350 amu) with an ionization energy of 70 eV. Ion source and quadrupole temperatures were 230 ℃ and 150 ℃, respectively.

4.3.7 Identification of volatile metabolites and data processing

Tentative chromatographic peak identification was made by library matching using the NIST 08 MS Library. In addition, retention indices (RIs) were calculated for each peak with reference to the normal alkanes C6-C20 series.[201] Calculated RIs were then compared with those stored in a NIST database.[202] Compounds were considered identified when both mass spectra and RIs led to the same identification. A threshold of 10^6 was used as a peak ion current for any compound. Peaks below this size were deleted from the dataset. Relative peak area percentage (semi quantitative data) was calculated as a fraction of the total ion count (TIC) for each analyte.

4.3.8 Chemometric multivariate analysis

Peak alignment and data pretreatment procedures are detailed in Appendix B. The MVOC dataset was normalized using peak area percentage ((peak ion count/TIC)×100). Relative standard deviations (RSD%) were calculated for each volatile metabolite using both the peak area and a compositional dataset (grouping by functional group). Each MVOC having good precision (RSD <60%) in both peak ion count and peak area % with a minimum signal intensity of peak ion count $>10^6$ units was used for multivariate analysis, while other MVOC data was discarded. Ultimately, 78 MVOCs were selected, and their peak area data transformed to obtain a mean of zero and a standard deviation of one by applying autoscaling.[71] This data was then used for classifying and discriminating fungal isolates.

Multivariate analysis of variance (MANOVA) was performed using software from International Business Machines Corp. (SPSS statistics 19). The selected 78 volatile metabolites were divided into chemical groupings including alcohols, aldehydes, alkanes, alkenes, alkynes, benzene related group (BTEX), esters, furans, ketones, terpenes, organic acids, pyrazines, sesquiterpenes, and sesquiterpenoids. The autoscaled MVOC dataset from these 14 groupings (based on functional group) was used in order to determine differences in the isolates, and the controls' MVOC profiles. Fisher's least significant difference (LSD) (P=0.05) was performed to analyze variance and mean separation among the fungal isolates.

To classify *A. flavus* isolates and identify the volatile biomarkers associated with a specific sample class (specific isolate or control), partial least square discriminant analysis (PLS-DA) was used as a supervised classification method. Compared to the PCA

model, PLS-DA can maximize the covariance between the numerical value (**X** matrix) of targeted volatile metabolites and class assignment (**Y** matrix). The PLS-DA and PCA were performed using the software program SIMCA-P+ 11.0 (Umetrics, Umea, Sweden). The peak area data of MVOCs for different isolates and control were directly loaded in the software. The data were pretreated by log transformation and mean centering methods.

The quality of the models was evaluated by a cumulative fraction of X-variation modeled up to a specific component, R^2X (cum), and the cumulative fraction of Y-variation modeled up to the specific model, R^2Y (cum), where R^2Y (cum) is defined as the proportion of variance explained by the models and indicates goodness of fit. Cumulative Q^2 (sum) values explain the cross-validated predictive ability of the model. The seven cross-validation groups were used throughout to determine the number of components. The metabolites with the greatest variable importance in projection (VIP) values in the model and *P*-value (less than 0.05) in ANOVA or t-test were regarded as potential biomarkers.

4.4 Results and discussion

4.4.1 VOC profile of *A. flavus* and control

MVOCs can readily diffuse through biological systems into the gas phase, serving as signaling molecules for the identification of species or as an indicator of state of health. MVOC tracking has also been used in quality control.[203] For example, Lebrun et al.[204] used mango fruit volatiles as maturity markers to determine the optimal harvest maturity for the mango fruit. The possibility exists that fungi-specific biomarkers exist in their MVOCs profiles that could be used to identify the presence of the fungus.

MVOCs from both toxigenic and non-toxigenic *A. flavus* isolates were collected and analyzed after 7 days incubation on MEA media in order to identify candidate biomarkers associated with the production of aflatoxins. MVOC analysis resulted in the identification and quantification of a total of 202 different compounds. The selected 78 compounds used for chemometrics belonged to 14 chemical classes including 6 alcohols, 6 aldehydes, 6 alkanes, 7 alkenes, 1 alkyne, 5 BTEX, 2 esters, 4 furans, 9 ketones, 2 terpenes, 2 organic acids, 2 pyrazines, 22 sesquiterpenes, and 4 sesquiterpenoids (Table 4.2). The 78 volatile metabolites have an average relative standard deviation (n=12) of 40.4% (Peak Area) and 37.4% (Peak Area %). Several of the Table 4.2 compounds including 2-methyl-1-propanol (**2**), 2-methyl-1-butanol (**4**), 1-octene (**20**), 2-methyl-furan (**34**) and most of the sesquiterpenes have been commonly reported as MVOCs emitted from different fungal cultures.[205-207] It should be noted that 12 replications of each sample (same incubation time) were divided over two days of testing.

MEA media (the control) produced a significant amount of 1-butanol (**3**, 76.8%), while none was detected in the fungal cultures. The elimination of this compound can be used as an indicator for fungal growth when this media is used. In contrast, the production of ethanol, 2-methyl-1-propanol, and 2-methyl-1-butanol are associated with fungal growth. It has been reported that production of these alcohols during the exponential growth phase closely correlate with fungal growth.[140] Branched chain alcohols are associated with catabolism of the branched chain amino acids (leucine, isoleucine, and valine) and lipids.[208]

Table 4.2 Volatile organic compounds identified from isolates of *A. flavus* and control, expressed in peak area percentage

No.	Compound[a]	Ret. Time (min)	RI exp[b]	RI lit[c]	Control	Peak area % ± standard deviation (12 replicates)					
						Toxigenic isolates			Non-toxigenic isolates		
						3357	K73	5-38	K32	K35	21882
Alcohols											
1	Ethanol	4.652	451	448	-	0.22±0.11[d]	1.72±1.61	0.01±0	0.26±0.11	0.32±0.24	0.08±0.05
2	2-Methyl-1-propanol	9.093	613	607	-	2.36±1.53	6.98±1.88	0.08±0.04	3.44±1.15	0.95±0.6	0.26±0.14
3	1-Butanol	10.720	649	654	76.78±5.16	-	-	-	-	-	-
4	2-Methyl-1-butanol	13.978	720	729	-	0.68±0.53	2.21±0.96	0.02±0.01	0.77±0.32	-	0.06±0.04
5	1-Octen-3-ol	26.700	962	962	-	-	-	-	-	-	0.03±0.02
6	4-Propylresorcinol	28.153	986		-	2.16±1.37	-	0.24±0.08	0.47±0.17	0.41±0.31	0.18±0.03
Aldehydes											
7	Butanal	7.328	566	570	1.13±0.43	-	-	-	-	-	-
8	2-Methyl-propanal	7.518	541	544	0.96±0.67	-	-	-	-	-	-
9	3-Methyl-butanal	10.069	634	632	3.1±0.62	-	-	-	-	-	-
10	2-Methyl-butanal	10.567	645	639	0.96±0.32	-	-	-	-	-	-
11	Benzaldehyde	24.970	933	925	3.1±1.36	-	-	-	-	-	-
12	Undecanal	41.971	1293	1286	0.13±0.05	-	-	-	-	-	-
Alkane											
13	Pentane	5.442	500	500	-	0.2±0.17	0.38±0.12	0.02±0.02	0.47±0.12	0.22±0.08	0.03±0.01
14	Hexane	8.376	600	600	-	-	-	-	0.29±0.17	0.16±0.09	0.03±0.01
15	Heptane	12.946	698	700	-	-	-	-	0.68±0.32	-	-
16	1,2-Dimethyl-3-pentyl-4-Propylcyclohexane	43.311	1358		-	-	-	-	2.26±0.77	-	0.26±0.11
17	Heptadecane	48.409	1693	1700	1.12±0.18	-	-	-	2.64±0.91	-	-
18	2,6,10,14-Tetramethyl-Pentadecane	48.544	1704	1712	0.21±0.05	0.48±0.21	0.88±0.51	-	1.3±0.14	0.5±0.22	0.11±0.04
Alkene											
19	1,4-Pentadiene	5.548	461	464	-	0.43±0.26	7.12±2.68	0.25±0.16	1.08±0.27	2.39±1.07	0.35±0.1
20	1-Octene	17.038	785	785	-	0.47±0.34	0.29±0.15	0.06±0.02	0.78±0.37	0.17±0.08	-
21	2,4,4,6,6,8,8-Heptamethyl-1-nonene	43.174	1351	1325	0.19±0.11	3.12±2.5	3.85±1.99	-	4.65±1.03	3.91±1.31	0.76±0.21

Table 4.2 (Continued)

22	(Z)-3-Hexadecene	43.312	1358		0.18±0.13	2.31±1.66	2.91±1.09	0.06±0.05	-	2.56±0.95	-
23	2,4,4,6,6,8,8-Heptamethyl-2-nonene	43.783	1381	1343	0.43±0.26	5.12±3.39	7.39±3.32	0.17±0.08	6.51±2.36	6.27±2.45	0.88±0.22
24	2,2,6,6-Tetramethyl-4-methylene-heptane	44.375	1414		-	-	-	-	1.07±0.37	-	-
25	4,5,9,10-Dehydro-Isolongifolene	46.611	1554	1544	-	0.86±0.31	0.97±0.44	0.37±0.2	1.07±0.26	0.77±0.27	0.22±0.13
Alkyne											
26	2-Methyl-1-octen-3-yne	27.536	976	981	-	0.3±0.22	-	-	0.72±0.14	0.14±0.08	0.11±0.03
BTEX											
27	Benzene	10.906	653	649	-	0.29±0.11	0.45±0.18	0.01±0.01	0.41±0.1	0.43±0.2	0.05±0.02
28	Toluene	15.482	752	755	0.66±0.45	1.3±0.78	1.14±0.43	0.02±0.01	6.04±2.04	3.38±2.26	0.28±0.08
29	P-xylene	20.467	852	855	-	0.14±0.07	0.06±0.08	0±0	0.62±0.38	0.34±0.17	0.02±0.01
30	O-xylene	21.000	862	862	-	0.29±0.22	0.22±0.11	0.02±0.02	1.67±1.33	0.94±0.56	0.04±0.02
31	Styrene	21.907	879	870	1.12±0.74	1.23±0.8	1.99±1.17	0.05±0.02	8.13±2.71	3.46±1.28	0.8±0.33
Esters											
32	Ethyl chrysanthemumate	37.937	1160	1116	-	8.7±4.1	-	0.06±0.02	-	2.68±1.54	3.99±0.66
33	Anhydroserricornin	39.646	1204	1191	-	1.35±0.71	-	0.04±0.02	-	-	0.22±0.05
Furans											
34	2-Methyl-furan	8.587	602	594	-	7.23±3.66	8.9±1.79	0.13±0.05	4.38±1.39	3.56±0.99	0.35±0.1
35	Furfural	17.866	803	795	5.08±2.45	-	-	-	-	-	-
36	2-Propionylfuran	19.717	838	976	-	-	-	-	-	-	0.03±0.01
37	2-(5-methyl-furan-2-yl)-propionaldehyde	24.460	924	1055	-	1.87±0.47	0.3±0.21	0.05±0.02	-	0.49±0.28	1.54±0.31
Ketones											
38	acetone	5.007	486	488	2.15±0.55	-	6.4±5.94	0.08±0.05	11.86±3.39	0.78±0.31	0.23±0.13
39	2,3-butanedione	7.177	558	560	0.18±0.03	-	-	-	-	-	-
40	3-Methyl-2-pentanone	14.419	729	736	-	0.98±0.40	-	0.01±0	-	0.94±0.34	0.68±0.16
41	4-Methyl-3-hexanone	18.912	822	804	-	0.37±0.24	-	0.02±0.01	-	-	0.07±0.03
42	1-Cyclopropyl-1-propanone	26.058	951	-	-	-	-	-	-	-	0.1±0.06
43	5,5-Dimethyl-2,4-hexanedione	29.516	1008	1004	-	6.17±2.69	-	0.01±0.01	-	8.42±6.75	32.41±5.77
44	5-Hydroxy-2,2-dimethylhexan-3-one	31.119	1033	1030	-	-	-	-	-	-	3.32±0.74

Table 4.2 (Continued)

45	2,2-Dimethylheptane-3,5-dione	35.743	1105	1103	-	16.37±7.74	-	0.18±0.08	0.2±0.09	7.42±4.63	19.69±4.06
46	3,3,6-Trimethyl-2,5-heptanedione	37.805	1156	1159	-	10.9±5.11	0.58±0.46	0.29±0.13	-	4.52±2.1	3.99±0.65
Terpenes											
47	α-Pinene	24.994	933	941	-	0.08±0.02	0.13±0.03	-	0.36±0.18	0.25±0.18	0.03±0.01
48	D-Limonene	30.712	1026	1020	0.16±0.05	0.18±0.05	0.28±0.09	0.02±0.01	0.44±0.16	0.3±0.07	0.3±0.08
Organic acids											
49	2-Methyl-Propanoic acid,	14.614	733	732	0.46±0.22	-	-	-	-	-	-
50	2-Tetradecyl ester-methoxyacetic acid	49.245	1758	-	0.14±0.08	-	-	-	1.00±0.66	-	-
Pyrazine											
51	2,5-Dimethyl-pyrazine	22.527	891	892	0.54±0.2	-	-	-	-	-	-
52	3-Ethyl-2,5-dimethyl-pyrazine,	32.947	1060	1063	0.14±0.05	-	-	-	-	-	-
Sesquiterpenes											
53	δ-Elemene	43.206	1352	1394	-	-	-	0.95±0.15	-	-	-
54	α-Cubebene	43.488	1367	1366	-	-	0.89±0.5	0.65±0.04	-	0.61±0.37	0.71±0.29
55	Ylangene	44.012	1393	1392	-	-	-	-	-	-	0.42±0.18
56	(-)-Aristolene	44.141	1399	1403	0.15±0.06	-	2.47±1.19	7.51±0.7	-	1.63±0.37	-
57	β-Elemene	44.259	1407	1398	0.61±0.35	-	10.81±2.78	26.09±4.35	21.53±3.58	8.31±2.02	-
58	Isoledene	44.385	1414	1419	0.03±0.02	1.03±0.53	1.43±0.55	0.79±0.08	-	1.3±0.32	0.57±0.31
59	β-Gurjurene	44.778	1439	1430	-	-	-	2.46±0.34	-	1.4±0.21	-
60	β-Cubebene	44.874	1444	1435	-	0.86±0.51	1.65±0.21	0.9±0.14	-	-	1.14±0.53
61	Caryophyllene	44.953	1449	1430	-	0.33±0.12	0.42±0.06	1.92±0.19	1.01±0.18	0.35±0.06	0.32±0.17
62	Bicyclo(4.4.0)dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	45.229	1466	1464	-	1.01±0.27	1.44±0.38	-	-	1.3±0.29	0.83±0.42
63	Calarene	45.265	1468	1442	-	-	-	5.29±0.24	-	-	-
64	Valencene	45.513	1484	1486	-	-	-	2.61±0.25	-	-	0.54±0.63
65	β-Selinene	45.563	1487	1491	-	-	-	-	3.46±0.53	-	-
66	α-Selinene	45.586	1488	1498	-	1.11±0.38	2.74±0.88	5.25±0.73	-	1.97±0.43	2.27±0.7
67	α-Farnesene	45.703	1495	1492	-	-	5.41±0.86	-	-	2.82±1.18	3.83±1.62
68	α-Cadinene	45.877	1507	1505	-	-	-	8.55±1.41	-	14.78±5.73	8.38±2.41

Table 4.2 (Continued)

69	Cubenene	46.046	1519	1516	-	1.67±0.76	4.73±1.24	9.85±2.08	1.80±0.58	2.27±0.15	2.60±1.36
70	γ-Cadinene	46.124	1524	1517	-	-	-	7.52±1.99	3.00±0.93	-	-
71	γ-Muurolene	46.290	1536	-	-	12.2±10.42	4.37±1.35	12.93±2.39	-	1.01±0.29	2.18±1.34
72	δ-Cadinene	46.324	1538	1524	-	4.89±4.47	6.56±2.03	9.71±6.19	2.41±3.49	4.04±1.14	3.27±2.01
73	β-Germacrene	46.422	1545	-	-	-	-	3.09±0.32	-	-	-
74	β-Panasinsene	46.487	1550	-	-	-	1.05±0.3	0.55±0.11	1.68±0.29	-	0.48±0.14
75	β-Cadinene	46.618	1559	-	-	1.19±0.48	1.94±0.29	1.83±0.13	1.14±0.26	1.06±0.16	0.86±0.5
Sesquiterpenoids											
76	Cadala-1(10),3,8-triene	46.907	1579	-	-	-	-	0.14±0.03	-	-	0.09±0.04
77	4aH-cycloproazulen-4a-ol, decahydro-1,1,4,7-Tetramethyl-	47.239	1603	1571	-	-	-	0.22±0.08	-	-	-
78	Cubenol	47.720	1640	1651	-	-	-	0.34±0.06	-	-	-

[a] Compound identification is based on a comparison of RI value and mass spectra using the NIST database

[b] RI exp is the Kovats retention index determined experimentally using a DB-1 non-polar stationary phase

[c] RI lit is the Kovats retention index value obtained from the NIST Chemistry WebBook

[d] Quantitative data is the average peak area percentag

e and standard deviation of 12 replications of each isolate.

MEA medium also produces low levels of several aldehydes including butanal (**7**), 3-methylbutanal (**9**), 2-methyl-butanal (**10**), and benzaldehyde (**11**), which were not detected in any fungal culture. This result agreed with Roze et al.[208] who reported that 2-methylbutanal, 3-methylbutanal, and 2-methylpropanal served as precursors for the synthesis of the corresponding branched chain alcohols and therefore may have been consumed by the growing fungus. Acetone (**38**) is produced by most bacteria and fungi species. Furans, including 3-methyl-furan (**34**) are produced by many fungal species and have been suggested as potential markers for mold growth in cereals.[209]

A substantial number of volatile sesquiterpenes are emitted from fungal cultures. Sesquiterpenes are usually released by fungi during the transition from exponential growth to the stationary growth phase. They are cyclized by different sesquiterpene cyclases starting from farnesol-pyrophosphate. The sesquiterpenes we report here have also been reported for other species of the phylum Ascomycota. Different fungal species often have multiple sesquiterpenes in common. For example, caryophyllene (**61**) was also detected from *Phialophora fastigata* [213] and α-farnesene (**67**) was also found in *Aspergillus fumigatus.*[209] From *Aspergillus terreus,* the sesquiterpene γ-cadinene (**70**) has been reported.[214] The genus *Penicillium* and *Aspergillus* both belongs to the phylum Ascomycota and family trichocomaceae. *Penicillium* is also known to produce many sesquiterpenes such as β-elemene (**57**), α-selinene (**66**), β-panasinsene (**74**), and β-gurjurene (**59**).[215]

As already mentioned, the production of MVOCs depends on the species, the substrate, and environmental conditions.[216] For this reason, it is unlikely that consistent fungal detection can be based on the detection of any single compound. However,

detection may be possible in the patterns of several compounds. In order to control some of the variables we maintained standardized fungi growing conditions (30 ℃, dark, and MEA media) because fungal MVOCs production is known to be affected by environmental stresses such as temperature, UV-radiation,[217] infection,[218] and herbivore attack of the growth media.[219]

Roze *et al.*[208] determined that a block in aflatoxin biosynthesis or disruption of the global regulator *veA* (velvet gene), which coordinates the biosynthesis of secondary metabolites, also affects MVOC profiles. Several studies have been carried out to find unique biomarkers associated with mycotoxins production.[206, 220, 221] For example, Zeringue *et al.*[206] found that aflatoxigenic strains of *A. flavus* produced several sequiterpenes that were not detected in the emissions of non-aflatoxigenic strains of *A. flavus*. Results presented here also suggest that fungal isolate identification information lies in the production of sesqiterpenes; however, we found that these compounds were produced by both toxigenic and non-toxigenic fungi.

Volatile terpenes have also been linked to the formation of the trichothecene class of mycotoxins from *Fusarium sporotrichoides* growth on cereal grains.[220] MVOC profiles variations among isolates of *A. flavus* have been investigated in this study in order to identify volatile biomarkers associated with aflatoxin production. Result show consistent quantitative MVOCs variations of volatiles among the isolates; however, all biological species produce MVOCs. These non-fungus MVOC will increase the complexity of any MVOC analysis from samples collected in the field. The combination of MVOC quantification followed by chemometric multivariate analysis described here for laboratory samples could one day be a powerful protocol for the field identification of

aflatoxigenic fungal isolates; however, representative field MVOCs must be collected and analyzed in order to provide discrimination models.

4.4.2 Investigation of VOC patterns from *A. flavus* isolates and relationship between chemical classes

A chemical class comparison was used for isolate identification. MVOCs were divided into 14 chemical classes based on their functional groups. The advantage of chemical class comparison is that this approach could be utilized with electronic nose detection technology. Many electronic nose detection principals are based on the interaction of sensing materials to the specific chemical functional groups. MANOVA was performed first to test the null hypothesis that there is no significant difference between any of the six isolates and the control. Eighty-four samples from the control and isolates were divided into seven groups (control, NRRL 3357, K73, 5-3B, K32, K35 and NRRL 21882). Data from groups of aldehydes, organic acids, pyrazines, and sesquiterpenoids did not follow the normal distributions required for MANOVA and therefore were not considered further. Thus ten chemical classes and their relative quantities (peak area percentage) were chosen for the statistical analysis.

A comparison of MVOC classes using peak area% are shown in Figure 4.2. The null hypothesis is that there is no significant difference of MVOCs emission among the isolates and control. The null hypothesis was rejected ($p<0.001$) for each pairwise isolate/control comparison when using the ten functional group MVOCs. These results could be used to discriminate at the isolate level, however, our primary goal is discrimination of aflatoxigenic from nonaflatoxigenic grouping of isolates.

