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Biomedically important pathogenic fungi detection with volatile biomarkers

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Abstract

Volatile chemical profiles collected from the headspace of *Aspergillus fumigatus* (a pathogenic fungus that causes invasive pulmonary aspergillosis, allergic bronchopulmonary aspergillosis and chronic fungal sinusitis) grown on media with the connective tissue protein elastin, found in lung tissue, contained a large abundance of the sesquiterpene farnesene (3,7,11-trimethyl-1,3,6,10-dodecatetraene) and, depending on extraction time and sorbent material, other farnesene isomers and sesquiterpenes such as bisabolene (methyl-4-(6-methylhepta-1,5-dien-2-yl)cyclohex-1-ene). When human lung cells were cultured externally and infected with *A. fumigatus*, farnesene was also detected in each model lung system. Volatiles measured from cultured nasal lavage collected from a patient diagnosed with chronic fungal sinusitis, a condition frequently caused by *A. fumigatus*, revealed the presence of another pathogenic fungus, less frequently responsible for sinusitis, *Epicoccum nigrum*. The volatile profile of *E. nigrum* differed markedly from that of *A. fumigatus* with no sesquiterpenes detected.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

1.1. Diseases caused by *Aspergillus* and current diagnostic measures

Aspergillosis is a group of infectious diseases caused by members of the fungal genus *Aspergillus* and includes *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger* and *A. terreus*. Aspergillosis commonly leads to allergic bronchopulmonary aspergillosis, pulmonary aspergilloma and invasive aspergillosis. These and similar fungal pathogens are also associated with fungal sinusitis, a nagging, pervasive condition that is difficult to successfully treat. *A. fumigatus* is the most invasive and life threatening, especially for immunocompromized patients [1] including those who received bone marrow and transplants, patients with HIV, cystic fibrosis, chronic obstructive pulmonary disease, chronic granulomatous disease, severe asthma with fungal sensitivity, and many others [2].

Typically, an infected individual experiences problematic respiratory symptoms that lead to physician consultation and examination. Physicians usually rely on costly lung x-ray examinations and biopsies to provide proof of infection; however, successful treatment of the advanced infection stage that is responsible for noticeable symptoms is rare. The mortality rate of patients diagnosed as *A. fumigatus* positive following x-ray and biopsy is as high as 50–100% [3]. A method for early disease detection is needed and this may be an ideal candidate for a diagnostic procedure based on breath metabolomics.

1.2. Fungal volatiles as a means for disease detection

Headspace extraction with sorbent materials followed by GCMS analysis has proven to be a successful method for the identification of volatile compounds produced by microorganisms including fungi. Typically these sorbent materials bind volatiles via adsorption or absorption in a static, dynamic or hybrid system.

³ The paper is written in memory of G K Podila.

1.2.1. Solid phase microextraction. An extraction device that utilizes a polydimethylsiloxane (PDMS)-coated fiber, often with one or more additional sorbent materials [4], solid phase microextraction (SPME), has been utilized by several researchers to investigate the volatile compounds generated by fungi. Preti *et al* [5] demonstrated that volatile components could be directly extracted from infected sinus mucus headspace with SPME. