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Multivariate Analysis of Microbial Volatile Organic Compounds for Aflatoxigenic *A. flavus* Detection

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***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 contamination. MVOCs profiles of different *A. flavus* isolates have been identified using a headspace solid phase microextraction gas chromatography mass spectrometry (HS-SPME-GCMS) technique. Multivariate 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 HS-SPME-GCMS combined with PLS-DA is a powerful method for fungal contamination identification and potential biomarkers discovery.**

***Keywords* – Aspergillus Flavus, Microbial Volatile Organic Compound, Multivariate Analysis, Partial Least Square Discriminant Analysis.**

I. 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.[1] These aflatoxins are considered to be among the world's most potent naturally occurring carcinogens and a powerful toxin.[2] *A. flavus* has the ability to survive on many organic nutrient sources including corn, cotton, stored grains, dead insects, and plant debris.[3] 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.[4] Extensive efforts have been made worldwide to detect and quantify aflatoxins,[5-9] to develop control strategies for aflatoxigenic *A. flavus*,[10-13] and to study the biosynthetic mechanism of aflatoxins.[14-15]

Many techniques including high-performance liquid chromatography mass spectrometry (HPLC-MS) [16-17], enzyme linked immune-sorbent assay (ELISA) [18-19], and multiplex polymerase chain reactions (multiplex PCR) [20] 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.[21-25] 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).[26] 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*.[22]

Substantial efforts have been exerted on the development of electronic nose technology for fast detection or identification of fungal contamination on food [27-30] or in an indoor environment [31-32]. 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,[33] temperature,[34] humidity,[35] and media.[36]

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.[37] When coupled with GC-MS analysis, it has been shown to provide accurate results, producing calibration curves with good fits over relevant concentrations.[38] This strategy has been well established for collecting MVOCs from species of *Aspergillus*,[39] *Penicillium*,[40] and *Fusarium*.[41] 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),[28, 42-43] linear discriminant analysis (LDA),[44-45] partial least squares projections to latent structures (PLS) analysis,[46] and CA[22, 26] 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 strains of *A. flavus* grown on malt extract agar (MEA) media, 2) discriminate aflatoxigenic and non-aflatoxigenic strains by performing multivariate analysis on MVOCs data, 3) identify volatile biomarkers specific to the group of aflatoxigenic strains.

II. Material and Methods

2.1. 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). Tween 20 solution was purchased from Thermo Fisher Scientific (Pittsburgh, PA).

2.2. Fungal species

The *A. flavus* strains used in this study are listed in Table 1. The aflatoxigenic strain NRRL 3357 and a non-aflatoxigenic strain 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 strain 5-38 was isolated from pig feed in Maben, MS. Aflatoxigenic strain K73 and non-aflatoxigenic strains K35 and K32 were collected from corn sampled in Sunflower County, MS.

Table 1. *A. flavus* strains used in the study.

Aflatoxigenic strains	Non-aflatoxigenic strains
NRRL 3357	NRRL 21882
5-38	K35
K73	K32

2.3 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 minutes. All the fungal isolates were cultured in a Petri dish (Fisher Scientific Inc.) containing MEA 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, 5-38 and K73) *A. flavus* strains, three non-aflatoxigenic (NRRL 21882, K32, K35) *A. flavus* strains 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 Fig. 1.

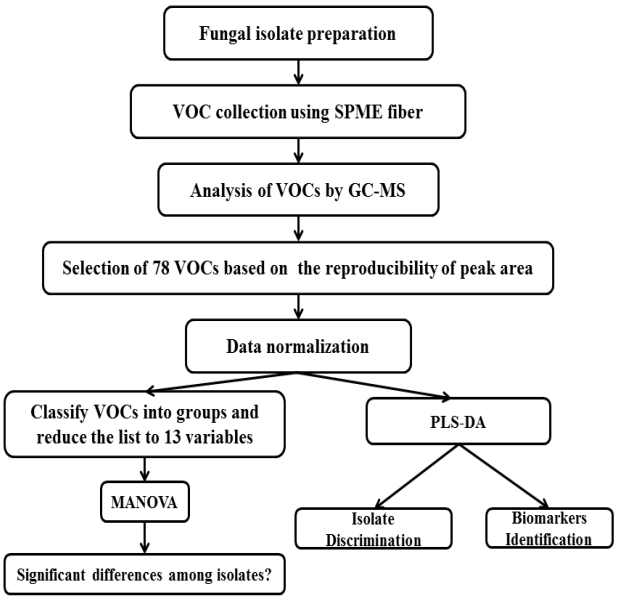


Fig.1. Summary of the experiment and data analysis procedures

2.4 Sampling of MVOCs

MVOCs were collected using an 85 µm carboxen/ divinylbenzene/polydimethylsiloxane (CAR/DVB/PDMS) SPME fiber which has excellent VOCs absorption characteristics [47], 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.

2.5 Aflatoxin production confirmation

Aflatoxin production was confirmed by VICAM Aflacheck which has a 10 ppb limit of detection. 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 minutes. A positive result could be confirmed if no test line appeared after 5 minutes. Three replications of the Aflacheck 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 strains (K73, NRRL 3357 and ASP 5-38) tested positive and each of the non-aflatoxigenic strains (NRRL 21882, K32, K35) tested negative for aflatoxins production on all 18 tests.

2.6 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.

2.7 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.[48] Calculated RIs were then compared with those stored in a NIST database.[49] 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.

2.8 Chemometric multivariate analysis

Peak alignment and data pretreatment procedures are detailed in supporting information description. 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.[50] This data was then used for classifying and discriminating fungal strains.

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 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 were performed using the software program SIMCA-P+ 11.0 (Umetrics, Umea, Sweden). The peak area data of MVOCs for different strains and control was directly loaded in the software. The data was 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.

III. Results and Discussion

3.1 MVOC 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.[51] For example, Lebrun et al. [52] used mango fruit volatiles as maturity markers to determine the optimal harvest maturity for the mango fruit. The 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* strains 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 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 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. [53-55] It should be noted that 12 replications of each sample (same incubation time and condition) were divided over two days of testing.

Table 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
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

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
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 percentage and standard deviation of 12 replications of each isolate.

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.[56] Branched chain alcohols are associated with catabolism of the branched chain amino acids (leucine, isoleucine, and valine) and lipids.[57]

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. [57] 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 [58].

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* [59] and α-farnesene (**67**) was also found in *Aspergillus fumigatus*.[58] From *Aspergillus terreus*, the sesquiterpene γ-cadinene (**70**) has been reported.[60] 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**).[61]

As already mentioned, the production of MVOCs depends on the species, the substrate, and environmental conditions.[62] 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 [63], infection [64], and herbivore attack of the growth media [65].

Roze et al. [57] 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. For example, Zeringue et al. [54] 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 sesquiterpenes, 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.[66] MVOC profiles variations among strains of *A. flavus* have been investigated in this study in order to identify volatile biomarkers associated with aflatoxin production. Result shows consistent quantitative MVOCs variations of volatiles among the strains, however, all biological species produce MVOCs. These non-fungus MVOCs will increase the complexity of any MVOC analysis from samples collected in the field. The combination of MVOCs quantification followed by multivariate analysis described here for laboratory samples could one day be a powerful protocol for the field identification of aflatoxigenic fungal strains, however, representative field MVOCs must be collected and analyzed in order to provide discrimination models.

3.2 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 strains and the control. Eighty-four samples from the control and strains were divided into seven groups (control, NRRL 3357, K73, 5-38, 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 Fig. 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 strain level, however, our primary goal is discrimination of aflatoxigenic from non-aflatoxigenic grouping of strains.

An LSD and Duncan test were performed to evaluate the effect of each strain 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% (5-38) of the TIC. The amount of sesquiterpenes collected from the 5-38 strain is significantly higher than that of other strains ($P<0.001$); in contrast, sesquiterpenes emissions from strains of K73 and K35 are not significantly different ($P=0.334$). Strains 3357 and NRRL 21882 also have similar sesquiterpene production ($P=0.148$). We observed that strain 21882 released much higher percentage (60%) ketones than that of other strains ($P<0.001$) 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 strains. A much larger amount of esters were extracted from strain 3357 compared to other strains and the control. Alcohols dominate the control (MEA agar) primarily because of high 1-butanol concentration.