An LSD and Duncan test were performed to evaluate the effect of each isolate on the emission abundance of a specific chemical class. Sesquiterpenes were the major chemical class collected by SPME fibers from all the cultures studied, where content can reach from 23% (3357) to 97% (5-3B) of the TIC. The amount of sesquiterpenes collected from the 5-3B isolate is significantly higher than that of other isolates ($P<0.001$); in contrast, sesquiterpenes emissions from isolates of K73 and K35 are not significantly different ($P=0.334$). Isolates 3357 and NRRL 21882 also have similar sesquiterpene production ($P=0.148$). We observed that isolate 21882 released much higher percentage (60%) ketones than that of other isolates ($P<0.001$) due to the collection of a large amount of 5,5-dimethyl-2,4-hexanedione (32.4%). Relatively higher percentages of alkanes, BTEX, and terpenes were observed in isolate K32 compared to other isolates. A much larger amount of esters were extracted from isolate 3357 compared to other isolates and the control. Alcohols dominate the control (MEA agar) primarily because of high 1-butanol concentration.

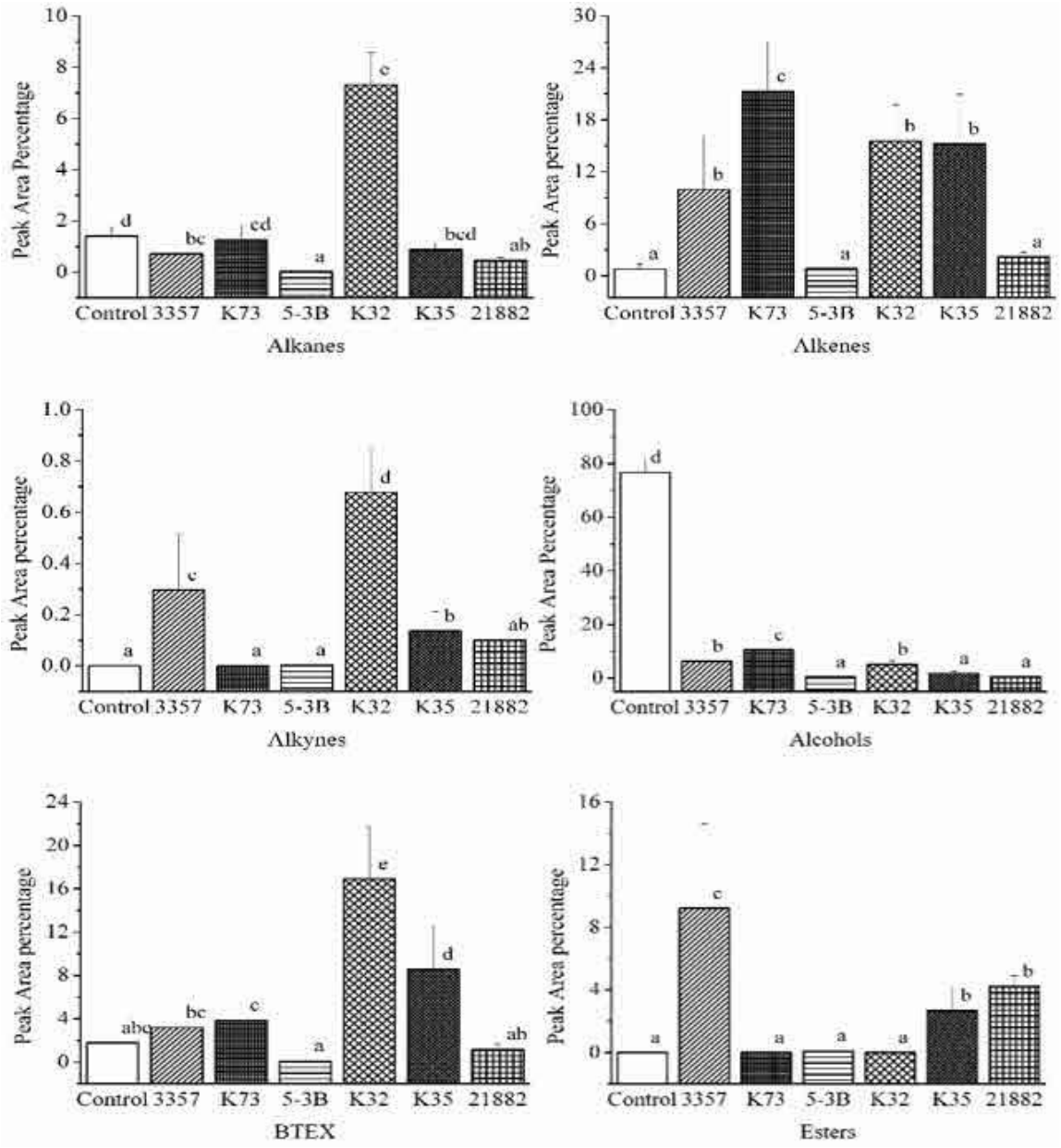


Figure 4.2 VOC patterns of A. flavus isolates.

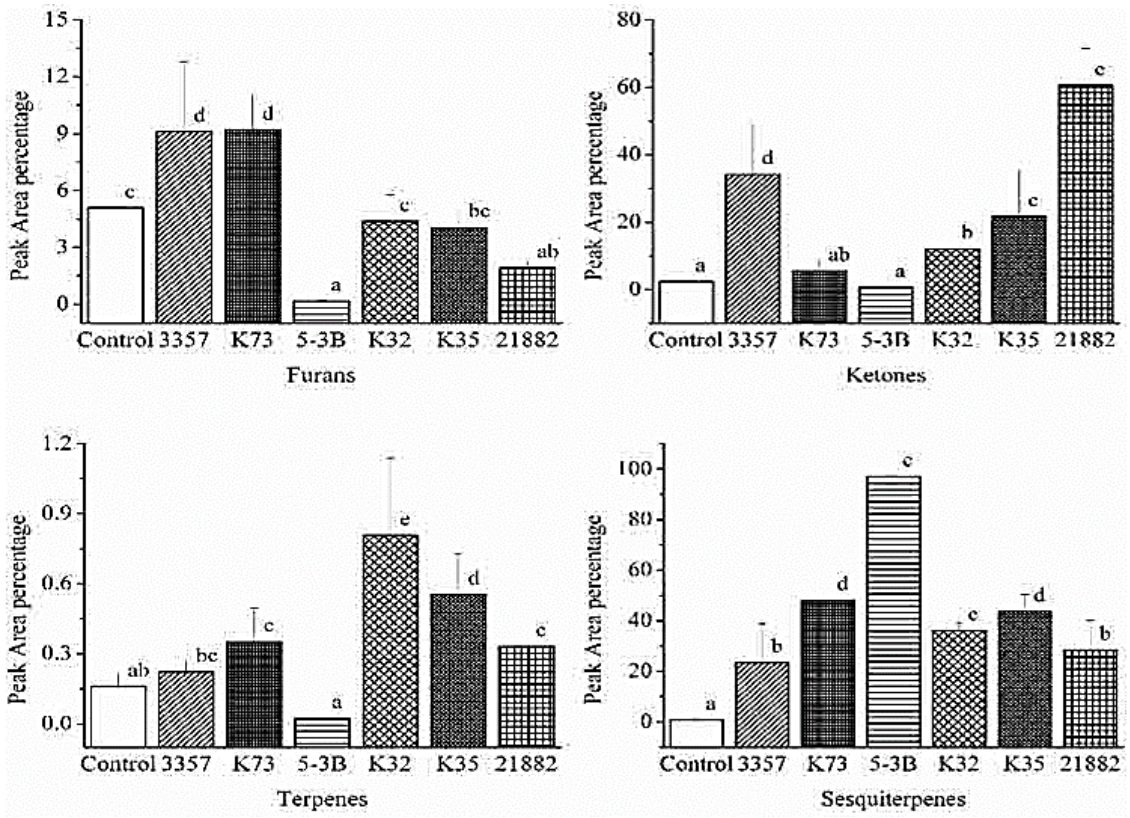


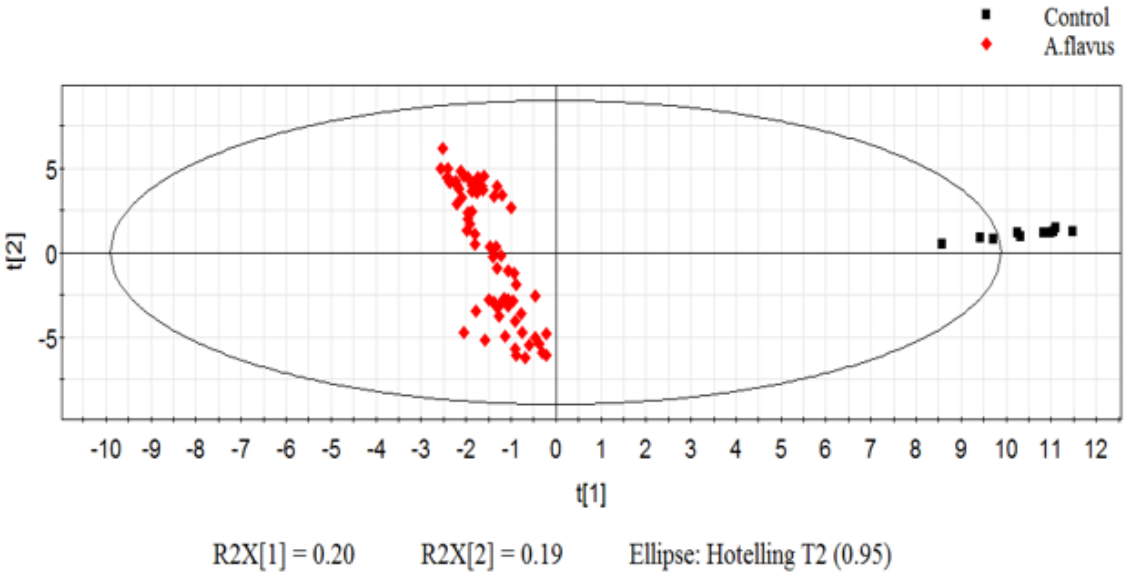
Figure 4.2 (Continued)

Mean (+standard deviation; n=12) peak area percentage of chemical classes from six A. flavus isolates and control measured over two collections. (5-h sampling time).Partial least squares discriminant analysis (PLS-DA) for aflatoxigenic and non-aflatoxigenic *A. flavus* and the identification of key biomarkers
The letters over each bar present the significant differences at P<0.05 among the isolates and control emissions (LSD and Duncan test). Different letters (a, b, c, d) show the significant differences

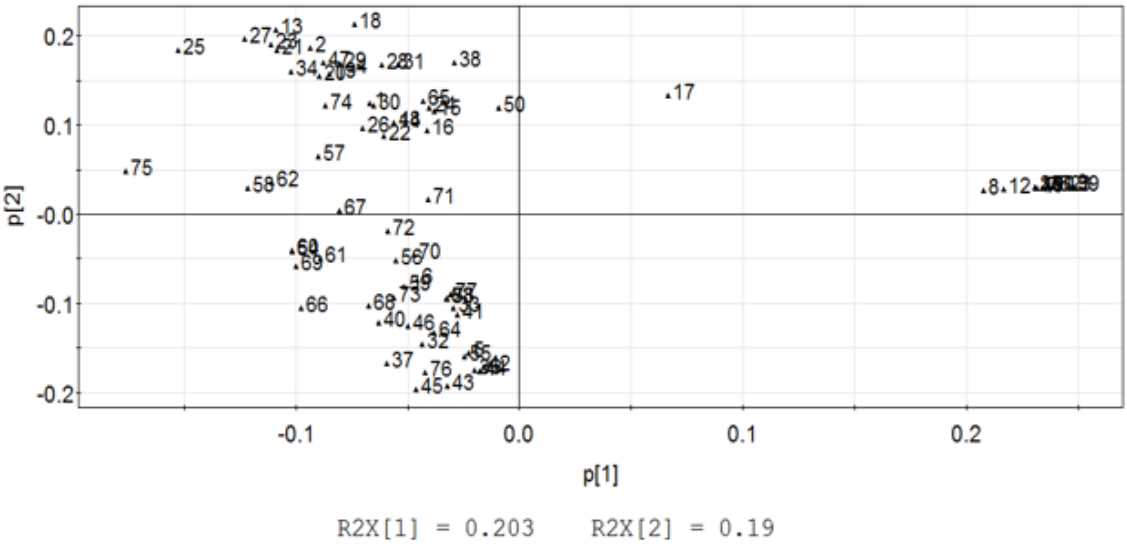
The goal of this discrimination study was to answer these questions: 1) Is it possible to discriminate aflatoxigenic and non-aflatoxigenic samples according to their volatile metabolites (MVOC) profiles? And 2) is it possible to identify biomarkers uniquely associated with these two fungal types? To answer these questions, PLS-DA was performed using the MVOC profiles of the six *A. flavus* isolates. With this method, the data are modeled in the way similar to PCA, but in combination with a discriminant

analysis. PLS-DA can be considered an extension of PCA and LDA using latent variables with the associated noise reduction of the PLS model. PLS can be utilized as a regression technique for modeling the association between X and Y in order to study complicated and approximate relationships. In this treatment, 82 samples (6 isolates × 12 replicates + the 10 controls) and the peak area of the 78 identified volatile metabolites formed an 82×78 matrix. This matrix was set as predictor variable X, and the class of six *A. flavus* isolates and control was treated as variable Y. The data were pretreated using log transformation and mean centering methods.

The first step is to discriminate *A. flavus* cultures from the control (MEA medium alone). The discrimination was successfully achieved with 2 principal component (R^2X = 0.394, R^2Y = 0.987 and Q^2 = 0.985). A quantitative measure of the goodness of fit is given by the parameter R^2, which explains variation. The predictive ability, on the other hand, is given by the goodness of prediction parameter Q^2. Generally, a $Q^2 > 0.5$ is regarded as good and a $Q^2 > 0.9$ as excellent. Figure 4.3A is the score plots of PLS-DA, where fungal isolates and control are easily separated.



(A)



(B)

Figure 4.4 PLS-DA score plot (A) and loading plot (B) comparing the log transformed peak area data of the identified MVOCs from the control and isolates of *A. flavus*.

The number in the loading plot represents the MVOC number listed in Table 4.1

Specific compounds, only produced by the control or by *A. flavus,* can be found using the loading plot (Figure 4.3B). The PLS-DA loading plot complements the score plot and can be used to identify possible volatile markers. Each data point represents one volatile metabolite and shows one relationship among each isolate. Using the loading plot as a guide, the potential biomarkers can be assigned a Variables Importance in the Projection (VIP) value. High ranking VIPs (Figure 4.4) are those MVOCs that are the farthest from the center of the loading plot. The VIP values summarize the importance of variables both to explain X (MVOCs) and to correlate to Y (isolates and control). MVOCs with VIP values greater than 1 are considered to be important X variables, while VIP values less than 0.5 are unimportant variables. The interval between 1 and 0.5 is of moderate importance. For example, 1-butanol (**3**), butanal (**7**), 2-methylpropanal (**8**), 3-methylbutanal (**9**), 2-methylbutanal (**10**), benzaldehyde (**11**), undecanal (**12**) and furfural (**35**) were only detected from control samples. They were clearly located in the control region of the score plot and are the MVOCs that are farthest from the center of the loading plot.

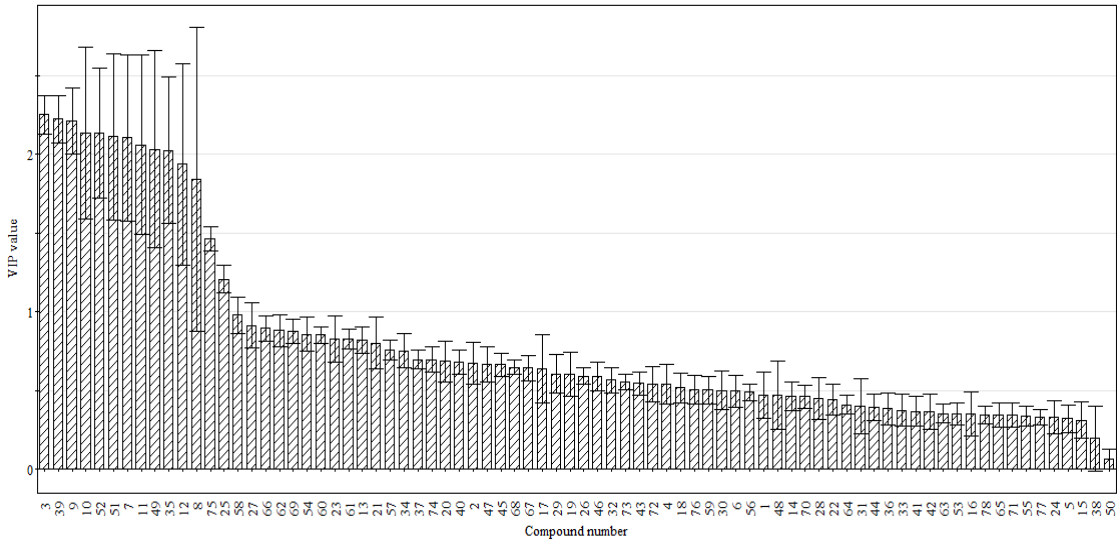
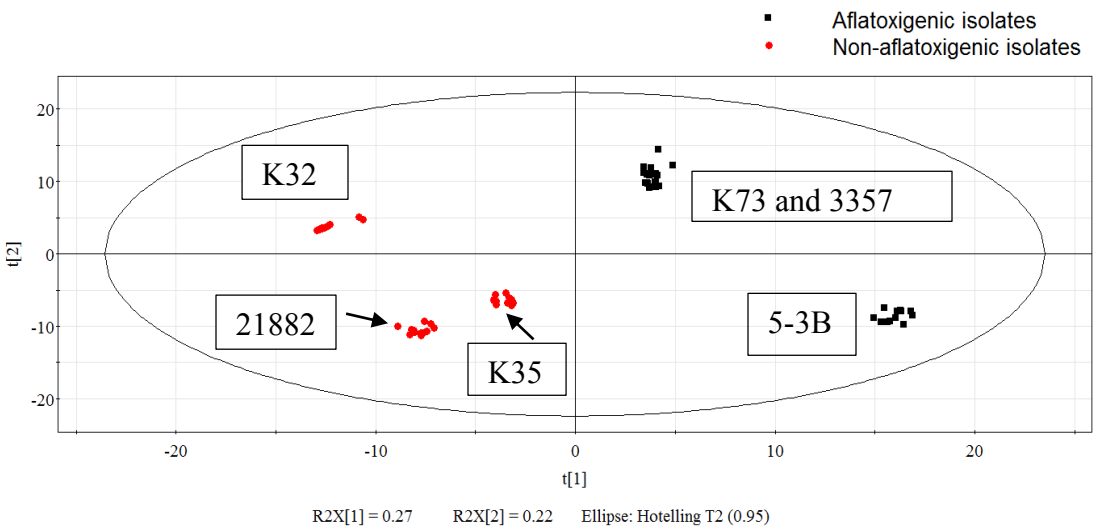


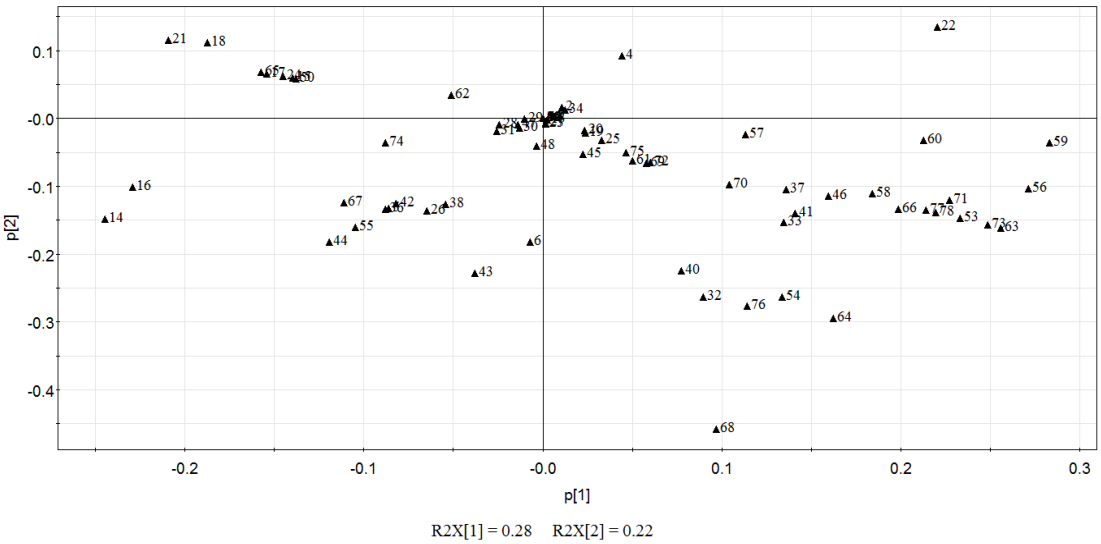
Figure 4.5 Variables importance in the projection (VIPs) for discriminating *A. flavus* from the control (media only).

The compound number used in the plot represents the volatile metabolites number listed in Table 4.1. The error bars are the standard deviations of VIP values (12 replicates).

The next step is to discriminate the aflatoxigenic from the non-aflatoxigenic isolates of *A. flavus* using the method (PLS-DA). The separation was successfully achieved with 3 principal components ($R^2X = 0.70$, $R^2Y = 0.99$ and $Q^2 = 0.99$). The distribution of 72 samples using the first and second components of this statistical analysis is presented in Figure 4.5A. The six isolates are grouped into 5 clearly defined clusters – 2 clusters the aflatoxigenic and 3 clusters for non-aflatoxigenic: the clusters can be grouped by aflatoxigenic isolates (black cubes) and non-aflatoxigenic isolates (red circles). The toxigenic isolate 5-3B is located on the positive region of t[1] and the negative region of t[2] because of a high sesquiterpene content. Isolate 5-3B is still easily differentiated from the samples of non-toxigenic isolates.



(A)



(B)

Figure 4.6 PLS-DA score plot (A) and loading plot (B) using the MVOC profiles log transformed data for aflatoxigenic (black cubic) and non-aflatoxigenic (red circle) isolates of *A. flavus*.

The number in loading plot represents the volatile metabolites number listed in Table 4.1.

The loading plot (Figure 4.5B) of the PLS-DA model enables visualization of the specific MVOCs that contribute the most to the discrimination (farthest from the center)

of the toxigenic and non-toxigenic isolates. Volatile metabolites presented in one category with a VIP value above 1 were selected for the student t test to examine the significant of difference between toxigenic and non-toxigenic isolate samples (Table 4.3).

Table 4.3 Possible volatile biomarkers for discrimination of toxigenic and non-toxigenic *A. flavus* isolates

No.[a]	Compound name	VIP[b]	Peak area ($\times 10^6$)[c]		P value[d]
			Toxic	Nontoxic	
14	hexane	2.249	0	3.43	<0.001
60	β-cubebene	2.011	2.46	5.21	<0.001
68	α-cadinene	1.995	225	44.9	<0.001
16	1,2-dimethyl-3-pentyl-4-propylcyclohexane	1.758	0	2.36	<0.001
22	(Z)-3-hexadecene	1.736	3.97	0.942	0.298
56	(-)-aristolene	1.489	210	0.841	<0.001
63	calarene	1.419	147	0	<0.001
73	β-germacrene	1.379	83.9	0	<0.001
71	γ-muurolene	1.349	363	10.6	<0.001
59	β-gurjurene	1.340	358	18.0	<0.001

[a] Compound numbers listed are same as numbers listed in Table 4.1
[b] VIP indicates the importance of variable both to explain X and correlate Y using SIMCA P+ software
[c] The relative quantities of biomarkers (peak area mean) are listed
[d] P value is obtained by performing student's T test.

Among the potential biomarkers listed in Table 4.3, the sesquiterepenes class of chemicals (compound **60**, **68**, **56**, **63**, **73**, **71**, and **59**) were the most highly represented. Thus as a group, sesquiterpenes may provide the chemical "fingerprints" required when

discriminating aflatoxigenic and non-aflatoxigenic isolates when grown on different substrates. Among these sesquiterpenes biomarkers observed in this study, β-Cubebene (**60**) has been reported to be released by edible mushroom *Piptoporus betulinus*.[222] α-Cadinene (**68**)was also emitted by *Resinicium bicolor,* which is a plant pathogen infecting trees named "Oregon pine".[223] γ-Muurolene (71) has been identified as a component of essential oil from *Melaleuca* species of Australian shrubs and trees [224] and damiana plants.[225] It has also been reported as an important MVOC produced by the fungus *Aspergillus ustus*[193] and the bacteria *E. coli.*[226]

In summary, the discrimination of toxigenic and non-toxigenic *A. flavus* based on volatile metabolites (MVOC profiles) has been successfully achieved using the PLS-DA model where $R^2X = 0.70$, $R^2Y = 0.99$, and $Q^2 = 0.99$. This is an indication that rapid identification of aflatoxigenic fungi is possible using SPME fiber collection of MVOCs emitted by fungi grown in a controlled environment. However, it should be emphasized that MVOC profiles will change with varied media and growth conditions, including temperature, humidity, and UV radiation. For optimal field results the methods describe here must be repeated under field conditions. With this strategy, fungal discrimination can be accomplished because PLS-DA provides a key projection of latent variables that focus on class separation (discrimination).

4.5 Conclusion

In conclusion, we determined that it is possible to discriminate aflatoxigenic from non-aflatoxigenic *A. flavus* using variations in volatile metabolite profiles under controlled growth conditions. This is the first attempt to differentiate *A. flavus* at the isolate level. The variation of volatile composition in chemical classes among the *A.*

flavus isolates is also considered significant when performing MANOVA statistical analysis.

Multivariate chemometric analysis was successfully used for the analysis of the MVOC profiles. Supervised PLS-DA was applied to discriminate aflatoxigenic and non-aflatoxigenic isolates. Using the loading plot and variable important analysis (VIP), potential biomarkers were identified for the non- and aflatoxin-producing isolates. We believe that the methods described here will be very helpful for further investigation of biomarkers related to aflatoxin biosynthesis and *A. flavus* isolate identification. The specific biomarkers presented here will not be relevant under all growth conditions. However similar methods can be applied to identify key markers under likely field conditions.

The combination of multivariate chemometric analysis and head space SPME GC-MS analysis is a powerful tool for fungal volatile metabolomics research. Our results show that MVOC profiling by GC-MS could be complementary to traditional molecular techniques used in fungal contamination identification.

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APPENDIX A

SUPPLEMENTARY MATERIALS FOR CHAPTER II

Table A.1 Volatile metabolites profiles of *A. flavus* K73 obtained using three types of SPME fibers CAR/PDMS, DVB/PDMS, and DVB/CAR/ PDMS with 6 replications each.

No.	Volatile organic compounds	Retention time (min)		Molecular formula	Peak area RSD (%)			Peak Area percentage RSD (%)		
		Min	Max		CAR	DVB CAR	DVB	CAR	DVB CAR	DVB
1	Ethanol	4.58	4.636	C_2H_6O	62.87	32.91		62.17	35.44	
2	Acetone	4.963	5.028	C_3H_6O	56.61	115.58	108.58	51.79	88.85	109.40
3	Isopropyl Alcohol	5.131	5.189	C_3H_8O	59.05	77.96		59.27	92.13	
4	1,4-Pentadiene	5.508	5.574	C_5H_8	48.84	61.92		40.43	39.97	
5	Acetic acid, methyl ester	5.737	5.811	$C_3H_6O_2$	48.73	88.44		35.22	84.65	
6	2,3-Butanedione	7.139	7.21	$C_4H_6O_2$	28.94	74.26		65.10	99.87	
7	Butanal	7.287	7.408	C_4H_8O	119.73			115.45		
8	Propanal, 2-methyl-	7.503	7.627	C_4H_8O	109.65			108.74		
9	Propanoic acid, 2-methyl-, anhydride	8.272	8.344	$C_8H_{14}O_3$	68.03	31.14		64.10	65.44	
10	Furan, 2-methyl-	8.537	8.604	C_5H_6O	38.42	20.27		30.07	22.40	
11	1-Propanol, 2-methyl-	9.018	9.077	$C_4H_{10}O$	30.36	32.65		29.73	30.85	
12	Furan, tetrahydro-	9.679	9.735	C_4H_8O	82.35	14.07	14.07	121.16	80.52	82.11
13	Benzene	10.871	11.084	C_6H_6	90.91	10.40		97.05	14.63	
14	Thiazole	13.526	13.704	C_3H_3NS	64.35			71.01		
15	1-Butanol, 3-methyl-	13.803	13.85	$C_5H_{12}O$	52.59	51.27		57.73	74.59	
16	1-Butanol, 2-methyl-	13.926	13.961	$C_5H_{12}O$	37.84	34.34		41.79	32.29	
17	Methyl Isobutyl Ketone	14.43	14.542	$C_6H_{12}O$	57.96			75.35		
18	Toluene	15.481	15.521	C_7H_8	13.78	15.85		25.07	26.19	
19	1-Octene	17.046	17.076	C_8H_{16}	57.50	39.76		69.69	52.06	
20	p-Xylene	20.527	20.548	C_8H_{10}		16.95				
21	Ethylbenzene	21.002	21.058	C_8H_{10}		33.35				
22	Styrene	21.871	21.948	C_8H_8	23.81	17.31	19.27	35.66	24.46	19.73
23	Heptane, 2,2,6,6-tetramethyl-4-methylene-	29.162	29.176	$C_{12}H_{24}$	136.37	32.65	51.68	100.83	25.69	31.55
24	3-Heptene, 2,2,4,6,6-pentamethyl-	29.727	29.741	$C_{12}H_{24}$	121.50	35.33	49.42	85.92	24.41	30.42
25	D-Limonene	30.717	30.74	$C_{10}H_{16}$	63.41	39.42		37.54	35.77	
26	2-Pentene, 2,4,4-trimethyl-	31.432	31.443	C_8H_{16}	110.45	34.63	41.21	75.10	24.28	25.42

Table A.1 (Continued)

27	Cyclohexane, 1-methyl-3-pentyl-	32.229	32.242	$C_{12}H_{24}$	121.10	29.91	60.55	86.46	17.57	32.38
28	Nonane, 2,2,4,4,6,8,8-heptamethyl-	33.083	33.097	$C_{16}H_{34}$	103.07	32.21	42.73	69.22	18.83	26.15
29	Nonanal	34.716	34.785	$C_9H_{18}O$		72.07	193.84			
30	Heptane, 1,1'-oxybis-	35.115	35.127	$C_{14}H_{30}O$	103.09	18.64	53.46	73.40	21.38	30.85
31	1-Pentanol, 2,2,4-trimethyl-	35.450	35.460	$C_8H_{18}O$	120.06	17.62	38.79	89.50	26.62	58.17
32	Undecane	35.556	35.558	$C_{11}H_{24}$			130.13			
33	Maltol propionate	35.794	35.943	$C_9H_{10}O_4$	72.72			78.39		
34	4-Undecene, 7-methyl-	36.269	36.278	$C_{12}H_{24}$	125.44		57.48	98.37		32.16
35	3-Undecene, 6-methyl-, (E)-	36.542	36.549	$C_{12}H_{24}$	93.94			88.16		
36	Undecane, 2,6-dimethyl-	38.679	38.687	$C_{13}H_{28}$	73.49	37.15	43.48	48.04	37.04	20.03
37	Undecane, 3,6-dimethyl-	38.907	38.915	$C_{13}H_{28}$	75.99	50.08	30.51	46.91	70.12	52.50
38	unknown1	39.000	39.175		72.75	25.74		82.34	59.95	
39	Dodecane	39.550	39.555	$C_{12}H_{26}$	74.52	15.24	63.33	56.60	57.01	45.86
40	2,4,4,6,6,8,8-Heptamethyl-1-nonene	41.645	41.650	$C_{16}H_{32}$	58.31	26.76		45.24	53.46	
41	Cyclohexane, 1,2-dimethyl-3-pentyl-4-propyl-	41.853	41.856	$C_{16}H_{32}$	46.60			111.00		
42	unknown2	41.921	41.925		37.92	164.69	150.64	36.64	232.23	110.35
43	Tridecane	42.147	42.155	$C_{13}H_{28}$	42.54	37.59	43.87	32.93	47.78	24.79
44	Decane, 3-cyclohexyl-	42.388	42.393	$C_{16}H_{32}$	41.55	34.96	35.56	21.45	40.79	11.83
45	Tetradecane, 2,5-dimethyl-	42.602	42.610	$C_{16}H_{34}$	40.77	39.18	90.34	34.98	42.10	49.47
46	1,7-Dimethyl-4-(1-methylethyl)cyclodecane	42.672	42.678	$C_{15}H_{30}$	47.37	31.55		65.36	61.61	
47	2,2,4,4,5,5,7,7-Octamethyloctane	43.173	43.178	$C_{16}H_{34}$	25.43	24.72	49.42	7.82	32.97	17.71
48	1-Hexadecene	43.314	43.375	$C_{16}H_{32}$	32.01		15.94	22.93	244.95	55.51
49	Unknown3	43.376	43.38		25.77	40.86	82.11	50.60	68.29	45.61
50	α-Cubebene	43.489	43.494	$C_{15}H_{24}$	41.33	22.17	24.16	26.46	26.33	112.09
51	unknown4	43.616	43.619		24.61	21.58	26.34	14.78	34.00	13.51
52	2,4,4,6,6,8,8-Heptamethyl-2-nonene	43.782	43.787	$C_{16}H_{32}$	26.04	27.02	31.52	15.35	40.04	11.73
53	unknown5	43.899	43.902		41.64	72.62	22.41	36.56	90.19	29.06
54	Ylangene	44.011	44.035	$C_{15}H_{24}$	89.82		122.34	104.47		85.23
55	(-)-Aristolene	44.141	44.15	$C_{15}H_{24}$	52.95	22.09	27.94	54.42	29.92	30.30
56	β-Elemene	44.254	44.259	$C_{15}H_{24}$	26.84	24.67	30.21	27.09	23.81	7.82
57	Isoledene	44.378	44.383	$C_{15}H_{24}$	36.10	28.78	29.90	23.00	33.27	27.58

Table A.1 (Continued)

58	Hept-2-ene, 2,4,4,6-tetramethyl-	44.469	44.475	$C_{11}H_{22}$	80.34	28.77	32.49	163.90	32.18	32.01
59	1-Hexadecene	44.546	44.55	$C_{16}H_{32}$	30.15	22.13	88.90	22.67	54.91	49.53
60	1-Pentadecene, 2-methyl-	44.675	44.682	$C_{16}H_{32}$	28.11	24.35	32.27	21.11	31.40	18.29
61	(-)-Germacrene D	44.872	44.879	$C_{15}H_{24}$	47.83	33.71	42.90	49.21	13.92	21.46
62	β-Cubebene	45.062	45.067	$C_{15}H_{24}$	28.81	42.94	41.55	26.49	18.95	23.03
63	(+)-Epi-bicyclosesquiphellandrene	45.257	45.265	$C_{15}H_{24}$	22.38	29.20	63.55	16.96	31.70	29.31
64	α-Selinene	45.589	45.594	$C_{15}H_{24}$	34.70	46.50	44.50	39.62	26.71	42.65
65	α-Farnesene	45.701	45.702	$C_{15}H_{24}$	67.10			118.28		
66	β-Cadinene	45.75	45.759	$C_{15}H_{24}$	10.89		22.63	56.34		34.41
67	Copaene	45.876	45.88	$C_{15}H_{24}$			30.91			
68	Cedrene	46.046	46.068	$C_{15}H_{24}$	29.51	37.65	28.37	14.59	20.40	28.98
69	γ-Cadinene	46.187	46.196	$C_{15}H_{24}$	26.86		36.63	16.04		36.41
70	Calamenene	46.334	46.341	$C_{15}H_{24}$	27.30	52.40	48.77	23.69	45.13	29.04
71	β-Panasinsene	46.485	46.489	$C_{15}H_{24}$	35.66	59.31	30.26	22.39	67.48	60.30
72	π-Calacorene	46.621	46.63	$C_{15}H_{24}$	17.02	32.57	36.48	22.81	21.70	23.13
73	Isovaleric acid, nonyl ester	46.749	46.787	$C_{14}H_{28}O_2$	35.54	84.97	44.44	66.25	61.06	64.84
74	Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	47.091	47.095	$C_{16}H_{30}O_4$		35.35	35.25			
75	n-Nonadecanol-1	47.155	47.16	$C_{19}H_{40}O$	67.04	29.26	36.32	73.09	83.04	43.66
76	4,4,5,6-Tetramethyltetrahydro-1,3-oxazin-2-thione	47.48	47.508	$C_8H_{15}NOS$	68.05	44.50	63.53	76.67	51.75	68.02
77	Sulfurous acid, cyclohexylmethyl hexyl ester	47.611	47.624	$C_{13}H_{26}O_3S$	23.79	104.74	26.64	26.37	115.74	28.74
78	unknown6	47.823	47.83		60.83	16.50	57.40	71.70	15.02	28.48
79	1-Decanol, 2-hexyl-	48.012	48.063	$C_{16}H_{34}O$	42.61	21.96	34.29	59.42	30.49	18.84
80	Sulfurous acid, cyclohexylmethyl dodecyl ester	48.665	48.668	$C_{19}H_{38}O_3S$	29.48	16.73	21.05	44.32	32.84	21.12
81	6-Tridecene, 2,2,4,10,12,12-hexamethyl-7-(3,5,5-trimethylhexyl)-	49.114	49.116	$C_{28}H_{56}$	48.81	24.72	24.70	58.62	57.05	29.69
82	Unkonwn7	49.342	49.352		56.54	30.35	10.82	68.41	83.59	36.96
83	Nonadecane-2,4-dione	49.601	49.606	$C_{19}H_{36}O_2$	91.05	136.20	59.76	105.77	105.22	60.56
84	Octadecanal	49.667	49.683		60.15			32.28		
	Average RSD (%)				**56.7**	**40.9**	**49.8**	**56.7**	**52.1**	**39.6**

Table A.2 Volatile metabolites profiles and peak area raw data obtained using three types of SPME fibers CAR/PDMS, DVB/PDMS, and DVB/CAR/ PDMS with 6 replications each.

No. *	Peak Area ($\times 10^6$)																	
	CAR/PDMS						CAR/DVB/PDMS						DVB/PDMS					
	REP 1	REP 2	REP 3	REP 4	REP 5	REP 6	REP 1	REP 2	REP 3	REP 4	REP 5	REP 6	REP 1	REP 2	REP 3	REP 4	REP 5	REP 6
1	2.96	2.75	1.94	4.77	9.44	8.51	3.81	2.02	1.99	3.49	2.01	2.09	-	-	-	-	-	-
2	35.85	44.71	18.18	12.77	9.14	38.99	27.68	1.98	5.89	8.15	3.94	2.75	4.11	1.31	0.24	0.22	4.34	0.47
3	9.97	15.91	4.72	3.99	4.24	8.21	4.46	-	1.12	3.06	1.23	0.56	-	-	-	-	-	-
4	11.21	16.78	26.47	16.48	16.14	39.54	10.59	3.34	14.11	4.49	6.06	3.67	-	-	-	-	-	-
5	4.52	6.14	3.20	2.49	2.09	7.32	1.60	-	0.64	0.46	0.28	0.21	-	-	-	-	-	-
6	0.88	1.22	-	1.80	1.22	0.99	0.12	-	0.20	0.24	0.63	0.18	-	-	-	-	-	-
7	0.29	4.55	0.80	1.26	0.98	0.23	-	-	-	-	-	-	-	-	-	-	-	-
8	1.47	6.43	1.23	1.14	0.87	0.82	-	-	-	-	-	-	-	-	-	-	-	-
9	0.68	2.91	1.04	0.68	0.80	2.18	0.13	-	0.18	0.12	0.11	0.22	-	-	-	-	-	-
10	9.51	13.51	16.22	14.88	20.73	28.48	8.04	5.22	10.22	8.53	9.01	8.08	-	-	-	-	-	-
11	45.66	39.44	19.13	44.10	55.64	52.65	11.58	4.04	6.52	9.11	7.22	8.33	-	-	-	-	-	-
12	-	0.78	4.65	0.68	2.30	1.48	-	0.15	0.21	0.21	0.20	-	-	0.15	0.21	0.21	0.20	-
13	0.42	0.65	4.29	4.64	2.61	0.38	0.59	0.46	0.55	0.48	0.49	0.47	-	-	-	-	-	-
14	0.96	2.10	3.00	4.22	5.70	1.18	-	-	-	-	-	-	-	-	-	-	-	-
15	4.21	1.68	3.05	4.89	8.94	6.00	1.31	-	1.04	1.18	0.42	0.36	-	-	-	-	-	-
16	9.21	4.16	6.36	10.14	13.94	10.81	3.91	1.30	4.22	3.87	2.82	2.81	-	-	-	-	-	-
17	0.75	0.18	1.20	0.73	0.47	0.39	-	-	-	-	-	-	-	-	-	-	-	-
18	1.89	2.65	2.47	2.82	2.20	2.55	0.95	1.07	1.47	1.25	1.27	1.38	-	-	-	-	-	-
19	0.51	0.51	2.15	2.35	1.66	1.15	0.12	0.13	0.17	0.27	0.19	0.32	-	-	-	-	-	-
20	-	-	-	-	-	-	-	0.10	0.11	0.14	0.14	-	-	-	-	-	-	-
21	-	-	-	-	-	-	0.11	0.17	0.28	0.29	0.29	0.21	-	-	-	-	-	-

Table A.2 (Continued)