3.3 Partial least squares discriminant analysis (PLS-DA) for aflatoxigenic and non-aflatoxigenic A. flavus and the identification of key biomarkers

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* strains. With this method, the data is 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 was pretreated using log transformation and mean centering methods.

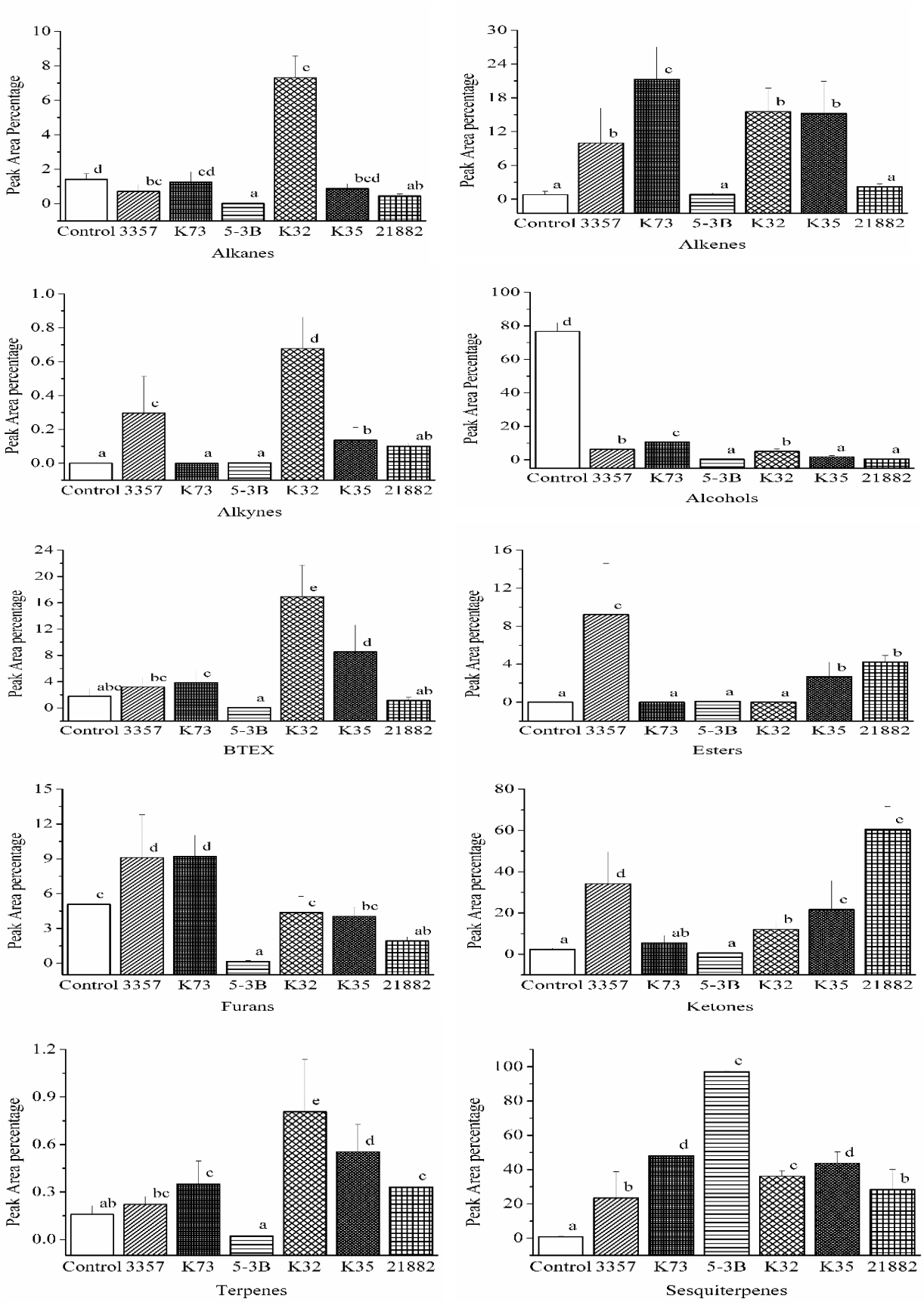
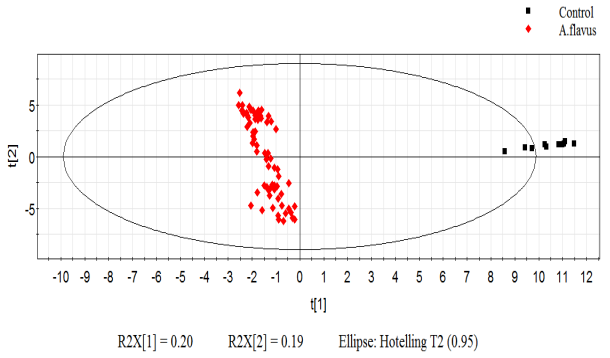
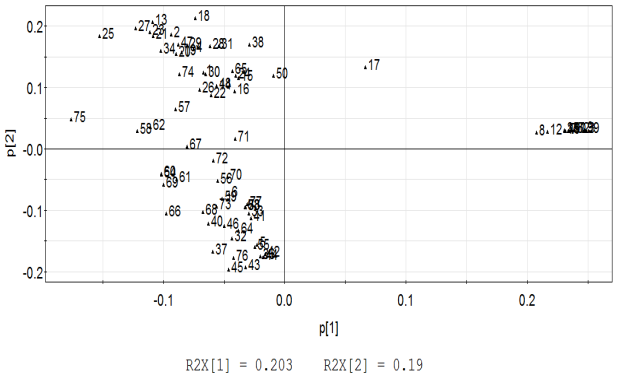