22	3.84	5.19	4.63	5.35	3.63	2.74	2.49	2.22	2.97	2.75	3.08	1.93	1.54	1.30	1.23	1.67	1.98	1.29
23	2.10	1.28	1.21	2.07	0.65	12.28	0.29	0.24	0.58	0.33	0.35	0.37	1.63	3.13	1.68	2.45	4.23	0.88
24	4.48	2.88	2.47	4.76	1.49	21.72	0.61	0.42	1.11	0.54	0.68	0.67	3.51	6.98	4.05	5.44	9.06	1.99
25	0.33	0.15	0.22	0.43	0.31	0.82	0.23	0.12	0.35	0.28	0.17	0.16	-	-	-	-	-	-
26	5.91	3.88	3.26	5.37	1.79	23.34	0.94	0.61	1.51	0.68	1.15	0.88	4.49	8.26	5.20	7.22	9.77	2.79
27	2.27	1.18	0.88	2.23	0.80	9.81	0.37	0.22	0.48	0.23	0.37	0.31	1.45	2.87	1.81	2.64	5.13	0.91
28	8.91	5.20	4.44	7.76	2.26	29.40	1.19	0.71	1.67	0.81	1.22	0.94	7.37	11.53	8.09	10.42	14.94	3.35
29	-	-	-	-	-	-	0.49	0.00	0.20	1.10	0.20	0.99	9.45	0.99	1.19	0.27	59.19	1.19
30	3.48	1.10	1.18	2.91	0.65	9.13	0.33	0.28	0.46	0.32	0.36	0.42	2.82	3.91	3.68	3.64	6.96	0.94
31	1.67	0.36	0.36	1.24	0.39	5.05	0.22	0.22	0.26	0.24	0.25	0.15	1.01	1.23	1.06	1.43	-	0.38
32	-	-	-	-	-	-	-	-	-	-	-	-	1.10	0.55	0.99	0.43	7.86	2.19
33	0.90	1.20	0.11	2.04	0.36	0.99	-	-	-	-	-	-	-	-	-	-	-	-
34	1.30	0.16	0.19	0.90	0.18	3.54	-	-	-	-	-	-	0.50	0.54	0.46	0.82	1.24	0.20
35	1.16	2.63	0.12	0.75	0.13	2.78	-	-	-	-	-	-	-	-	-	-	-	-
36	4.20	1.61	1.59	3.20	1.32	7.53	0.37	0.41	0.66	0.26	0.72	0.43	2.73	3.24	3.36	3.85	5.80	1.31
37	1.60	0.69	0.55	1.22	0.76	3.30	0.26	0.13	0.42	-	0.41	0.15	1.04	1.43	1.27	1.51	-	0.62
38	0.50	1.94	4.13	3.25	7.72	2.74	-	0.15	0.14	0.12	0.13	0.22	-	-	-	-	-	-
39	1.56	0.27	0.50	1.39	0.51	2.41	0.11	-	0.14	0.12	0.15	0.11	2.20	1.96	3.30	1.91	4.96	0.35
40	2.25	0.67	0.54	1.16	1.09	2.38	-	0.38	0.55	0.31	0.60	0.42	-	-	-	-	-	-
41	-	-	-	0.71	0.72	1.51	-	-	-	-	-	-	-	-	-	-	-	-
42	2.16	0.81	0.78	1.60	1.36	1.53	19.71	-	0.37	-	-	0.30	2.68	1.21	1.46	1.06	15.38	0.96
43	3.01	1.04	1.23	2.29	1.69	3.06	0.76	0.73	0.39	0.75	0.64	0.24	2.38	2.61	3.87	3.24	5.08	1.17
44	6.36	3.36	3.53	5.60	4.07	9.19	0.97	1.93	2.13	0.89	2.15	1.60	4.71	5.36	7.07	6.91	9.89	3.57
45	1.35	0.86	0.37	1.00	1.39	1.60	0.27	0.63	0.78	0.30	0.72	0.59	0.78	1.13	1.32	1.19	4.76	0.92
46	1.53	-	1.03	0.37	0.90	1.67	-	0.31	0.28	0.15	0.38	0.36	-	-	-	-	-	-

Table A.2 (Continued)

47	5.13	4.56	3.54	4.94	3.74	6.94	1.38	2.34	2.50	1.40	2.12	1.79	5.75	5.74	7.67	7.01	14.20	3.68
48	4.03	5.18	2.26	3.73	2.68	5.22	1.97	-	-	-	-	-	3.37	3.39	4.71	4.53	-	4.40
49	3.97	-	2.38	3.22	2.78	4.51	-	3.79	3.63	2.41	1.68	1.51	3.54	3.73	5.03	4.20	17.25	5.11
50	1.38	1.25	0.68	1.78	0.90	2.21	0.63	0.75	1.08	0.82	0.87	0.59	0.35	-	0.31	0.49	-	-
51	21.11	15.55	16.25	19.64	13.70	26.40	7.67	11.42	10.13	6.03	9.32	8.34	19.63	23.64	28.62	26.17	32.08	14.46
52	10.29	7.07	7.86	9.70	6.93	13.14	3.66	6.02	4.91	2.62	4.38	4.01	9.85	11.52	14.31	13.07	18.34	7.00
53	1.68	0.92	0.59	1.35	1.96	2.12	0.28	1.62	0.65	0.31	0.60	0.58	0.77	1.15	1.51	1.50	1.23	1.17
54	1.86	-	0.77	0.61	-	4.25	-	-	-	-	-	-	3.55	0.95	2.57	1.22	12.36	0.99
55	3.99	7.83	2.03	3.58	3.47	2.48	1.53	1.64	1.61	0.82	1.60	1.68	3.92	3.60	3.56	3.40	3.62	1.43
56	18.54	15.90	11.62	20.22	9.40	16.82	9.28	7.90	10.46	4.66	7.83	9.59	13.78	17.13	20.01	19.54	27.26	11.59
57	1.83	1.26	0.71	1.75	1.34	2.31	0.69	0.99	1.19	0.49	1.04	0.93	0.98	0.91	1.57	1.76	1.30	0.89
58	-	-	0.36	-	-	1.31	0.42	0.40	0.48	0.18	0.49	0.46	0.39	0.42	0.83	0.79	-	0.47
59	1.03	0.97	0.47	0.81	0.57	1.04	-	0.42	0.45	0.24	0.39	0.40	0.44	0.54	0.83	0.73	2.51	0.38
60	2.23	1.31	1.03	1.76	1.68	2.21	0.86	1.03	1.11	0.50	0.98	1.06	1.48	1.49	2.53	2.36	2.94	1.41
61	1.97	3.48	0.96	1.50	1.53	1.51	2.29	1.13	1.89	0.88	1.57	1.36	1.63	1.91	1.49	2.41	4.11	1.94
62	1.73	2.13	0.98	2.12	1.21	1.94	2.47	0.84	1.93	0.88	1.40	1.30	1.34	1.27	2.03	2.75	3.37	1.53
63	1.84	1.77	0.96	1.79	1.63	2.04	1.01	0.94	1.42	0.60	1.22	1.45	0.63	0.79	1.25	1.12	2.64	0.68
64	4.96	8.07	4.54	6.70	3.52	3.57	5.12	1.41	4.15	1.73	3.45	2.62	2.63	2.50	2.42	4.04	2.90	0.58
65	2.44	5.96	-	1.44	-	2.18	-	-	-	-	-	-	-	-	-	-	-	-
66	2.65	-	2.40	2.33	3.05	2.54	-	-	-	-	-	-	9.69	10.25	9.07	7.81	14.43	12.33
67	-	-	-	-	-	-	-	-	-	-	-	-	34.70	52.21	35.86	60.27	24.47	44.43
68	4.33	3.81	2.09	3.61	3.02	5.27	6.69	4.51	8.27	3.00	4.98	3.73	3.06	3.63	4.28	6.49	4.30	3.63
69	0.78	0.64	0.36	0.65	0.63	0.89	-	-	-	-	-	-	0.62	0.64	1.01	1.55	0.96	0.83
70	20.04	14.29	9.63	11.93	11.21	15.93	12.64	6.91	15.93	1.22	10.46	11.31	2.62	3.67	4.04	7.44	8.51	3.42
71	1.54	1.31	0.68	1.71	1.55	2.34	1.83	0.60	3.08	-	1.46	1.03	0.90	0.48	0.76	1.18	-	0.86

Table A.2 (Continued)

72	2.21	1.80	1.94	2.81	1.95	2.37	3.20	1.14	2.29	2.21	1.75	1.97	0.96	1.20	2.11	1.83	2.49	1.25
73	1.19	0.93	0.46	-	1.27	0.76	4.21	1.71	1.12	0.72	0.79	0.90	0.80	0.31	0.58	0.93	-	0.37
74	-	-	-	-	-	-	1.70	1.12	1.91	0.74	0.91	1.32	0.38	0.29	0.73	0.61	-	0.65
75	1.04	1.73	0.79	0.64	2.68	0.53	-	0.61	-	0.58	0.74	1.06	2.07	0.88	2.10	1.18	1.56	1.01
76	1.72	6.80	5.56	2.03	2.43	1.44	1.83	7.77	7.09	3.67	6.48	4.21	5.71	4.67	8.97	2.52	3.28	1.07
77	1.14	0.86	0.75	0.86	1.38	1.21	1.09	-	10.13	6.03	1.14	1.10	1.11	0.99	1.88	1.33	1.33	0.97
78	1.21	0.40	1.46	0.66	2.42	0.92	2.15	1.63	2.04	1.35	1.68	1.93	0.65	1.14	1.61	1.38	3.12	1.08
79	0.68	1.07	1.66	0.51	1.53	0.96	1.44	1.13	0.90	1.12	0.90	1.49	1.48	1.11	2.10	1.81	2.64	1.16
80	4.70	4.28	5.66	2.91	6.67	3.60	5.31	5.18	3.63	5.54	4.72	6.14	5.31	8.26	7.00	6.10	9.35	6.43
81	1.63	1.65	3.53	4.73	4.36	1.77	1.87	-	3.03	2.22	1.62	2.06	4.78	4.23	8.30	6.15	6.40	5.31
82	0.35	0.52	0.91	0.54	1.31	0.36	1.28	0.98	-	0.86	0.60	-	1.80	1.47	1.60	1.57	1.61	1.97
83	0.30	0.26	3.54	2.16	1.73	0.58	3.70	0.80	0.33	0.35	0.41	0.32	1.29	6.24	2.35	2.83	3.18	1.76
84	-	-	-	-	-	-	-	-	-	-	-	-	2.91	3.16	3.08	1.55	6.73	1.53

* Compound number and names are identical to those in Table A.1

Table A.3 Volatile metabolites profiles and log transformed data obtained using three types of SPME fibers CAR/PDMS, DVB/PDMS, and DVB/CAR/ PDMS with 6 replications each.

	Log transformed data																	
No.*	CAR REP 1	CAR REP 2	CAR REP 3	CAR REP 4	CAR REP 5	CAR REP 6	DVB CAR REP 1	DVB CAR REP 2	DVB CAR REP 3	DVB CAR REP 4	DVB CAR REP 5	DVB CAR REP 6	DVB REP 1	DVB REP 2	DVB REP 3	DVB REP 4	DVB REP 5	DVB REP 6
1	6.47	6.44	6.29	6.68	6.97	6.93	6.58	6.31	6.30	6.54	6.30	6.32	-	-	-	-	-	-
2	7.55	7.65	7.26	7.11	6.96	7.59	7.44	6.30	6.77	6.91	6.60	6.44	6.61	6.12	5.39	5.34	6.64	5.67
3	7.00	7.20	6.67	6.60	6.63	6.91	6.65	-	6.05	6.49	6.09	5.75	-	-	-	-	-	-
4	7.05	7.22	7.42	7.22	7.21	7.60	7.03	6.52	7.15	6.65	6.78	6.56	-	-	-	-	-	-
5	6.66	6.79	6.50	6.40	6.32	6.86	6.20	-	5.80	5.66	5.44	5.33	-	-	-	-	-	-
6	5.95	6.09	-	6.26	6.09	6.00	5.07	-	5.30	5.38	5.80	5.25	-	-	-	-	-	-
7	5.46	6.66	5.90	6.10	5.99	5.36	-	-	-	-	-	-	-	-	-	-	-	-
8	6.17	6.81	6.09	6.06	5.94	5.91	-	-	-	-	-	-	-	-	-	-	-	-
9	5.83	6.46	6.02	5.83	5.90	6.34	5.11	-	5.26	5.07	5.03	5.34	-	-	-	-	-	-
10	6.98	7.13	7.21	7.17	7.32	7.45	6.91	6.72	7.01	6.93	6.95	6.91	-	-	-	-	-	-
11	7.66	7.60	7.28	7.64	7.75	7.72	7.06	6.61	6.81	6.96	6.86	6.92	-	-	-	-	-	-
12	-	5.89	6.67	5.83	6.36	6.17	-	5.18	5.32	5.32	5.30	-	-	5.18	5.32	5.32	5.30	-
13	5.62	5.81	6.63	6.67	6.42	5.58	5.77	5.66	5.74	5.68	5.69	5.67	-	-	-	-	-	-
14	5.98	6.32	6.48	6.62	6.76	6.07	-	-	-	-	-	-	-	-	-	-	-	-
15	6.62	6.22	6.48	6.69	6.95	6.78	6.12	-	6.02	6.07	5.62	5.56	-	-	-	-	-	-
16	6.96	6.62	6.80	7.01	7.14	7.03	6.59	6.12	6.63	6.59	6.45	6.45	-	-	-	-	-	-
17	5.87	5.25	6.08	5.86	5.67	5.59	-	-	-	-	-	-	-	-	-	-	-	-
18	6.28	6.42	6.39	6.45	6.34	6.41	5.98	6.03	6.17	6.10	6.10	6.14	-	-	-	-	-	-
19	5.71	5.70	6.33	6.37	6.22	6.06	5.07	5.10	5.24	5.43	5.28	5.50	-	-	-	-	-	-
20	-	-	-	-	-	-	-	5.01	5.03	5.14	5.16	-	-	-	-	-	-	-

Table A.3 (Continued)

21	-	-	-	-	-	-	5.04	5.23	5.44	5.47	5.47	5.32	-	-	-	-	-	-
22	6.58	6.71	6.67	6.73	6.56	6.44	6.40	6.35	6.47	6.44	6.49	6.29	6.19	6.11	6.09	6.22	6.30	6.11
23	6.32	6.11	6.08	6.32	5.81	7.09	5.46	5.39	5.77	5.52	5.54	5.57	6.21	6.50	6.23	6.39	6.63	5.94
24	6.65	6.46	6.39	6.68	6.17	7.34	5.79	5.62	6.05	5.73	5.83	5.83	6.55	6.84	6.61	6.74	6.96	6.30
25	5.51	5.18	5.34	5.63	5.49	5.92	5.37	5.07	5.54	5.45	5.23	5.20	-	-	-	-	-	-
26	6.77	6.59	6.51	6.73	6.25	7.37	5.97	5.78	6.18	5.83	6.06	5.95	6.65	6.92	6.72	6.86	6.99	6.45
27	6.36	6.07	5.94	6.35	5.90	6.99	5.57	5.34	5.68	5.36	5.57	5.49	6.16	6.46	6.26	6.42	6.71	5.96
28	6.95	6.72	6.65	6.89	6.35	7.47	6.08	5.85	6.22	5.91	6.09	5.97	6.87	7.06	6.91	7.02	7.17	6.52
29	-	-	-	-	-	-	5.69	-	5.29	6.04	5.31	5.99	6.98	6.00	6.08	5.42	7.77	6.07
30	6.54	6.04	6.07	6.46	5.81	6.96	5.52	5.45	5.66	5.50	5.56	5.62	6.45	6.59	6.57	6.56	6.84	5.97
31	6.22	5.56	5.56	6.09	5.60	6.70	5.35	5.35	5.41	5.38	5.40	5.17	6.00	6.09	6.03	6.16	-	5.57
32	-	-	-	-	-	-	-	-	-	-	-	-	6.04	5.74	6.00	5.64	6.90	6.34
33	5.96	6.08	5.03	6.31	5.56	6.00	-	-	-	-	-	-	-	-	-	-	-	-
34	6.11	5.21	5.27	5.95	5.25	6.55	-	-	-	-	-	-	5.70	5.73	5.66	5.91	6.09	5.29
35	6.06	6.42	5.08	5.88	5.12	6.44	-	-	-	-	-	-	-	-	-	-	-	-
36	6.62	6.21	6.20	6.51	6.12	6.88	5.56	5.62	5.82	5.41	5.85	5.63	6.44	6.51	6.53	6.59	6.76	6.12
37	6.20	5.84	5.74	6.09	5.88	6.52	5.41	5.12	5.62	-	5.61	5.17	6.02	6.15	6.11	6.18	-	5.79
38	5.70	6.29	6.62	6.51	6.89	6.44	-	5.17	5.14	5.07	5.11	5.34	-	-	-	-	-	-
39	6.19	5.43	5.70	6.14	5.71	6.38	5.04	-	5.14	5.08	5.18	5.04	6.34	6.29	6.52	6.28	6.70	5.55
40	6.35	5.83	5.73	6.06	6.04	6.38	-	5.58	5.74	5.49	5.78	5.63	-	-	-	-	-	-
41	-	-	-	5.85	5.86	6.18	-	-	-	-	-	-	-	-	-	-	-	-
42	6.33	5.91	5.89	6.20	6.13	6.19	7.29	-	5.57	-	-	5.47	6.43	6.08	6.16	6.02	7.19	5.98
43	6.48	6.02	6.09	6.36	6.23	6.49	5.88	5.86	5.59	5.88	5.80	5.37	6.38	6.42	6.59	6.51	6.71	6.07
44	6.80	6.53	6.55	6.75	6.61	6.96	5.99	6.29	6.33	5.95	6.33	6.20	6.67	6.73	6.85	6.84	7.00	6.55
45	6.13	5.93	5.57	6.00	6.14	6.20	5.44	5.80	5.89	5.47	5.86	5.77	5.89	6.05	6.12	6.08	6.68	5.96

Table A.3 (Continued)

46	6.19	-	6.01	5.57	5.96	6.22	-	5.48	5.44	5.17	5.58	5.56	-	-	-	-	-	-
47	6.71	6.66	6.55	6.69	6.57	6.84	6.14	6.37	6.40	6.15	6.33	6.25	6.76	6.76	6.88	6.85	7.15	6.57
48	6.61	6.71	6.35	6.57	6.43	6.72	6.30	-	-	-	-	-	6.53	6.53	6.67	6.66	-	6.64
49	6.60	-	6.38	6.51	6.44	6.65	-	6.58	6.56	6.38	6.22	6.18	6.55	6.57	6.70	6.62	7.24	6.71
50	6.14	6.10	5.83	6.25	5.95	6.35	5.80	5.88	6.03	5.91	5.94	5.77	5.54	-	5.49	5.69	-	-
51	7.32	7.19	7.21	7.29	7.14	7.42	6.88	7.06	7.01	6.78	6.97	6.92	7.29	7.37	7.46	7.42	7.51	7.16
52	7.01	6.85	6.90	6.99	6.84	7.12	6.56	6.78	6.69	6.42	6.64	6.60	6.99	7.06	7.16	7.12	7.26	6.85
53	6.23	5.96	5.77	6.13	6.29	6.33	5.45	6.21	5.82	5.50	5.78	5.76	5.89	6.06	6.18	6.18	6.09	6.07
54	6.27	-	5.88	5.78	-	6.63	-	-	-	-	-	-	6.55	5.98	6.41	6.08	7.09	5.99
55	6.60	6.89	6.31	6.55	6.54	6.39	6.19	6.21	6.21	5.91	6.21	6.22	6.59	6.56	6.55	6.53	6.56	6.16
56	7.27	7.20	7.07	7.31	6.97	7.23	6.97	6.90	7.02	6.67	6.89	6.98	7.14	7.23	7.30	7.29	7.44	7.06
57	6.26	6.10	5.85	6.24	6.13	6.36	5.84	5.99	6.08	5.69	6.02	5.97	5.99	5.96	6.20	6.25	6.12	5.95
58	-	-	5.56	-	-	6.12	5.62	5.60	5.68	5.25	5.69	5.67	5.59	5.63	5.92	5.90	5.78	5.67
59	6.01	5.99	5.67	5.91	5.75	6.02	-	5.63	5.65	5.37	5.59	5.60	5.65	5.73	5.92	5.86	6.40	5.58
60	6.35	6.12	6.01	6.25	6.22	6.34	5.94	6.01	6.05	5.70	5.99	6.02	6.17	6.17	6.40	6.37	6.47	6.15
61	6.29	6.54	5.98	6.18	6.19	6.18	6.36	6.05	6.28	5.95	6.20	6.13	6.21	6.28	6.17	6.38	6.61	6.29
62	6.24	6.33	5.99	6.33	6.08	6.29	6.39	5.92	6.29	5.95	6.15	6.12	6.13	6.10	6.31	6.44	6.53	6.18
63	6.26	6.25	5.98	6.25	6.21	6.31	6.00	5.97	6.15	5.78	6.09	6.16	5.80	5.90	6.10	6.05	6.42	5.83
64	6.70	6.91	6.66	6.83	6.55	6.55	6.71	6.15	6.62	6.24	6.54	6.42	6.42	6.40	6.38	6.61	6.46	5.76
65	6.39	6.78	-	6.16	-	6.34	-	-	-	-	-	-	-	-	-	-	-	-
66	6.42	-	6.38	6.37	6.48	6.40	-	-	-	-	-	-	6.99	7.01	6.96	6.89	7.16	7.09
67	-	-	-	-	-	-	-	-	-	-	-	-	7.54	7.72	7.55	7.78	7.39	7.65
68	6.64	6.58	6.32	6.56	6.48	6.72	6.83	6.65	6.92	6.48	6.70	6.57	6.49	6.56	6.63	6.81	6.63	6.56
69	5.89	5.80	5.56	5.81	5.80	5.95	-	-	-	-	-	-	5.79	5.81	6.01	6.19	5.98	5.92
70	7.30	7.16	6.98	7.08	7.05	7.20	7.10	6.84	7.20	6.09	7.02	7.05	6.42	6.56	6.61	6.87	6.93	6.53

Table A.3 (Continued)

71	6.19	6.12	5.83	6.23	6.19	6.37	6.26	5.78	6.49	-	6.16	6.01	5.95	5.68	5.88	6.07	-	5.93
72	6.35	6.26	6.29	6.45	6.29	6.37	6.50	6.06	6.36	6.34	6.24	6.29	5.98	6.08	6.32	6.26	6.40	6.10
73	6.08	5.97	5.67	-	6.10	5.88	6.62	6.23	6.05	5.86	5.90	5.95	5.90	5.50	5.77	5.97	-	5.57
74	-	-	-	-	-	-	6.23	6.05	6.28	5.87	5.96	6.12	5.58	5.46	5.86	5.78	-	5.81
75	6.02	6.24	5.90	5.80	6.43	5.73	-	5.78	-	5.77	5.87	6.02	6.32	5.94	6.32	6.07	6.19	6.00
76	6.24	6.83	6.74	6.31	6.39	6.16	6.26	6.89	6.85	6.56	6.81	6.62	6.76	6.67	6.95	6.40	6.52	6.03
77	6.06	5.94	5.88	5.93	6.14	6.08	6.04	-	7.01	6.78	6.06	6.04	6.05	6.00	6.27	6.13	6.12	5.99
78	6.08	5.60	6.17	5.82	6.38	5.96	6.33	6.21	6.31	6.13	6.22	6.28	5.81	6.06	6.21	6.14	6.49	6.03
79	5.83	6.03	6.22	5.71	6.19	5.98	6.16	6.05	5.95	6.05	5.95	6.17	6.17	6.05	6.32	6.26	6.42	6.06
80	6.67	6.63	6.75	6.46	6.82	6.56	6.72	6.71	6.56	6.74	6.67	6.79	6.73	6.92	6.85	6.79	6.97	6.81
81	6.21	6.22	6.55	6.67	6.64	6.25	6.27	-	6.48	6.35	6.21	6.31	6.68	6.63	6.92	6.79	6.81	6.72
82	5.54	5.72	5.96	5.73	6.12	5.55	6.11	5.99	-	5.94	5.78	-	6.25	6.17	6.20	6.20	6.21	6.29
83	5.48	5.42	6.55	6.33	6.24	5.76	6.57	5.91	5.52	5.54	5.62	5.51	6.11	6.80	6.37	6.45	6.50	6.24
84	-	-	-	-	-	-	-	-	-	-	-	-	6.46	6.50	6.49	6.19	6.83	6.18