Fig.2. VOC patterns of *A. flavus* isolates. Mean (+standard deviation; n=12) peak area percentage of chemical classes from six *A. flavus* isolates and control measured over two collections(5-h sampling time). The letters over each bar present the significant differences at $P<0.05$ among the isolates and control emissions (LSD and Duncan test). Different letters (a, b, c, d) show the significant differences.

The 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. Fig. 3a is the score plots of PLS-DA where fungal strains and control are easily separated. Specific compounds, only produced by the control or *A. flavus* can be found using the loading plot (Fig. 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 strain.



(a)



(b)

Fig.3. 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 2.

Using the loading plot as a guide, the potential biomarkers can be assigned a variables importance in the projection (VIP) value. High ranking VIPs (Fig. 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.

The next step is to discriminate the aflatoxigenic from the non-aflatoxigenic strains 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 Fig. 5a. The six strains are grouped into 5 clearly defined clusters – 2 clusters the aflatoxigenic and 3 clusters for non-aflatoxigenic: the clusters can be grouped by aflatoxigenic strains (black cubes) and non-aflatoxigenic strains (red circles). The toxigenic strain 5-3B is located on the positive region of t[1] and the negative region of t[2] because of a high sesquiterpene content. Strain 5-3B is still easily differentiated from the samples of non-toxigenic strains.