* Compound number and names are identical to those in Table A.1

Table A.4 MVOCs identified from *A.flavus* 5-3B growth on different medium expressed in peak area

No.	Compound name	Chemical class	Retention time (min)		Medium (peak area × 10^5)				
			Mean[a]	STD[b]	CDA	CSA	CDL	MEA	CMA
1	Ethanol	alcohol	4.595	0.043	15.9±7.29[c]	590.21±239.37	2440.89±2796.9	50.61±31.82	188.42±92.03
2	Acetone	ketone	5.022	0.031	-	281.67±97.01	141.78±155.48	266.07±150.62	71.06±24.65
3	Butane, 2-methyl-	alkane	5.036	0.014	45.37±9.07	-	-	-	-
4	Isopropyl Alcohol	alcohol	5.149	0.007	4470.76±600.92	311.57±65.79	-	78.41±46.3	57.03±7.79
5	Furan	furan	5.296	0.024	-	-	308.56±155.76	-	-
6	Pentane	alkane	5.462	0.028	587.95±42.37	-	47.93±17.47	-	-
7	1,4-Pentadiene	alkene	5.547	0.020	-	700.43±153.48	465.53±75.88	211.04±103.06	12.73±2.91
8	Acetic acid, methyl ester	organic acid	5.751	0.009	-	458.35±115.33	-	42.94±20.93	-
9	Propanal, 2-methyl-	aldehyde	6.231	0.078	-	-	11.66±8.95	-	-
10	1-Propanol	alcohol	6.486	0.048	11.71±4.94	151.74±59.84	85.96±106.79	-	-
11	2,3-Butanedione	ketone	7.180	0.028	-	-	42.15±33.1	-	-
12	Butanal	aldehyde	7.357	0.047	-	-	16.31±8.41	13.49±16.15	-
13	2-Butanone	ketone	7.559	0.070	56.56±7.22	-	126.15±180.14	19.92±21.85	-
14	Furan, 3-methyl-	furan	8.173	0.025	-	-	42.48±14.56	-	-
15	Propanoic acid, 2-methyl-, anhydride	organic acid	8.313	0.025	48.24±10.16	-	-	13.82±9.4	-
16	3-Buten-2-ol, 2-methyl-	alcohol	8.311	0.020	-	37.38±19.28	47.19±27.55	-	4.55±1.31
17	Ethyl Acetate	ester	8.409	0.024	992.03±165.31	64±26.09	-	-	-
18	Furan, 2-methyl-	furan	8.567	0.021	131.7±41.4	359.53±45.32	1136.12±317.39	172.2±66.17	11.71±3.08
19	1-Propanol, 2-methyl-	alcohol	9.064	0.031	-	595.23±192.33	579.97±506.88	427.71±129.85	-
20	Furan, tetrahydro-	furan	9.709	0.021	20.77±4.59	-	-	19.8±16.31	13.07±4.5
21	Benzene	aromatic hydrocarbons	10.888	0.018	-	-	-	21.64±19.67	3.53±1.24
22	1-Butanol	alcohol	10.885	0.062	16.49±5.49	77.85±42.54	167.19±52.94	-	-
23	Furan, 2,5-dimethyl-	Furan	12.846	0.016	12.54±3.28	-	33.1±9.95	-	-
24	Heptane	alkane	12.973	0.077	-	6.13±1.42	9.2±5.99	-	-
25	3-Buten-1-ol, 3-methyl-	alcohol	13.626	0.050	-	128.43±38.71	35.92±44.35	28.58±18.4	-
26	1-Butanol, 3-methyl-	alcohol	13.782	0.027	-	73.04±22.25	615.08±856.14	47.94±25.21	-
27	1-butanol, 2-methyl	alcohol	13.951	0.018	-	79.77±20.4	348.89±523.16	91.03±34.45	-
28	Methyl Isobutyl Ketone	ketone	14.464	0.041	67.17±8.01	-	-	6.18±3.58	-
29	Toluene	aromatic hydrocarbons	15.506	0.027	12.9±4.35	24.72±7.95	100.55±27.21	24.3±3.35	8.63±1.71
30	1-Octene	alkene	17.048	0.011	-	20±4.16	-	13.88±7.98	-
31	Acetic acid, butyl ester	organic acid	17.464	0.037	22.91±9.76	-	28.91±15.31	-	-
32	Octane	alkane	17.629	0.012	-	8.67±4.39	30.57±22.66	-	-
33	Ethylbenzene	aromatic hydrocarbons	20.385	0.015	17.21±3.62	4.02±1.15	184.85±58.56	-	-
34	p-Xylene	aromatic hydrocarbons	20.825	0.012	-	8.78±0.99	605.31±178.84	-	-

Table A.4 (Continued)

35	2-Heptanone	ketone	21.323	0.024	18.47±3.88	-	95.69±40.68	-	-
36	Styrene	aromatic hydrocarbons	21.984	0.101	-	37.67±8.33	91.57±15.71	42.3±10.07	23.68±10.62
37	o-Xylene	aromatic hydrocarbons	22.093	0.010	-	-	96.05±28.18	-	-
38	Nonane	alkane	22.967	0.007	-	-	14.86±10.42	-	-
39	Cyclopentane, (2-methylbutylidene)-	alkene	24.451	0.005	-	-	88.96±78.41	-	-
40	Propanoic acid, 3-ethoxy-, ethyl ester	organic acid	26.417	0.021	-	-	102.92±51.74	-	-
41	Octane, 2,5,6-trimethyl-	alkane	26.667	0.036	-	-	18.69±17.82	-	1.81±0.56
42	Benzene, 1,2,3-trimethyl-	aromatic hydrocarbons	27.102	0.019	26.25±27.14	-	12.83±9.05	-	-
43	β-myrcene	alkene	27.933	0.027	-	-	49.41±36.06	-	-
44	Benzene, 1-ethyl-2-methyl-	aromatic hydrocarbons	28.073	0.015	-	-	45.45±16.85	-	-
45	Decane	alkane	28.951	0.003	-	-	18.31±8.89	-	1.69±0.74
46	Heptane, 2,2,6,6-tetramethyl-4-methylene-	alkene	29.167	0.005	-	-	-	32.63±44.5	2.55±0.7
47	3-Heptene, 2,2,4,6,6-pentamethyl-	alkene	29.732	0.005	-	-	-	63±76.55	4.04±1.58
48	1-Hexanol, 2-ethyl-	alcohol	29.853	0.046	-	-	204.52±257.54	-	-
49	Benzene, 1-methyl-4-(1-methylethyl)-	aromatic hydrocarbons	30.096	0.027	17.02±3.95	17.75±2.69	21.58±16.29	-	-
50	D-Limonene	aromatic hydrocarbons	30.733	0.040	-	34.91±3.24	334.45±548.07	3.76±2.38	8.23±2.14
51	2-Pentene, 2,4,4-trimethyl-	alkene	31.435	0.004	14.24±3.69	-	-	72.57±80.15	7.05±2.91
52	Hexadecane,2,6,10,14-tetramethyl-	alkane	31.688	0.005	0±0	19.97±2.33	-	-	14.27±2.33
53	Cyclohexane, 1-methyl-3-pentyl-	alkane	32.234	0.005	39.03±4.12	-	-	28.62±34.65	2.81±1.1
54	Dodecane	alkane	33.053	0.009	14.13±2.11	46.36±15.97	46.55±14.76	96.62±99.59	30.9±5.98
55	Undecane, 3,6-dimethyl-	alkane	33.537	0.007	9.36±2	18.39±2.88	-	-	13.45±2.86
56	Undecane, 2,8-dimethyl-	alkane	34.510	0.005	-	20.2±2.97	-	-	14.49±3.55
57	Nonanal	aldehyde	34.663	0.012	-	-	43.66±18.71	-	-
58	Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl-	alkene	34.710	0.015	-	21.79±9.41	-	-	-
59	1,6-Octadien-3-ol, 3,7-dimethyl-	alcohol	34.761	0.008	-	-	27.22±12.06	-	-
60	Heptane, 1,1'-oxybis-	ether	35.119	0.004	-	-	-	30.73±31.68	9.64±2.23
61	1-Pentanol, 2,2,4-trimethyl-	alcohol	35.453	0.004	-	-	-	15.13±18.16	2.8±0.68
62	Undecane	alkane	35.557	0.001	12.06±0.64	-	13.01±6.15	-	-
63	2,2-Dimethylheptane-3,5-dione, keto form	ketone	35.810	0.015	-	10.12±2.82	40.61±25.83	10.44±13.1	5.38±0.64
64	4-Undecene, 7-methyl-	alkene	36.271	0.003	-	-	-	12.62±11.85	1.81±0.64
65	3-Undecene, 6-methyl-, (E)-	alkene	36.544	0.003	-	-	-	-	3.12±1.15
66	Undecane, 2,6-dimethyl-	alkane	38.682	0.003	89.05±146.74	-	-	13.55±10.3	7.73±4.76

Table A.4 (Continued)

67	Decanal	aldehyde	39.186	0.045	6.65±0.86	14.93±8.36	237.75±178.25	11.07±8.25	8.58±2.82
68	Dodecane	alkane	39.545	0.030	-	6.03±2.55	-	-	5.54±2.15
69	1,3,7-Octatriene, 3,7-dimethyl-	alkene	40.716	0.004	12.36±2.45	-	13.54±11.91	13.49±7.87	0±0
70	2,4,4,6,6,8,8-Heptamethyl-1-nonene	alkane	41.647	0.002	-	-	27.07±8.35	9.81±4.57	5.72±4.08
71	Hexadecane	alkane	41.853	0.002	-	-	-	13.75±5.21	4.42±1.67
72	delta-selinene	sesquiterpene	41.949	0.020	-	-	17.91±13.36	20.56±8.75	3.33±1.9
73	Heptacosane	alkane	42.160	0.002	19.56±6.76	-	11.21±10.14	53.5±22.23	9.73±4.97
74	6,7-Dimethyl-1,2,3,5,8,8a-hexahydronaphthalene	sesquiterpene	42.380	0.011	-	38.07±17.19	29.69±32.23	10.95±4.47	20.75±10.79
75	Tetradecane, 2,5-dimethyl-	alkane	42.606	0.003	-	-	-	11.02±5.22	5.35±2.74
76	1,7-Dimethyl-4-(1-methylethyl)cyclodecane	alkane	42.675	0.002	-	-	-	-	4.82±3.36
77	Humulene	sesquiterpene	42.829	0.002	-	-	11.15±5.65	-	-
78	Bicyclogermacrene	sesquiterpene	43.031	0.008	-	-	14.66±12.78	48.07±12.22	5.7±3.75
79	2,2,4,4,5,5,7,7-Octamethyloctane	alkane	43.176	0.002	396.28±87.88	-	-	-	17.33±9.11
80	δ-Elemene	sesquiterpene	43.204	0.008	-	92.82±33.47	562.72±343.28	38.51±12.33	-
81	(Z)-3-hexadecene	alkene	43.313	0.001	84.9±22.87	19.73±5.31	-	33.72±8.69	14.96±7
82	trans-α-Bergamotene	sesquiterpene	43.379	0.002	281.78±76.95	38.73±25.84	76.7±37.31	13.67±5.65	16.12±8.52
83	α-Cubebene	sesquiterpene	43.489	0.002	100.11±27	81.03±18.27	541.68±440.8	187.74±46.2	-
84	3-Hexadecene, (Z)-	alkene	43.619	0.004	61.29±13.82	155.34±56.66	68.58±35.09	91.63±23.86	75.67±34.33
85	trans-7-Hexadecene	alkene	43.781	0.002	-	83.67±30.86	44.2±28.74	14.35±5.98	37.69±16.72
86	2,4,4,6,6,8,8-Heptamethyl-2-nonene	alkene	43.900	0.001	620.91±98.73	-	-	18.71±16.8	8.94±5.36
87	Ylangene	sesquiterpene	43.988	0.020	10572.19±1341.27	96.19±32.13	813.31±345.08	38.95±20.63	-
88	(-)-Aristolene	sesquiterpene	44.106	0.005	15239.56±2280.15	944.4±250.79	13881.22±6677.65	154.16±41.38	27.34±8.56
89	β-Elemene	sesquiterpene	44.253	0.007	266±64.17	2769.61±1203.22	18305.47±8626.08	15.32±5.53	46.49±20.43
90	Isoledene	sesquiterpene	44.383	0.004	-	73.97±10.09	422.01±318.33	8.37±6.73	6.03±2.1
91	Hept-2-ene, 2,4,4,6-tetramethyl-	alkene	44.472	0.002	290.3±31.61	-	-	-	3.51±1.93
92	.beta.-Humulene	sesquiterpene	44.502	0.004	-	89.92±34.4	365.74±193.86	8.16±2.46	-
93	1-Hexadecene	alkene	44.547	0.002	276±71.04	-	-	17.01±4.78	4.26±1.94
94	β-Selinene	sesquiterpene	44.575	0.036	-	-	344.09±176.45	-	-
95	α-Farnesene	sesquiterpene	44.690	0.011	4353.73±846	63.08±26.82	317.15±208.13	-	7.95±3.88
96	α-Gurjunene	sesquiterpene	44.778	0.003	646.95±138.46	186.76±79.07	5032.01±2940.87	18.25±8.73	-
97	β-Cubebene	sesquiterpene	44.871	0.002	2067.09±469.92	61.2±18.36	942.5±513.86	-	5.61±3.25
98	Caryophyllene	sesquiterpene	44.952	0.002	1248.95±382.82	216.16±108.68	1948.14±1086.96	-	-
99	γ-Gurjunene	sesquiterpene	45.029	0.029	-	-	1090.95±1165.25	16.87±4.86	-

Table A.4 (Continued)

100	1H-Cycloprop[e]azulene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.beta.,7b.alpha.)]-	sesquiterpene	45.060	0.003	311.24±49.92	125.5±28.98	706.1±478.51	-	-
101	Bicyclo(4.4.0)dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	sesquiterpene	45.110	0.002	3409.79±679.45	-	441.36±268.66	16.72±3.74	3.42±1.65
102	Calarene	sesquiterpene	45.266	0.002	-	536.12±179.33	4823.74±3306.65	-	4.09±2.69
103	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	sesquiterpene	45.363	0.001	1648.79±302.7	74.56±21.11	760.75±132.07	-	-
104	Valencene	sesquiterpene	45.511	0.002	1694.25±458.45	425.11±215.33	2675.07±2334.26	52.25±18.13	1.74±0.71
105	α-Selinene	sesquiterpene	45.590	0.002	10480.97±2747.3	535.97±115.4	3142.61±2099.02	30.04±20.16	-
106	α-Farnesene	sesquiterpene	45.742	0.004	1662.43±320.14	1078.53±553.2	13975.38±9283.55	-	3.58±1.06
107	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1π4aπ8aπ-(π-	sesquiterpene	45.920	0.002	-	-	2644.83±2058.27	-	-
108	Spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11-methylene-, (-)-	sesquiterpene	45.973	0.010	4724.53±913.76	2375.34±803.93	-	36.88±10.88	-
109	Cubenene	sesquiterpene	46.030	0.049	2868.64±817.09	504.45±234.97	9414.43±6260.95	-	4.51±1.6
110	γ-Cadinene	sesquiterpene	46.120	0.005	837.17±209.38	-	4986.89±3756.35	6.59±1.77	-
111	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.beta.)]-	sesquiterpene	46.181	0.003	9186.14±5596.53	99.98±39.71	1255.05±890.33	-	1.46±0.44
112	γ-Muurolene	sesquiterpene	46.292	0.007	8230.41±2357.63	-	16965.62±12078.89	138.37±37.77	-
113	δ-Cadinene	sesquiterpene	46.329	0.004	1503.41±176.83	3195.96±1441.01	11705.52±8149.9	-	6.42±1.85
114	β-Germacrene	sesquiterpene	46.421	0.003	605.78±126.3	334.85±117.26	2428.04±1666.32	15.21±5.42	-
115	β-Panasinsene	sesquiterpene	46.489	0.008	1507.55±381.97	160.96±65.9	1218.25±852.87	21.81±3.71	-
116	β-Cadinene	sesquiterpene	46.616	0.002	163.7±39.94	307.11±178.31	4221.52±3499.69	9.23±3.28	-
117	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl	sesquiterpene	46.711	0.007	74.4±26.35	-	346.53±220.96	-	-
118	Copaene	sesquiterpene	46.841	0.003	105.9±36.33	12.71±6.85	151.51±120.15	-	-
119	Cadala-1(10),3,8-triene	alkene	46.905	0.005	-	15.11±6.38	174.8±49.59	-	-
120	Isolongifolene, 4,5,9,10-dehydro-	sesquiterpene	46.955	0.002	92.11±20.58	20.76±6.98	156.64±110.37	-	-
121	πGuaiene	sesquiterpene	47.015	0.001	161.82±17.12	-	159.94±111.83	-	-
122	Cadina-1(10),6,8-triene	alkene	47.141	0.003	60.67±16.91	34.94±14	299.1±144.84	12.34±8.27	-
123	4aH-cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl-	sesquiterpene	47.234	0.006	52.99±13.69	24.37±9.99	194.66±118.71	-	-

Table A.4 (Continued)

124	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,7.beta.,8a.alpha.)]-	sesquiterpene	47.360	0.003	68.89±16.55	-	102.35±83.18	33.29±22.65	-
125	4,4,5,6-Tetramethyltetrahydro-1,3-oxazin-2-thione	ether	47.475	0.003	158.34±43.55	20.42±8.09	274.57±169.52	-	-
126	9-Methyl-S-octahydrophenanathracene	alkene	47.598	0.004	-	49.13±18.73	497.64±329.24	10.35±2.46	8.27±5.12
127	Cedrene	sesquiterpene	47.716	0.002	138.27±32.65	41.94±15.33	580.94±376.27	-	-
128	πVatirenene	sesquiterpene	47.741	0.005	27.97±10.61	-	-	11.79±7.17	-
129	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, [1aR-(1a.alpha.,4a.beta.,7.alpha.,7a.beta.,7 b.alpha.)]-	sesquiterpene	47.863	0.003	54.63±13.58	-	170.09±111.79	-	-
130	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]-	sesquiterpene	47.993	0.012	52.76±19.91	37.84±15.42	437.68±298.11	10.7±4.56	-
131	.alpha.-Cadinol	alcohol	48.145	0.003	272.05±66.88	23.31±6.58	370.49±256.44	-	2.86±2.72
132	Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	aromatic hydrocarbons	48.345	0.008	-	68.7±22.8	603.4±64.59	-	13.85±5

[a] Mean of the retention time

[b] standard deviation of the retention time

[c] mean and standard deviation of the peak area for each compound in different growth media

Table A.5 15 selected MVOCs quantity variation expressed in peak area from *A. flavus* 5-38 caused by spores' suspension concentration

Sample name	MVOCs (Peak area $\times 10^7$)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
H1	1.53	2.72	2.46	4.47	0.69	1.29	0.48	13.02	56.26	15.30	16.18	46.37	3.54	2.35	5.08
H2	2.86	7.87	4.93	7.14	2.60	2.67	0.62	13.70	43.80	12.35	10.80	27.69	3.54	1.46	2.42
H3	2.50	5.62	3.91	4.87	1.78	2.10	0.59	15.87	53.92	17.23	15.93	41.90	4.65	2.38	4.61
H4	3.81	8.64	5.68	4.99	1.78	2.14	0.96	36.99	125.52	57.60	45.81	107.96	15.70	9.94	15.32
H5	2.06	8.40	5.05	6.27	2.12	2.11	0.64	26.62	89.68	30.70	19.93	55.67	7.11	2.93	6.05
H6	1.40	8.69	4.36	4.52	2.01	2.12	0.65	9.45	27.21	7.96	8.23	23.90	2.80	1.62	2.17
M1	2.62	7.43	4.78	5.43	2.96	2.70	0.56	19.74	82.79	23.64	17.80	49.42	5.87	2.40	4.34
M2	2.64	14.19	6.89	6.74	2.58	2.92	0.81	19.43	53.93	19.23	19.70	51.03	6.07	2.59	5.06
M3	2.59	4.94	5.16	6.73	2.84	3.14	0.65	31.05	142.60	54.79	51.22	112.84	11.43	6.32	13.50
M4	2.25	6.04	4.77	6.97	2.33	2.96	0.77	26.23	120.11	35.96	29.77	71.39	7.63	3.59	7.25
M5	2.78	6.62	4.33	5.42	1.50	1.88	0.59	13.75	57.68	0.53	0.42	39.63	3.72	2.48	4.60
M6	2.84	8.63	5.57	4.82	1.91	2.06	0.56	19.77	62.89	21.75	25.59	55.82	8.73		5.93
L1	1.11	13.25	6.32	4.49	2.21	2.18	0.56	13.43	43.60	13.25	10.90	31.34	3.99	1.60	2.56
L2	2.04	10.04	5.31	4.72	2.16	2.54	0.68	9.35	28.93	10.15	9.37	24.22	2.84	1.54	2.58
L3	1.38	7.55	3.79	4.57	1.71	2.22	0.67	10.02	38.98	11.81	11.39	31.84			3.26
L4	2.10	8.96	6.04	6.69	2.37	2.89	0.67	10.56	38.45	11.14	8.47	23.72	2.75	1.25	2.25
L5	1.43	6.87	3.95	3.83	1.14	1.46	0.53	9.82	29.14	2.58	1.36	33.54	2.97	1.98	3.67
L6	1.30	6.68	3.41	6.64	2.00	2.74	0.68	8.81	36.58	8.75	5.65	16.31	1.97	0.92	1.55
T1	2.05	0.90	2.89	10.16	3.15	3.33	0.59	12.44	52.40	13.10	20.98	44.62	3.62	2.30	4.42
T2	1.41	0.96	1.58	6.31	1.23	1.93	0.97	20.46	95.11	26.70	40.66	72.85	6.83	3.86	8.88
T3	1.85	0.76	1.92	10.12	3.10	3.22	0.68	3.11	19.15	2.91	5.14	8.11	0.89	0.59	1.05
T4	1.57	0.77	2.87	14.47	4.21	4.94	0.88	23.94	112.92	27.74	37.42	48.79	6.21	3.34	6.93

Table A.5 (Continued)

T5	1.62	0.95	2.24	6.77	2.21	2.67	0.98	7.41	33.41	6.51	7.72	17.37	1.92	1.26	1.88
T6	0.96	1.10	2.08	7.18	2.32	2.61	0.92	22.85	113.50	24.49	32.46	71.99	7.01	3.50	6.39

Sample name H1-6= high concentration spores' suspension treated sample replication 1-6
Sample name M1-6= medium concentration spores' suspension treated sample replication 1-6
Sample name L1-6= low concentration spores' suspension treated sample replication 1-6
Sample name T1-6= toothpick method inoculated sample replication 1-6
MVOCs 1-15 are: 1. Ethanol, 2. 1, 4-Pentadiene, 3. 2-methylfuran, 4. 2-methyl-1-Propanol, 5. 3-methylbutanol, 6. 2-methylbutanol, 7. Toluene, 8. (-)-Aristolene, 9. β-Elemene, 10. α-Farnesene, 11. Cubenene, 12. δ-Cadinene, 13. β-Germacrene, 14. β-Panasinsene, 15. β-Cadinene.

Table A.6 MVOCs profiles of *A. flavus* 5-38 growth on CDA, CSA and CDL medium.

No.	Compound name	R.T. (min)	Growth media (peak area percentage)																	
			CDA						CSA						CDL					
			REP 1	REP 2	REP 3	REP 4	REP 5	REP 6	REP 1	REP 2	REP 3	REP 4	REP 5	REP 6	REP 1	REP 2	REP 3	REP 4	REP 5
1	Ethanol	4.595	0.27	0.10	0.11	0.10	0.16	0.22	7.40	2.25	9.40	5.43	5.14	5.79	27.19	8.98	3.87	71.91	10.09
2	Acetone	5.022	-	-	-	-	-	-	2.85	3.09	2.88	2.08	1.59	4.42	1.33	0.51	0.47	4.13	0.65
3	Butane, 2-methyl-	5.036	0.39	0.44	0.49	0.32	0.51	0.58	-	-	-	-	-	-	-	-	-	-	-
4	Isopropyl Alcohol	5.149	-	-	-	-	-	-	2.79	2.19	3.63	2.85	3.19	4.04	-	-	-	-	-
5	Furan	5.296	48.11	34.91	51.93	46.53	46.03	40.74	-	-	-	-	-	-	3.26	5.28	3.71	1.52	1.66
6	Pentane	5.462	-	-	-	-	-	-	-	-	-	-	-	-	0.77	0.33	0.46	0.35	0.48
7	1,4-Pentadiene	5.547	5.82	6.29	6.28	5.43	6.13	5.33	6.48	7.96	8.08	8.71	4.57	6.23	5.67	3.93	4.77	3.88	5.02
8	Acetic acid, methyl ester	5.751	-	-	-	-	-	-	4.59	4.33	5.49	4.10	2.84	6.16	-	-	-	-	-
9	Propanal, 2-methyl-	6.231	-	-	-	-	-	-	-	-	-	-	-	-	0.26	0.04	0.07	0.14	0.07
10	1-Propanol	6.486	-	-	-	-	-	-	1.68	0.60	2.46	1.50	1.31	1.55	1.46	0.16	0.08	2.46	0.13
11	2,3-Butanedione	7.180	0.11	0.13	0.12	0.05	0.20	0.09	-	-	-	-	-	-	0.79	0.23	0.12	0.77	0.20
12	Butanal	7.357	-	-	-	-	-	-	-	-	-	-	-	-	0.27	0.07	0.10	0.22	0.16
13	2-Butanone	7.559	-	-	-	-	-	-	-	-	-	-	-	-	4.29	0.27	0.04	1.57	0.14
14	Furan, 3-methyl-	8.173	0.60	0.58	0.60	0.56	0.63	0.43	-	-	-	-	-	-	0.64	0.50	0.35	0.33	0.30
15	Propanoic acid, 2-methyl-, anhydride	8.313	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	3-Buten-2-ol, 2-methyl-	8.311	0.47	0.49	0.62	0.37	0.56	0.37	0.22	0.74	0.35	0.36	0.20	0.38	0.90	0.36	0.38	0.17	0.56
17	Ethyl Acetate	8.409	-	-	-	-	-	-	0.51	0.84	0.93	0.67	0.20	0.69	-	-	-	-	-
18	Furan, 2-methyl-	8.567	11.05	8.73	10.93	10.57	11.15	7.09	3.36	3.29	4.09	4.11	3.01	3.72	13.33	14.48	12.81	6.81	9.38
19	1-Propanol, 2-methyl-	9.064	1.22	1.19	1.46	0.74	1.29	2.00	5.03	3.25	8.81	7.33	5.47	5.82	10.30	1.40	1.88	12.21	3.21
20	Furan, tetrahydro-	9.709	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	Benzene	10.888	0.16	0.24	0.21	0.26	0.22	0.15	-	-	-	-	-	-	-	-	-	-	-
22	1-Butanol	10.885	-	-	-	-	-	-	0.82	0.26	0.79	0.42	1.47	0.91	1.90	1.43	1.08	2.28	-
23	Furan, 2,5-dimethyl-	12.846	0.24	0.17	0.19	0.15	0.17	0.07	-	-	-	-	-	-	0.37	0.36	0.46	0.22	0.24
24	Heptane	12.973	0.10	0.12	0.10	0.10	0.16	0.18	0.05	0.07	0.08	0.06	0.04	0.07	0.09	0.03	0.04	0.15	0.15
25	3-Buten-1-ol, 3-methyl-	13.626	-	-	-	-	-	-	1.35	0.81	1.68	1.77	1.09	0.99	0.30	0.10	0.07	1.14	0.19
26	1-Butanol, 3-methyl-	13.782	-	-	-	-	-	-	0.69	0.44	0.99	1.00	0.62	0.65	5.54	1.14	0.87	21.10	2.11

Table A.6 (Continued)

27	1-butanol, 2-methyl	13.951	-	-	-	-	-	-	0.69	0.48	1.07	0.94	0.79	0.82	1.97	0.89	0.38	12.79	1.42
28	Methyl Isobutyl Ketone	14.464	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	Toluene	15.506	0.71	0.66	0.63	0.70	0.79	0.55	0.15	0.27	0.26	0.38	0.19	0.23	1.15	0.57	0.90	1.23	1.17
30	1-Octene	17.048	0.12	0.19	0.12	0.07	0.16	0.11	0.22	0.22	0.25	0.21	0.15	0.15	-	-	-	-	-
31	Acetic acid, butyl ester	17.464	-	-	-	-	-	-	-	-	-	-	-	-	0.52	0.10	0.24	0.27	0.31
32	Octane	17.629	0.13	0.26	0.17	0.16	0.26	0.39	0.08	0.15	0.09	0.11	0.05	0.03	0.23	0.06	0.16	0.61	0.48
33	Ethylbenzene	20.385	-	-	-	-	-	-	0.04	0.05	0.04	0.05	0.02	-	2.28	0.87	1.95	1.83	2.31
34	p-Xylene	20.825	0.14	0.20	0.17	0.12	0.21	0.19	0.08	0.10	0.09	0.09	0.08	-	6.93	3.03	6.52	6.10	7.68
35	2-Heptanone	21.323	-	-	-	-	-	-	-	-	-	-	-	-	1.48	0.34	0.91	0.99	1.06
36	Styrene	21.984	0.24	0.19	0.13	0.20	0.19	0.17	0.43	0.42	0.46	0.36	0.36	0.23	0.97	0.67	1.08	0.87	0.99
37	o-Xylene	22.093	-	-	-	-	-	-	-	-	-	-	-	-	0.84	0.55	1.14	1.01	1.27
38	Nonane	22.967	-	-	-	-	-	-	-	-	-	-	-	-	0.20	0.01	0.06	0.24	0.23
39	Cyclopentane, (2-methylbutylidene)-	24.451	-	-	-	-	-	-	-	-	-	-	-	-	0.25	1.82	1.67	0.37	0.33
40	Propanoic acid, 3-ethoxy-, ethyl ester	26.417	-	-	-	-	-	-	-	-	-	-	-	-	1.76	0.33	1.10	0.83	1.12
41	Octane, 2,5,6-trimethyl-	26.667	-	-	-	-	-	-	-	-	-	-	-	-	0.48	0.02	0.11	0.12	0.20
42	Benzene, 1,2,3-trimethyl-	27.102	-	-	-	-	-	-	-	-	-	-	-	-	0.27	0.03	0.12	0.09	0.14
43	β-myrcene	27.933	0.07	0.33	0.17	0.09	0.13	0.78	-	-	-	-	-	-	0.51	0.05	0.21	0.88	0.81
44	Benzene, 1-ethyl-2-methyl-	28.073	-	-	-	-	-	-	-	-	-	-	-	-	0.51	0.19	0.48	0.44	0.65
45	Decane	28.951	-	-	-	-	-	-	-	-	-	-	-	-	0.23	0.06	0.14	0.19	0.30
46	Heptane, 2,2,6,6-tetramethyl-4-methylene-	29.167	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A.6 (Continued)

47	3-Heptene, 2,2,4,6,6-pentamethyl-	29.732	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	1-Hexanol, 2-ethyl-	29.853	-	-	-	-	-	-	-	-	-	-	-	-	0.38	0.04	0.09	4.62	5.10
49	Benzene, 1-methyl-4-(1-methylethyl)-	30.096	-	-	-	-	-	-	0.16	0.22	0.18	0.20	0.15	0.16	0.18	0.03	0.09	0.37	0.40
50	D-Limonene	30.733	0.13	0.24	0.16	0.14	0.17	0.18	0.38	0.38	0.37	0.33	0.32	0.31	13.13	0.43	0.78	1.05	1.34
51	2-Pentene, 2,4,4-trimethyl-	31.435	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
52	Hexadecane,2,6,10,14-tetramethyl-	31.688	0.14	0.18	0.15	0.12	0.18	0.08	0.18	0.22	0.18	0.18	0.23	0.19	-	-	-	-	-
53	Cyclohexane, 1-methyl-3-pentyl-	32.234	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
54	Dodecane	33.053	0.39	0.46	0.36	0.35	0.38	0.40	0.31	0.48	0.37	0.75	0.52	0.36	0.55	0.28	0.37	0.48	0.65
55	Undecane, 3,6-dimethyl-	33.537	0.13	0.18	0.13	0.13	0.15	0.12	0.17	0.20	0.16	0.17	0.23	0.17	-	-	-	-	-
56	Undecane, 2,8-dimethyl-	34.510	0.09	0.13	0.08	0.08	0.10	0.08	0.19	0.22	0.18	0.20	0.25	0.18	-	-	-	-	-
57	Nonanal	34.663	-	-	-	-	-	-	-	-	-	-	-	-	0.58	0.16	0.32	0.54	0.58
58	Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl-	34.710	-	-	-	-	-	-	0.30	0.30	0.22	0.28	0.10	0.10	-	-	-	-	-
59	1,6-Octadien-3-ol, 3,7-dimethyl-	34.761	-	-	-	-	-	-	-	-	-	-	-	-	0.36	0.11	0.21	0.26	0.42
60	Heptane, 1,1'-oxybis-	35.119	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
61	1-Pentanol, 2,2,4-trimethyl-	35.453	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62	Undecane	35.557	-	-	-	-	-	-	-	-	-	-	-	-	0.18	0.05	0.09	0.12	0.21
63	2,2-Dimethylheptane-3,5-dione, keto form	35.810	0.12	0.12	0.12	0.11	0.12	-	0.10	0.15	0.12	0.09	0.08	0.08	0.27	0.35	0.22	0.00	0.78
64	4-Undecene, 7-methyl-	36.271	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
65	3-Undecene, 6-methyl-, (E)-	36.544	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
66	Undecane, 2,6-dimethyl-	38.682	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A.6 (Contiued)

67	Decanal	39.186	0.19	0.42	0.36	0.22	0.27	3.88	0.13	0.21	0.15	0.27	0.02	0.12	4.64	3.98	0.90	1.14	1.23
68	Dodecane	39.545	0.06	0.07	0.06	0.07	0.07	0.08	0.05	0.06	0.05	0.11	0.04	0.06	-	-	-	-	-
69	1,3,7-Octatriene, 3,7-dimethyl-	40.716	-	-	-	-	-	-	-	-	-	-	-	-	0.33	0.17	0.07	0.05	0.06
70	2,4,4,6,6,8,8-Heptamethyl-1-nonene	41.647	0.13	0.11	0.10	0.11	0.17	0.13	-	-	-	-	-	-	0.39	0.24	-	0.20	0.26
71	Hexadecane	41.853	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
72	delta-selinene	41.949	-	-	-	-	-	-	-	-	-	-	-	-	0.38	0.11	-	0.10	0.14
73	Heptacosane	42.160	-	-	-	-	-	-	-	-	-	-	-	-	0.26	0.05	-	0.05	0.08
74	6,7-Dimethyl-1,2,3,5,8,8a-hexahydronaphthalene	42.380	0.10	0.22	0.13	0.21	0.22	0.29	0.28	0.32	0.22	0.71	0.35	0.40	0.77	0.07	-	0.24	0.11
75	Tetradecane, 2,5-dimethyl-	42.606	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
76	1,7-Dimethyl-4-(1-methylethyl)cyclodecane	42.675	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
77	Humulene	42.829	-	-	-	-	-	-	-	-	-	-	-	-	0.21	0.07	0.08	0.09	0.10
78	Bicyclogermacrene	43.031	-	-	-	-	-	-	-	-	-	-	-	-	0.34	0.03	0.03	0.17	0.15
79	2,2,4,4,5,5,7,7-Octamethyloctane	43.176	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
80	δ-Elemene	43.204	2.93	4.42	4.64	3.14	3.54	5.09	0.63	0.80	0.49	1.27	1.26	1.12	6.89	1.52	2.62	9.69	7.40
81	(Z)-3-hexadecene	43.313	-	-	-	-	-	-	0.17	0.19	0.13	0.00	0.22	0.27	-	-	-	-	-
82	trans-α-Bergamotene	43.379	0.64	0.71	1.28	0.77	0.83	0.87	0.23	0.25	0.16	0.88	0.39	0.41	1.05	0.23	0.54	1.13	0.88
83	α-Cubebene	43.489	1.95	3.70	2.56	2.20	2.70	3.80	0.64	0.85	0.54	0.91	1.02	0.92	4.33	0.99	1.80	11.02	8.95
84	3-Hexadecene, (Z)-	43.619	0.52	1.00	0.95	1.11	1.10	1.32	1.25	1.29	0.95	2.55	1.45	1.83	0.88	0.19	-	0.99	0.69
85	trans-7-Hexadecene	43.781	0.40	0.66	0.54	0.57	0.78	0.73	0.68	0.68	0.50	1.38	0.78	0.99	0.65	0.09	0.20	0.75	0.53
86	2,4,4,6,6,8,8-Heptamethyl-2-nonene	43.900	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A.6 (Continued)

87	Ylangene	43.988	4.63	7.11	7.33	5.64	6.26	6.28	0.64	0.74	-	-	1.25	1.23	8.97	3.04	-	10.23	10.29
88	(-)-Aristolene	44.106	83.34	114.68	114.15	101.13	101.02	120.01	7.54	8.26	6.32	9.96	12.61	11.97	134.92	65.08	82.56	217.12	194.38
89	β-Elemene	44.253	127.52	163.14	162.34	132.97	140.64	187.76	17.76	24.96	14.11	26.44	46.58	36.35	201.29	73.74	116.05	277.82	246.38
90	Isoledene	44.383	-	2.78	2.61	1.84	0.00	3.40	0.64	0.72	0.62	0.77	0.88	0.81	4.30	0.73	1.34	7.73	7.00
91	Hept-2-ene, 2,4,4,6-tetramethyl-	44.472	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
92	.beta.-Humulene	44.502	-	3.11	3.33	2.55	2.80	2.72	0.63	0.72	0.47	1.01	1.30	1.26	3.93	1.25	2.18	5.91	5.02
93	1-Hexadecene	44.547	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
94	β-Selinene	44.575	4.03	2.49	2.78	2.08	2.20	2.99	0.48	-	-	-	-	-	3.14	1.10	-	5.15	4.37
95	α-Farnesene	44.690	-	-	-	-	-	-	-	0.42	0.29	0.69	0.89	0.86	-	0.98	1.93	5.52	4.26
96	α-Gurjunene	44.778	29.98	52.67	45.82	38.65	42.76	51.35	1.18	1.73	0.93	1.80	2.98	2.58	54.74	17.41	23.12	83.91	72.42
97	β-Cubebene	44.871	4.43	7.43	7.40	5.73	5.76	8.07	0.48	0.56	0.41	0.55	0.86	0.81	9.09	2.42	-	14.34	11.85
98	Caryophyllene	44.952	23.06	18.91	19.15	15.40	28.88	18.63	1.35	1.64	1.03	2.01	3.84	3.09	20.73	6.70	10.35	32.11	27.52
99	γ-Gurjunene	45.029	-	8.47	15.37	9.99	-	16.13	-	-	-	-	-	-	7.56	2.45	1.75	30.24	12.55
100	1H-Cycloprop[e]azulene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.beta.,7b.alpha.)]-	45.060	-	5.39	-	-	-	-	1.07	1.23	0.88	1.18	1.69	1.48	8.27	1.79	-	-	11.13
101	Bicyclo(4.4.0)dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	45.110	-	3.08	-	2.63	-	3.63	-	-	-	-	-	-	3.45	1.21	-	7.44	5.55
102	Calarene	45.266	25.23	37.71	33.94	32.55	30.07	45.08	4.67	3.79	3.75	5.04	8.36	6.55	41.04	14.75	22.05	93.48	69.87
103	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	45.363	-	-	-	-	-	-	0.63	0.52	-	-	0.99	0.84	-	-	-	8.54	6.67
104	Valencene	45.511	12.57	16.96	15.20	18.08	14.81	21.31	3.73	2.50	2.15	3.56	7.80	5.78	15.16	7.28	10.54	62.35	38.43

Table A.6 (Continued)

105	α-Selinene	45.590	14.09	19.38	18.56	10.59	15.36	23.68	5.13	4.46	4.33	4.84	7.36	6.04	29.56	9.13	13.90	58.83	45.70
106	α-Farnesene	45.742	67.77	122.49	100.75	97.02	92.81	148.03	7.84	7.96	5.33	8.67	20.01	14.91	118.42	46.55	64.49	263.42	205.89
107	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1π4aπ8aπ-(π-	45.920	-	16.27	15.62	15.91	13.35	21.97	-	-	-	-	-	-	21.04	7.41	9.82	56.55	37.42
108	Spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11-methylene-, (-)-	45.973	-	-	-	-	-	-	24.96	16.04	16.08	20.17	36.69	28.58	-	-	-	-	-
109	Cubenene	46.030	47.36	44.96	38.91	52.38	37.64	62.21	4.34	3.17	2.96	4.41	9.26	6.12	59.25	43.23	52.91	190.07	125.26
110	γ-Cadinene	46.120	20.99	27.06	20.55	31.26	29.48	42.77	-	-	-	-	-	-	30.62	18.91	23.99	107.29	68.54
111	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.beta.)]-	46.181	5.17	9.22	8.73	7.89	-	10.86	0.75	0.61	-	-	1.48	1.16	10.13	3.85	5.51	24.87	18.39
112	γ-Muurolene	46.292	-	74.03	46.98	-	-	154.57	-	-	-	-	-	-	190.15	47.70	71.63	187.06	351.74
113	δ-Cadinene	46.329	91.67	57.60	59.82	113.56	88.87	0.00	28.78	22.35	16.83	26.32	56.22	41.26	-	63.60	76.71	210.86	-
114	β-Germacrene	46.421	15.06	14.25	12.75	14.04	16.45	17.65	2.70	2.39	2.55	-	5.10	4.01	17.82	8.80	11.91	48.04	34.84
115	β-Panasinsene	46.489	-	5.61	4.64	6.35	-	7.63	1.47	0.94	-	1.15	2.60	1.88	7.33	5.53	6.69	25.61	15.76
116	β-Cadinene	46.616	11.10	16.65	13.27	15.52	12.26	21.65	2.76	1.62	1.49	2.20	6.07	4.28	21.00	17.37	18.63	97.66	56.42
117	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl	46.711	1.84	1.49	1.10	1.59	-	2.17	-	-	-	-	-	-	2.95	1.45	1.69	6.79	4.44
118	Copaene	46.841	-	0.69	0.47	0.90	0.55	1.12	0.09	0.06	-	-	0.22	0.14	0.90	0.54	0.70	3.38	2.05
119	Cadala-1(10),3,8-triene	46.905	1.59	0.75	0.72	1.14	0.80	1.36	0.12	0.08	-	-	0.23	0.17	1.64	1.27	1.36	2.48	1.99
120	Isolongifolene, 4,5,9,10-dehydro-	46.955	-	-	-	-	-	-	0.16	0.17	0.23	0.34	0.20	0.15	0.91	-	-	2.84	0.95
121	πGuaiene	47.015	0.66	1.04	0.82	0.95	0.80	1.25	-	-	-	-	-	-	1.28	0.55	0.68	3.15	2.34
122	Cadina-1(10),6,8-triene	47.141	1.35	1.50	1.70	1.61	1.78	1.77	0.31	0.24	0.20	0.34	0.58	0.44	2.43	1.58	2.09	5.18	3.69