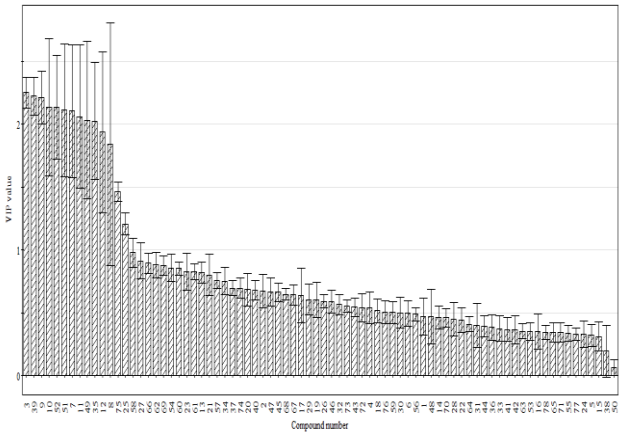
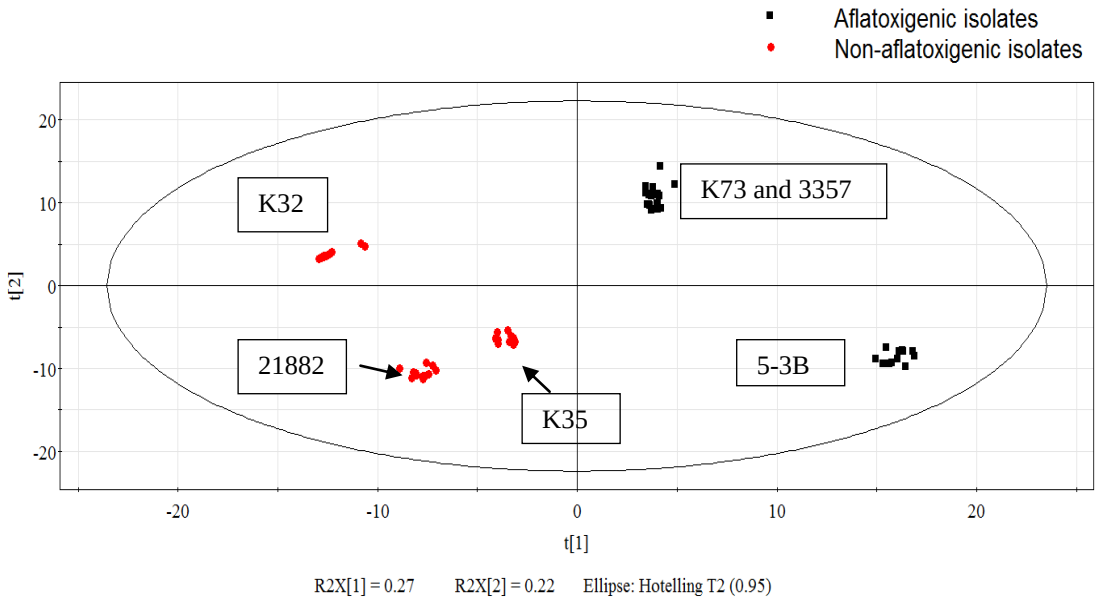


Fig.4. Variables importance in the projection (VIPs) for discriminating *A. flavus* from the control (media only). The compound number used in the plot represents the volatile metabolites number listed in Table 2. The error bars are the standard deviations of VIP values (12 replicates).

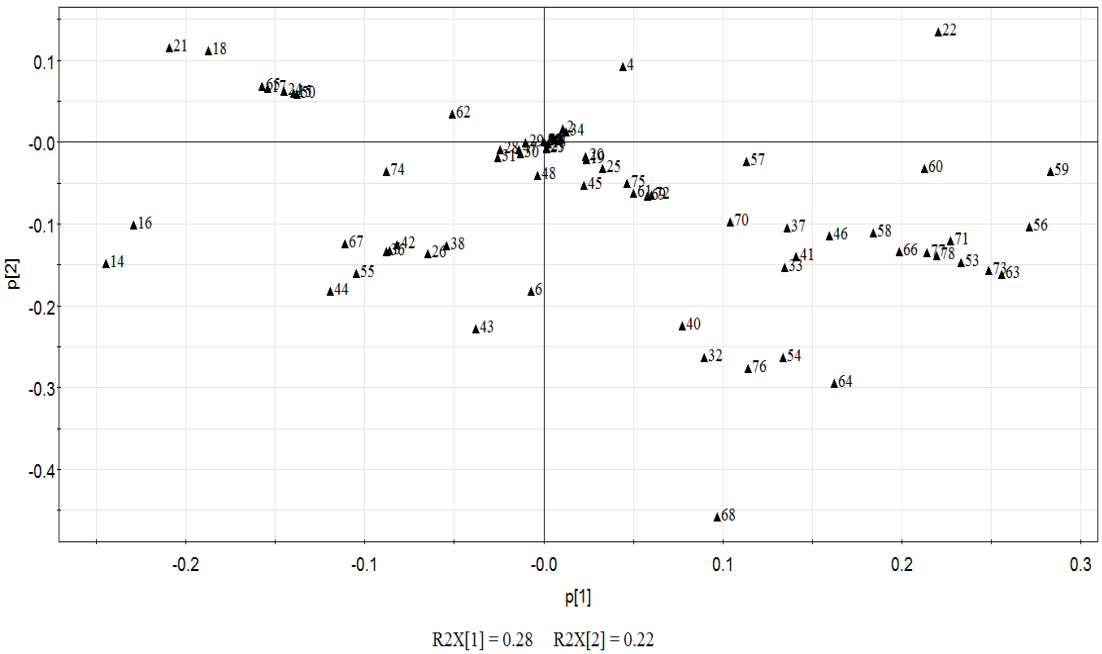
The loading plot (Fig. 5b) of the PLS-DA model enables visualization of the specific MVOCs that contribute the most to the discrimination (farthest from the center) of the toxigenic and non-toxigenic strains. Volatile metabolites presented in one category with a VIP value above 1 were selected for the student T test to examine the significant of difference between toxigenic and non-toxigenic strain samples (Table 3).

Among the potential biomarkers listed in Table 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 strains 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*

[67]. α-Cadinene was also emitted by *Resinicium bicolor* which is a plant pathogen infecting trees named "Oregon pine".[68] γ-Muurolene (**71**) has been identified as a component of essential oil from *Melaleuca* species of Australian shrubs and trees [69] and damiana plants.[70] It has also been reported as an important MVOC produced by the fungus *Aspergillus ustus* [35] and the bacteria *E. coli*.[71]



(a)



(b)

Fig.5. 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) strains of *A. flavus*. The number in loading plot represents the volatile metabolites number listed in Table 2.

Table 3: Possible volatile biomarkers for discrimination of toxigenic and non-toxigenic *A. flavus* isolates

No.	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.

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).

IV. 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 strain level. The variation of volatile composition in chemical classes among the *A. flavus* strains 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 strains. We believe that the methods described here will be very helpful for further investigation of biomarkers related to aflatoxin biosynthesis and *A. flavus* strain 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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was born in Ann Arbor Michigan, USA on March 7th, 1963. He graduated with a B.S. in chemistry from Albion College in Albion, Michigan in 1985, and a Ph.D. in chemistry from the University of Texas at Austin, in 1991. His graduate work focused on synthetic organic fluorine chemistry, as did his postdoctoral work at Clemson University.

From 1994 to 1998 he worked at the Naval Research Laboratory (NRL), Washington DC, on the development of absorbent polymer-film-based chemical sensors. In February 2003, Dr. Mlsna co-founded Seacoast Science. He joined the chemistry faculty at Mississippi State University in 2009. His work has been presented at scientific conferences and published in journals and books on numerous occasions.

While at Seacoast Dr. Mlsna was awarded several grants from government agencies for the development of chemical microsensor systems. In 2008 Seacoast was nominated for an SBIR achievement award and in 2007 won a Most Innovative Product (MIP) award for chemical sensor products related to security applications. Dr. Mlsna has published 65 papers, 5 book chapters and has 8 patents. He received the Mississippi State University department's Outstanding Faculty Award in 2012 (voted by students).

Richard E. Baird, Ph.D.

was born in Pittsburgh Pennsylvania, USA on March 2, 1956. He graduated with a B.S. in Forestry Management and Forest Pathology/Mycology from West Virginia University in 1978 and 1980, respectively. His interest in fungal physiology and taxonomy caused him to pursue and complete a Ph.D. in Botany/Mycology at University of Tennessee.

He developed a diverse background of knowledge working with many agricultural crop and forest ecosystem fungi. He assisted farmers with field crop problems, diagnostic support, and development of control recommendations for over 10 years working in Indiana with Purdue University and then with the University of Georgia. He joined the Entomology and Plant Pathology faculty at Mississippi State University in 1999. His mycological efforts continued with research to control agricultural plant pathogens and the role of fungi in forest ecosystems. These studies involved molecular and traditional approaches for understanding genetic variability of important plant pathogens such as *Aspergillus flavus* and *Macrophomina phaseolina*, monographic investigations of macrofungi such as stipitate hydnums, and development of novel taxonomic tools for determining microscopic fungal taxa. Over 150 research papers, extension bulletins, book chapters or books have been published by Dr. Baird.