Table A.6 (Continued)

123	n-Nonadecanol-1	47.157	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
124	4aH-cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl-	47.234	0.48	0.63	0.54	0.49	-	0.89	0.15	0.19	0.18	0.31	0.41	0.22	2.02	0.88	0.83	3.71	2.30
125	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,7.beta.,8a.alpha.)]-	47.360	0.41	0.72	0.66	0.37	0.52	0.49	-	-	-	-	-	-	0.76	0.54	0.34	2.43	1.05
126	4,4,5,6-Tetramethyltetrahydro-1,3-oxazin-2-thione	47.475	0.64	0.72	0.63	0.68	0.49	0.99	0.22	0.13	0.12	-	0.32	0.23	2.27	1.08	1.83	5.50	3.05
127	9-Methyl-S-octahydrophenanathracene	47.598	1.47	1.57	1.29	1.79	1.06	2.31	0.46	0.26	0.31	0.59	0.75	0.59	3.37	3.11	2.61	10.60	5.18
128	Cedrene	47.716	-	-	-	-	-	-	0.46	0.28	0.27	0.38	0.69	0.44	5.60	2.92	2.34	11.80	6.38
129	πVatirenene	47.741	1.25	1.39	1.12	1.38	1.15	2.01	-	-	-	-	-	-	-	-	-	-	-
130	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, [1aR-(1a.alpha.,4a.beta.,7.alpha.,7a.beta.,7b.alpha.)]-	47.863	0.00	0.26	0.19	0.23	0.00	0.43	-	-	-	-	-	-	1.65	0.86	0.65	3.47	1.88
131	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]-	47.993	0.58	0.71	0.47	0.37	0.45	0.68	0.43	0.24	0.22	0.32	0.63	0.43	4.13	2.21	1.44	9.03	5.07
132	.alpha.-Cadinol	48.145	0.62	0.44	0.30	0.50	0.44	0.87	0.26	0.15	0.18	0.23	0.34	0.25	3.29	1.96	1.22	7.78	4.27
133	Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	48.345	3.26	2.39	2.52	3.21	1.63	3.32	0.60	0.43	0.51	0.71	1.06	0.82	6.37	6.71	5.43	6.40	5.26

Table A.7 MVOCs profiles of *A.flavus* 5-38 grown on MEA and CMA medium (Continued from Table A.6)

No.	Compound name	R.T. (min)	MEA						CMA					
			REP1	REP2	REP3	REP4	REP5	REP6	REP1	REP2	REP3	REP4	REP5	REP6
1	Ethanol	4.595	0.30	0.27	0.19	0.48	0.94	0.85	3.19	2.62	2.21	0.96	1.16	1.17
2	Acetone	5.022	3.59	4.47	1.82	1.28	0.91	3.90	0.49	0.54	0.61	0.66	1.16	0.82
3	Butane, 2-methyl-	5.036	-	-	-	-	-	-	-	-	-	-	-	-
4	Isopropyl Alcohol	5.149	1.00	1.59	0.47	0.40	0.42	0.82	0.45	0.55	0.67	0.57	0.54	0.64
5	Furan	5.296	-	-	-	-	-	-	-	-	-	-	-	-
6	Pentane	5.462	-	-	-	-	-	-	-	-	-	-	-	-
7	1,4-Pentadiene	5.547	1.12	1.68	2.65	1.65	1.61	3.95	0.09	0.11	0.13	0.12	0.15	0.17
8	Acetic acid, methyl ester	5.751	0.45	0.61	0.32	0.25	0.21	0.73	-	-	-	-	-	-
9	Propanal, 2-methyl-	6.231	-	-	-	-	-	-	-	-	-	-	-	-
10	1-Propanol	6.486	-	-	-	-	-	-	-	-	-	-	-	-
11	2,3-Butanedione	7.180	-	-	-	-	-	-	-	-	-	-	-	-
12	Butanal	7.357	0.03	0.45	0.08	0.13	0.10	0.02	-	-	-	-	-	-
13	2-Butanone	7.559	0.15	0.64	0.12	0.11	0.09	0.08	-	-	-	-	-	-
14	Furan, 3-methyl-	8.173	-	-	-	-	-	-	-	-	-	-	-	-
15	Propanoic acid, 2-methyl-, anhydride	8.313	0.07	0.29	0.10	0.07	0.08	0.22	-	-	-	-	-	-
16	3-Buten-2-ol, 2-methyl-	8.311	-	-	-	-	-	-	0.05	0.03	0.04	0.03	0.06	0.06
17	Ethyl Acetate	8.409	-	-	-	-	-	-	-	-	-	-	-	-
18	Furan, 2-methyl-	8.567	0.95	1.35	1.62	1.49	2.07	2.85	0.11	0.08	0.11	0.09	0.14	0.17
19	1-Propanol, 2-methyl-	9.064	4.57	3.94	1.91	4.41	5.56	5.26	-	-	-	-	-	-
20	Furan, tetrahydro-	9.709	0.00	0.08	0.47	0.07	0.23	0.15	0.17	0.09	0.12	0.08	0.19	0.14
21	Benzene	10.888	0.04	0.07	0.43	0.46	0.26	0.04	0.06	0.03	0.03	0.02	0.04	0.04
22	1-Butanol	10.885	-	-	-	-	-	-	-	-	-	-	-	-
23	Furan, 2,5-dimethyl-	12.846	-	-	-	-	-	-	-	-	-	-	-	-
24	Heptane	12.973	-	-	-	-	-	-	-	-	-	-	-	-
25	3-Buten-1-ol, 3-methyl-	13.626	0.10	0.21	0.30	0.42	0.57	0.12	-	-	-	-	-	-
26	1-Butanol, 3-methyl-	13.782	0.42	0.17	0.30	0.49	0.89	0.60	-	-	-	-	-	-
27	1-butanol, 2-methyl	13.951	0.92	0.42	0.64	1.01	1.39	1.08	-	-	-	-	-	-
28	Methyl Isobutyl Ketone	14.464	0.07	0.02	0.12	0.07	0.05	0.04	-	-	-	-	-	-
29	Toluene	15.506	0.19	0.27	0.25	0.28	0.22	0.26	0.11	0.08	0.06	0.08	0.10	0.09
30	1-Octene	17.048	0.05	0.05	0.22	0.23	0.17	0.12	-	-	-	-	-	-
31	Acetic acid, butyl ester	17.464	-	-	-	-	-	-	-	-	-	-	-	-
32	Octane	17.629	-	-	-	-	-	-	-	-	-	-	-	-
33	Ethylbenzene	20.385	-	-	-	-	-	-	-	-	-	-	-	-
34	p-Xylene	20.825	-	-	-	-	-	-	-	-	-	-	-	-
35	2-Heptanone	21.323	-	-	-	-	-	-	-	-	-	-	-	-
36	Styrene	21.984	0.38	0.52	0.46	0.54	0.36	0.27	0.36	0.29	0.08	0.31	0.23	0.15
37	o-Xylene	22.093	-	-	-	-	-	-	-	-	-	-	-	-
38	Nonane	22.967	-	-	-	-	-	-	-	-	-	-	-	-

Table A.7 (Continued)

39	Cyclopentane, (2-methylbutylidene)-	24.451	-	-	-	-	-	-	-	-	-	-	-	-
40	Propanoic acid, 3-ethoxy-, ethyl ester	26.417	-	-	-	-	-	-	-	-	-	-	-	-
41	Octane, 2,5,6-trimethyl-	26.667	-	-	-	-	-	-	0.03	0.01	0.01	-	0.02	0.02
42	Benzene, 1,2,3-trimethyl-	27.102	-	-	-	-	-	-	-	-	-	-	-	-
43	β-myrcene	27.933	-	-	-	-	-	-	-	-	-	-	-	-
44	Benzene, 1-ethyl-2-methyl-	28.073	-	-	-	-	-	-	-	-	-	-	-	-
45	Decane	28.951	-	-	-	-	-	-	0.03	0.02	0.01	0.01	0.01	0.01
46	Heptane, 2,2,6,6-tetramethyl-4-methylene-	29.167	0.21	0.13	0.12	0.21	0.06	1.23	0.03	0.02	-	0.02	0.03	0.02
47	3-Heptene, 2,2,4,6,6-pentamethyl-	29.732	0.45	0.29	0.25	0.48	0.15	2.17	0.04	0.03	0.02	0.05	0.06	0.04
48	1-Hexanol, 2-ethyl-	29.853	-	-	-	-	-	-	-	-	-	-	-	-
49	Benzene, 1-methyl-4-(1-methylethyl)-	30.096	-	-	-	-	-	-	-	-	-	-	-	-
50	D-Limonene	30.733	0.03	0.02	0.02	0.04	0.03	0.08	0.12	0.08	0.06	0.07	0.08	0.07
51	2-Pentene, 2,4,4-trimethyl-	31.435	0.59	0.39	0.33	0.54	0.18	2.33	0.07	0.05	0.03	0.09	0.11	0.07
52	Hexadecane,2,6,10,14-tetramethyl-	31.688	-	-	-	-	-	-	0.18	0.15	0.12	0.13	0.14	0.13
53	Cyclohexane, 1-methyl-3-pentyl-	32.234	0.23	0.12	0.09	0.22	0.08	0.98	0.03	0.02	0.01	0.03	0.04	0.03
54	Dodecane	33.053	0.89	0.52	0.44	0.78	0.23	2.94	0.32	0.27	0.21	0.37	0.36	0.33
55	Undecane, 3,6-dimethyl-	33.537	-	-	-	-	-	-	0.18	0.15	0.11	0.12	0.12	0.12
56	Undecane, 2,8-dimethyl-	34.510	-	-	-	-	-	-	0.21	0.17	0.11	0.14	0.12	0.13
57	Nonanal	34.663	-	-	-	-	-	-	-	-	-	-	-	-
58	Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl-	34.710	-	-	-	-	-	-	-	-	-	-	-	-
59	1,6-Octadien-3-ol, 3,7-dimethyl-	34.761	-	-	-	-	-	-	-	-	-	-	-	-
60	Heptane, 1,1'-oxybis-	35.119	0.35	0.11	0.12	0.29	0.06	0.91	0.11	0.10	0.06	0.12	0.11	0.08
61	1-Pentanol, 2,2,4-trimethyl-	35.453	0.17	0.04	0.04	0.12	0.04	0.50	0.03	0.02	-	0.04	0.03	0.02
62	Undecane	35.557	-	-	-	-	-	-	-	-	-	-	-	-
63	2,2-Dimethylheptane-3,5-dione, keto form	35.810	0.13	0.02	0.02	0.09	0.02	0.35	0.06	0.06	0.06	0.05	0.04	0.06
64	4-Undecene, 7-methyl-	36.271	0.12	0.26	0.01	0.08	0.01	0.28	0.02	0.01	-	0.03	0.02	0.01
65	3-Undecene, 6-methyl-, (E)-	36.544	-	-	-	-	-	-	0.04	0.03	0.02	0.05	0.03	0.02
66	Undecane, 2,6-dimethyl-	38.682	0.16	0.07	0.06	0.12	0.08	0.33	0.06	0.05	0.03	0.16	0.10	0.06
67	Decanal	39.186	0.16	0.03	0.05	0.14	0.05	0.24	0.09	0.14	0.08	0.07	0.05	0.08
68	Dodecane	39.545	-	-	-	-	-	-	0.06	0.06	0.03	0.09	0.05	0.04
69	1,3,7-Octatriene, 3,7-dimethyl-	40.716	0.23	0.07	0.05	0.12	0.11	0.24	-	-	-	-	-	-
70	2,4,4,6,6,8,8-Heptamethyl-1-nonene	41.647	-	-	-	0.07	0.07	0.15	0.04	0.04	0.02	0.13	0.06	0.05
71	Hexadecane	41.853	0.22	0.08	0.08	0.16	0.14	0.15	0.03	0.04	0.03	0.08	0.04	0.05
72	delta-selinene	41.949	0.30	0.10	0.12	0.23	0.17	0.31	0.03	0.03	0.01	0.07	0.03	0.03
73	Heptacosane	42.160	0.64	0.34	0.35	0.56	0.41	0.92	0.09	0.09	0.04	0.19	0.09	0.08
74	6,7-Dimethyl-1,2,3,5,8,8a-hexahydronaphthalene	42.380	0.13	0.09	0.04	0.10	0.14	0.16	0.17	0.18	0.08	0.41	0.22	0.18
75	Tetradecane, 2,5-dimethyl-	42.606	0.15	0.00	0.10	0.04	0.09	0.17	0.03	0.08	0.02	0.09	0.04	0.07
76	1,7-Dimethyl-4-(1-methylethyl)cyclodecane	42.675	-	-	-	-	-	-	0.03	0.03	0.01	0.11	0.05	0.04
77	Humulene	42.829	-	-	-	-	-	-	-	-	-	-	-	-

Table A.7 (Continued)

78	Bicyclogermacrene	43.031	0.51	0.46	0.35	0.49	0.37	0.69	0.03	0.04	0.02	0.13	0.07	0.06
79	2,2,4,4,5,5,7,7-Octamethyloctane	43.176	-	-	-	-	-	-	0.13	0.18	0.07	0.34	0.17	0.16
80	δ-Elemene	43.204	0.40	0.52	0.23	0.37	0.27	0.52	-	-	-	-	-	-
81	(Z)-3-hexadecene	43.313	0.40	0.00	0.24	0.32	0.28	0.45	0.13	0.16	0.06	0.28	0.13	0.13
82	trans-α-Bergamotene	43.379	0.14	0.13	0.07	0.18	0.09	0.22	0.12	0.15	0.06	0.32	0.16	0.15
83	α-Cubebene	43.489	2.11	1.56	1.63	1.96	1.37	2.64	-	-	-	-	-	-
84	3-Hexadecene, (Z)-	43.619	1.03	0.71	0.79	0.97	0.69	1.31	0.64	0.78	0.34	1.38	0.71	0.69
85	trans-7-Hexadecene	43.781	0.17	0.09	0.06	0.14	0.20	0.21	0.34	0.36	0.17	0.68	0.36	0.34
86	2,4,4,6,6,8,8-Heptamethyl-2-nonene	43.900	0.19	-	0.08	0.06	-	0.42	0.06	0.07	0.03	0.19	0.09	0.10
87	Ylangene	43.988	0.40	0.78	0.20	0.36	0.35	0.25	-	-	-	-	-	-
88	(-)-Aristolene	44.106	1.85	1.59	1.16	2.02	0.94	1.68	0.28	0.34	0.15	0.39	0.22	0.25
89	β-Elemene	44.253	0.18	0.13	0.07	0.18	0.13	0.23	0.39	0.51	0.21	0.83	0.39	0.45
90	Isoledene	44.383	-	-	0.04	-	-	0.13	0.07	0.06	0.03	0.09	0.05	0.05
91	Hept-2-ene, 2,4,4,6-tetramethyl-	44.472	-	-	-	-	-	-	0.03	0.04	0.01	0.07	0.03	0.03
92	.beta.-Humulene	44.502	0.10	0.10	0.05	0.08	0.06	0.10	-	-	-	-	-	-
93	1-Hexadecene	44.547	0.22	0.13	0.10	0.18	0.17	0.22	0.04	0.06	0.02	0.07	0.03	0.04
94	β-Selinene	44.575	-	-	-	-	-	-	-	-	-	-	-	-
95	α-Farnesene	44.690	-	-	-	-	-	-	0.07	0.09	0.03	0.15	0.07	0.07
96	α-Gurjunene	44.778	0.20	0.35	0.10	0.15	0.15	0.15	-	-	-	-	-	-
97	β-Cubebene	44.871	-	-	-	-	-	-	0.06	0.08	0.02	0.11	0.03	0.04
98	Caryophyllene	44.952	-	-	-	-	-	-	-	-	-	-	-	-
99	γ-Gurjunene	45.029	0.17	0.21	0.10	0.21	0.12	0.19	-	-	-	-	-	-
100	Bicyclo(4.4.0)dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	45.110	0.18	0.18	0.10	0.18	0.16	0.20	0.04	0.04	0.01	0.06	0.03	0.03
101	Calarene	45.266	-	-	-	-	-	-	0.02	0.05	0.02	0.09	0.03	0.04
102	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	45.363	-	-	-	-	-	-	-	-	-	-	-	-
103	Valencene	45.511	0.50	0.81	0.45	0.67	0.35	0.36	0.02	0.02	0.01	0.03	0.01	0.02
104	α-Selinene	45.590	0.24	0.60	-	0.14	-	0.22	-	-	-	-	-	-
105	α-Farnesene	45.742	-	-	-	-	-	-	0.05	0.04	0.00	0.04	0.02	0.03
106	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1π4aπ8aπ-(π-	45.920	-	-	-	-	-	-	-	-	-	-	-	-
107	Spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11-methylene-, (-)-	45.973	0.43	0.38	0.21	0.36	0.30	0.53	-	-	-	-	-	-
108	Cubenene	46.030	-	-	-	-	-	-	0.05	0.06	0.03	0.07	0.03	0.04
109	γ-Cadinene	46.120	0.08	0.06	0.04	0.06	0.06	0.09	-	-	-	-	-	-
110	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.beta.)]-	46.181	-	-	-	-	-	-	0.02	0.01	-	0.02	0.01	0.01
111	γ-Muurolene	46.292	2.00	1.43	0.96	1.19	1.12	1.59	-	-	-	-	-	-

Table A.7 (Continued)

112	δ-Cadinene	46.329	-	-	-	-	-	-	0.05	0.06	0.04	0.09	0.06	0.08
113	β-Germacrene	46.421	0.15	0.13	0.07	0.17	0.16	0.23	-	-	-	-	-	-
114	β-Panasinsene	46.489	0.22	0.18	0.19	0.28	0.19	0.24	-	-	-	-	-	-
115	β-Cadinene	46.616	0.12	0.09	0.05	-	0.13	0.08	-	-	-	-	-	-
116	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl	46.711	-	-	-	-	-	-	-	-	-	-	-	-
117	Copaene	46.841	-	-	-	-	-	-	-	-	-	-	-	-
118	Cadala-1(10),3,8-triene	46.905	-	-	-	-	-	-	-	-	-	-	-	-
119	Isolongifolene, 4,5,9,10-dehydro-	46.955	-	-	-	-	-	-	-	-	-	-	-	-
120	πGuaiene	47.015	-	-	-	-	-	-	-	-	-	-	-	-
121	Cadina-1(10),6,8-triene	47.141	0.10	0.17	0.08	0.06	0.27	0.05	-	-	-	-	-	-
122	n-Nonadecanol-1	47.157	-	-	-	-	-	-	-	-	-	-	-	-
123	4aH-cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl-	47.234	-	-	-	-	-	-	-	-	-	-	-	-
124	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,7.beta.,8a.alpha.)]-	47.360	0.17	0.68	0.56	0.20	0.24	0.14	-	-	-	-	-	-
125	4,4,5,6-Tetramethyltetrahydro-1,3-oxazin-2-thione	47.475	-	-	-	-	-	-	-	-	-	-	-	-
126	9-Methyl-S-octahydrophenanathracene	47.598	0.11	0.09	0.08	0.09	0.14	0.12	0.05	0.18	0.07	0.04	0.09	0.07
127	Cedrene	47.716	-	-	-	-	-	-	-	-	-	-	-	-
128	πVatirenene	47.741	0.12	0.04	0.15	0.07	0.24	0.09	-	-	-	-	-	-
129	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, [1aR-(1a.alpha.,4a.beta.,7.alpha.,7a.beta.,7b.alpha.)]-	47.863	-	-	-	-	-	-	-	-	-	-	-	-
130	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]-	47.993	0.07	0.11	0.17	0.05	0.15	0.10	-	-	-	-	-	-
131	.alpha.-Cadinol	48.145	-	-	-	-	-	-	0.02	0.02	0.00	0.08	0.01	0.01
132	Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	48.345	-	-	-	-	-	-	0.17	0.09	0.07	0.15	0.15	0.20

APPENDIX B

DATA ANALYSIS PROTOCOLS

B.1 Properties of MVOC data

MVOC data analysis can play a critical role in the discrimination and classification of fungal isolates. Some factors that should be considered before performing multivariate analysis:

1. Differences in orders of magnitude between measured MVOC concentrations are common, however, MVOCs present in high concentrations are not necessarily more important than those present at low concentration.

2. Some MVOCs show large fluctuations in concentration under identical experimental conditions. This is due to uninduced biological variation which can be attributed to phenotype variation. Thus not all data fluctuations can be attributed to random or systematic error.

3. MVOC data from fungus tends to be heteroscedastic, thus the assumption that the total uninduced variation resulting from biology sampling, and analytical measurements is symmetric around zero with equal standard deviation is not true. Deviations in data from heteroscedasticity can be reduced with data pretreatment.

4. Errors in experimental data also originates from the sampling, sample work-up and instrument errors.

B.2 Data pretreatment methods

In this section, data pretreatment methods used in this dissertation are demonstrated with reasons for each treatment discussed.

1. After analysis of a MVOC sample using GC-MS, the gas chromatogram was corrected using the background subtraction options in the Agilent GC-MS data analysis software. The difference between treated chromatograms and untreated chromatogram is

shown in Figure B.1. The MVOC data report was generated in Excel format by simply selecting the customized data analyzing methods using Agilent GC-MS data analysis software. Some important data information included retention time, peak area, peak area % and compound name as shown in Figure B.2.

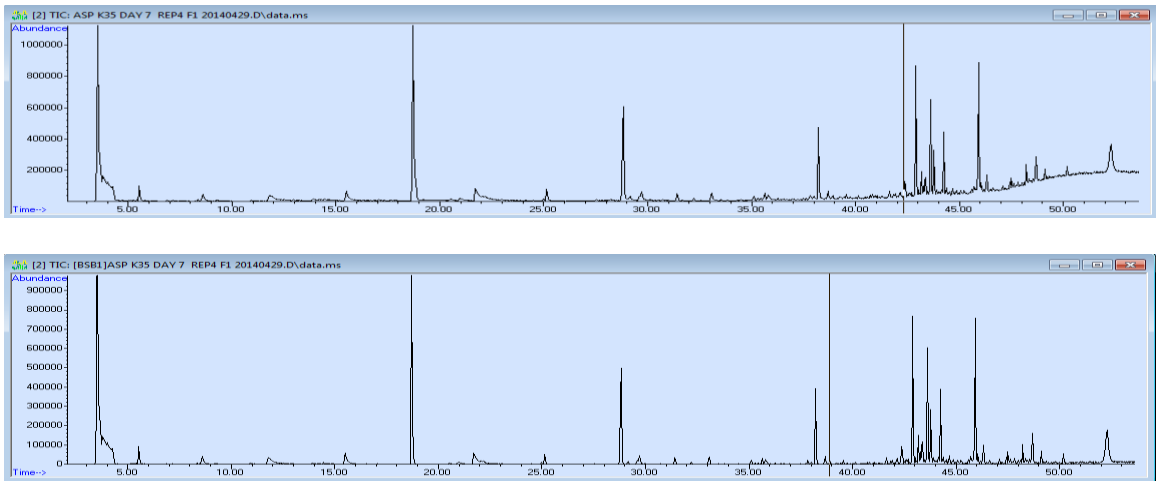

Figure B.1 TIC chromatogram comparison with (lower) and without (upper) background subtraction.

R.T.	Height	Area	Pct Max	Pct Total	Library/ID	Ref	CAS
3.534	1912525	1.01E+08	90.56	7.985	Carbon dioxide	80	000124-38-9
3.905	256663	16135701	14.53	1.281	Carbon dioxide	80	000124-38-9
3.942	254433	5610407	5.05	0.445	Carbon dioxide	80	000124-38-9
4.015	261128	25278674	22.76	2.007	Carbon dioxide	81	000124-38-9
4.157	226598	9829246	8.85	0.78	Carbon dioxide	81	000124-38-9
4.234	220994	15830033	14.25	1.257	Carbon dioxide	81	000124-38-9
4.438	3654	128477	0.12	0.01	Sulfur dioxide	357	007446-09-5
4.506	2928	145484	0.13	0.012	Sulfur dioxide	356	007446-09-5
4.637	9822	462314	0.42	0.037	Ethanol	95	000064-17-5
4.989	697152	22846972	20.57	1.814	Acetone	215	000067-64-1
5.159	51695	3522592	3.17	0.28	2-Propanone, 1-methoxy-	2037	005878-19-3
5.431	19350	665705	0.6	0.053	Pentane	705	000109-66-0
5.534	50092	2161555	1.95	0.172	1,3-Butadiene, 2-methyl-	457	000078-79-5
5.808	7082	466412	0.42	0.037	Oxirane, 2,3-dimethyl-	695	003266-23-7
5.91	4288	214479	0.19	0.017	Allene	48	000463-49-0
6.237	4499	364227	0.33	0.029	Cyclopropane, ethenylmethylen	1095	019995-92-7
6.37	2992	223350	0.2	0.018	Cyclopropane, ethenylmethylen	1095	019995-92-7
6.497	2286	119015	0.11	0.009	Cyclopropene, 3-methyl-3-vinyl-	1093	071153-30-5
6.847	14866	812825	0.73	0.065	Silanol, trimethyl-	2234	001066-40-6
7.165	11611	503467	0.45	0.04	Aziridine, 2,2-dimethyl-	617	002658-24-4
7.343	4315	217278	0.2	0.017	Furan, 2,5-dihydro-	542	001708-29-8

Figure B.2 Excel documents of MVOC data analyzed by Agilent GC-MS data analysis software.

R.T. = retention time, Height= peak height, Area= peak area, Pct Total= peak area percentage, Library/ID= compound name, Ref= reference number in NIST library and CAS= CAS number of the compound.

2. The retention time, peak area and compound name information were kept; then replication data, was saved in the same excel file for peak alignment (Figure B.3).

	5-3B REP1			5-3B REP2			5-3B REP3	
R.T.	Area	Compound Name	R.T.	Area	Compound Name	R.T.	Area	Compound Name
3.549	91536644	Carbon dioxide	3.552	86307643	Carbon dioxide	3.594	78402596	Carbon dioxide
4.662	249649	Ethanol	4.682	229304	Ethanol	4.69	2265282	Ethanol
5.041	3904280	Acetone	5.048	5401230	Acetone	5.102	3538122	Acetone
5.451	235041	Pentane	5.444	2645975	Pentane	5.493	2793856	Pentane
5.552	12304939	1,4-Pentadiene	5.556	14490612	1,4-Pentadiene	5.608	11149171	1,4-Pentadiene
5.825	921579	Acetic acid, hydrazide	5.822	1548557	Acetic acid, hydrazide	5.881	844174	Acetic acid, hydrazide
8.589	7190265	Furan, 2-methyl-	8.596	8149872	Furan, 2-methyl-	8.659	6467737	Furan, 2-methyl-
9.103	6234900	1-Propanol, 2-methyl-	9.112	5609604	1-Propanol, 2-methyl-	9.168	5535444	1-Propanol, 2-methyl-
10.923	803141	Benzene	10.92	578119	Benzene			
						13.826	4041776	1-Butanol, 3-methyl-
13.995	894104	1-Butanol, 2-methyl-	13.989	2381736	1-Butanol, 2-methyl-	13.988	5746267	1-Butanol, 2-methyl-, (.+,
14.449	1321941	2-Pentanone, 3-methyl-	14.469	584355	2-Pentanone, 3-methyl-	14.491	842422	2-Pentanone, 3-methyl-
15.534	1436034	Toluene	15.538	1661203	Toluene	15.559	1561764	Toluene
15.779	1714718	Butanoic acid, 2-methyl-, methyl	15.787	1604736	Butanoic acid, 2-methyl-, methyl ester			
17.049	3154480	1-Octene	17.045	5485998	1-Octene	17.073	3325908	1-Octene
			17.642	523896	Octane			
18.911	3159214	3-Hexanone, 4-methyl-	18.938	1248613	3-Hexanone, 4-methyl-	18.946	1910274	3-Hexanone, 4-methyl-
20.485	579202	p-Xylene	20.49	758703	p-Xylene	20.512	770185	p-Xylene
20.928	1883495	o-xylene	20.92	2402517	o-xylene	20.937	2615912	o-xylene

Figure B.3 Sample subset of the replication data of 5-3B in excel for peak alignment. For each replication data including R.T.(retention time), Area (peak area) and compound name.

3. Retention time differences were observed to vary around a mean. This can be attributed to instrument and personal error which requires accurate correction for proper MVOC analysis. Compounds with low repeatability, appearing in less than 40% of the replicates, were deleted from further consideration. The remaining MVOC peaks were matched in a row with identical compounds from replicate analysis with close retention times. The revised data for one particular isolate is shown in Figure B.4 where retention times and peak area are matched from 12 replicates. It should be mentioned that, compound names were not yet confirmed by this step.

								5-3B isolate					
Library/ID	Min	Max	REP1	REP2	REP3	REP4	REP5	REP6	REP7	REP8	REP9	REP10	REP11	REP12
Carbon dioxide	3.488	3.594	91536644	86307643	78402596	91446064	1.22E+08	1.09E+08	85159623	1.01E+08	93060154	84227133	1.18E+08	1.13E+08
Ethanol	4.59	4.69	249649	229304	2265282	442103	605603	550262	284789	471540	433286	266416	20447260	1198472
Acetone	4.986	5.102	3904280	5401230	3538122	5357861	3648276	11859910	5151292	6286711	4584994	3799382	7408808	10636522
Pentane	5.39	5.493	235041	2645975	2793856	2323812	2367890	3409850	202074	291569	319096	260131	275025	795373
1,4-Pentadiene	5.494	5.608	12304939	14490612	11149171	13920185	50033228	8392044	4872821	21774410	21784631	15136892	21625104	22231541
Acetic acid, hydrazide	5.805	5.881	921579	1548557	844174	2515084	3301204	4099436		1375936	373319		986076	1969208
Furan, 2-methyl-	8.522	8.659	7190265	8149872	6467737	8015972	15208750	7054550	9486332	13687642	13725443	8642705	15238392	15984241
1-Propanol, 2-methyl-	9.03	9.168	6234900	5609604	5535444	6826254	4526147	14109575	3570640	3553826	5188143	4081001	11038654	10632644
Benzene	10.857	10.939	803141	578119		561419	433497	763446	801836	1021136	959267	999215	855765	1776896
1-Butanol, 3-methyl-	13.749	13.826			4041776								15265216	1887341
1-Butanol, 2-methyl-	13.925	14.001	894104	2381736	5746267	1041969	1618162	2615724	1444202	963719	1232740	1847085	12008579	3422984
2-Pentanone, 3-methyl-	14.419	14.491	1321941	584355	842422	529974	339648	1734114	1378089	153999	768408	555167	325881	614845
Toluene	15.486	15.559	1436034	1661203	1561764	1036301	1301915	1678236	2195376	2092284	2153919	2393394	2094618	2940636
Butanoic acid, 2-methyl-,	15.779	15.795	1714718	1604736		1257661	773535	3603117						
Hexanal	16.532	16.547							924347	1226391	1580768	265996	728863	2919961
1-Octene	17.013	17.073	3154480	5485998	3325908	2890555	6378835	3171861	5253612	6591648	6279902	5700048	6311482	5361909
Octane	17.613	17.646		523896			517993	329167		302121	202722	1710867	228715	1184669
3-Hexanone, 4-methyl-	18.879	18.949	3159214	1248613	1910274	1239846	1121903	2711059	2837108	1402794	1817438	2148123	824594	1887342

Figure B.4 A subset of the revised MVOC data of isolate 5-3B with retention time range

(Min= minimum retention time and Max= maximum retention time) and peak area from each replicate.

4. Data from different isolates and controls were combined in one excel file where mean and standard deviation of retention time were calculated (Figure B.5). The file also contains the peak areas for each MVOC for each replicate.

	R.T.	R.T	R.T.	R.T				Blank										3357			
Library/ID	Min	Max	Mean	STD	REP1	REP2	REP3	REP4	REP5	REP6	REP7	REP8	REP9	REP10	REP1	REP2	REP3	REP4	REP5	REP6	REP7
Ethanol	4.58	4.636													108281	4E+06	390971	271698	515837	532153	501865
Acetone	4.963	5.055	5.007		2E+07	1E+07	9E+06	2E+07	3E+07	9E+06	3E+07	2E+07	1E+07	1E+07							
Pentane	5.438	5.466	5.441												334412	248272	188356	205695	509821	545346	334412
1,4-Pentadiene	5.501	5.574	5.548	0.030											908287	712708	335986	941568	789696	926107	792719
Propanal, 2-methyl-	6.334	6.389	6.370	0.015	1E+06	1E+06	1E+05	1E+06	1E+06	2E+05	2E+06	1E+06	5E+05	6E+05							
2,3-Butanedione	7.128	7.195	7.177	0.019	2E+06	1E+06	8E+05	1E+06	2E+06	1E+06	2E+06	2E+06	9E+05	2E+06							
Butanal	7.286	7.354	7.328	0.018	1E+07	7E+06	4E+06	1E+07	1E+07	4E+06	2E+07	2E+07	4E+06	6E+06							
Propanal, 2-methyl-	7.504	7.628	7.518	0.030	1E+07	2E+06	9E+05	1E+07	1E+07	2E+06	2E+07	2E+07	5E+06	3E+06							
Hexane	8.349	8.387	8.375833	0.009379																	
Furan, 2-methyl-	8.535	8.606	8.587	0.014											1E+07	1E+07	9E+06	2E+07	1E+07	1E+07	1E+07
1-Propanol, 2-methyl-	9.018	9.11	9.093	0.014											2E+06	5E+06	3E+06	3E+06	4E+06	7E+06	2E+06
Butanal, 3-methyl-	10.046	10.087	10.069	0.013	3E+07	2E+07	1E+07	2E+07	3E+07	2E+07	4E+07	3E+07	2E+07	3E+07							
Butanal, 2-methyl-	10.546	10.584	10.567	0.011	9E+06	9E+06	3E+06	1E+07	9E+06	2E+06	1E+07	9E+06	7E+06	7E+06							
1-Butanol	10.697	10.737	10.720	0.013	8E+08	6E+08	5E+08	7E+08	9E+08	4E+08	8E+08	7E+08	4E+08	5E+08							
Benzene	10.871	10.932	10.906	0.017											522729	789003	580022	732037	648188	567248	431195
Heptane	12.935	12.958	12.94617	0.006753																	
1-Butanol, 2-methyl-	13.926	13.989	13.97767	0.010254												124445		1E+06	2E+06	1E+06	1E+06
2-Pentanone, 3-methyl-	14.436	14.488	14.419	0.007											2E+06	2E+06	4E+06	1E+06	4E+06	4E+06	991711
Propanoic acid, 2-methyl-	14.567	14.729	14.6142	0.051581	3E+06	1E+06	4E+06	1E+06	2E+06	3E+06	7E+06	6E+06	3E+06	5E+06							
Toluene	15.482	15.555	15.482	0.011	2E+06	3E+06	2E+06	2E+06	2E+06	9E+06	8E+06	8E+06	7E+06	7E+06	2E+06	2E+06	1E+06	2E+06	2E+06	1E+06	3E+06

Figure B.5 Subset of the combined data from different isolates and control.

Data columns include compound name (Library ID), minimum retention time, maximum retention time, mean and standard deviation of retention time. 10 replicates of blank and 7 replicates of 3557 are only shown in this figure.

5. The MVOCs were grouped into 12 chemical classes based on their functional group (Figure B.6).

no.	Library/ID	R.T.	RSD	RI exp	RI lit
	alcohols				
1	Ethanol	4.652	0.034	451	448
2	1-Propanol, 2-methyl-	9.093	0.014	613	607
3	1-Butanol	10.720	0.013	649	654
4	1-Butanol, 2-methyl-	13.978	0.010	720	729
5	1-Octen-3-ol	26.700	0.028	962	962
6	4-propylresorcinol	28.153	0.004	986	
	aldehydes				
7	Butanal	7.328	0.018	566	570
8	Propanal, 2-methyl-	7.518	0.030	541	544
9	Butanal, 3-methyl-	10.069	0.013	634	632
10	Butanal, 2-methyl-	10.567	0.011	645	639
11	Benzaldehyde	24.970	0.016	933	925
12	Undecanal	41.971	0.009	1293	1286
	alkane				
13	Pentane	5.442	0.027	500	500
14	Hexane	8.376	0.009	600	600

Figure B.6 A subset of the grouped MVOC data format with experimental and literature RI value.

6. The retention index of each compound was calculated based on equation 1 using alkane standards. The literature retention index of each compound was obtained from a NIST webbook library. The compound names were confirmed using mass spectrum library search and RI value matchup.

$$I = 100 \times \left[n + (N - n)\left(\frac{t_{r(unkown)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}}\right)\right] \quad \text{(Equation B.1)}$$

I = Kovats retention index,

n = the number of carbon atoms in the smaller n-alkane,

N = the number of carbon atoms in the larger n-alkane,

t_r = the retention time.

7. The 78 identified compounds which have a low relative standard deviation (RSD < 60%) were selected as input for statistical software. The MVOC data was converted to the proper input format for SIMCA P+ software. The row input is the samples from different isolates and column input is peak area percentage values for different compounds as shown in Figure B.7.

A	B	C	D	E	F	G	H	I	J	K
ID		1	2	3	4	5	6	7	8	9
Chemical classes	Class	ethanol	2-methyl-1-propanol	1-butanol	2-methyl-1-butanol	1-Octen-3-ol	4-propylresorcinol	butanal	2-methyl-propanal	3-methyl-butanal
Blank1	Control	0	0	81.14437645	0	0	0	1.066620813	1.29446212	2.837885873
Blank2	Control	0	0	80.77328209	0	0	0	1.035453302	0.289275057	3.416385165
Blank3	Control	0	0	78.12786487	0	0	0	0.765048415	0.154011074	2.084196048
Blank4	Control	0	0	79.32837288	0	0	0	1.192207665	1.263876825	2.693460077
Blank5	Control	0	0	84.4224411	0	0	0	1.337119398	0.918391131	2.454330815
Blank6	Control	0	0	70.73400642	0	0	0	0.644617536	0.336491721	2.656006224
Blank7	Control	0	0	76.88295498	0	0	0	1.751561126	1.872188268	3.639361091
Blank8	Control	0	0	75.65825743	0	0	0	1.898474121	2.075952734	3.668975426
Blank9	Control	0	0	67.41451921	0	0	0	0.684642062	0.896237312	3.630799707
Blank10	Control	0	0	73.29361484	0	0	0	0.878195448	0.473722907	3.883862201
A3357A1	3357	0.028114814	0.457940089	0		0	2.258087148	0	0	0
A3357A2	3357	2.214835969	2.660791879	0	0.068837822	0	4.145265444	0	0	0
A3357A3	3357	0.121401948	0.879268366	0		0	3.244722613	0	0	0
A3357A4	3357	0.15528507	1.979130503	0	0.580472667	0	1.516691932	0	0	0
A3357A5	3357	0.197348828	1.684561321	0	0.714400103	0	3.886452436	0	0	0
A3357A6	3357	0.188223708	2.450689639	0	0.477012745	0	3.776513837	0	0	0
A3357A7	3357	0.305636184	1.464540945	0	0.87647029	0	0.887045589	0	0	0
A3357A8	3357	0.36655615	5.395700583	0	1.166084806	0	1.084748772	0	0	0
A3357A9	3357	0.28795031	4.024705639	0	1.034987186	0	0.909788139	0	0	0
A3357A10	3357	0.361097526	3.953864083	0	0.995525738	0	1.58083517	0	0	0
A3357A11	3357	0.182477911	1.051742779	0	0.506308632	0	2.632219429	0	0	0

Figure B.7 Subset of the input data (peak area percentage) format for different MVOCs from 6 isolates and a control.

APPENDIX C

SUPPLEMENTARY MATERIALS FOR CHAPTER IV

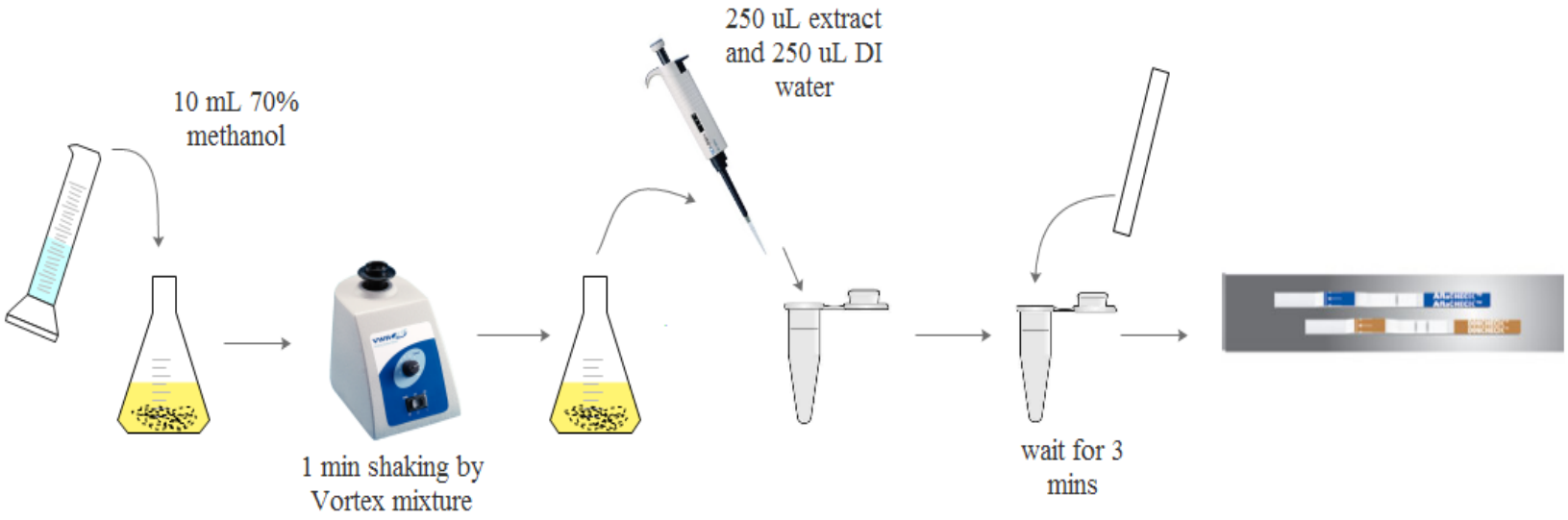

Figure C.1 Aflatoxins confirmation procedure using AflaCheck test kits (a summary of manufacturer instructions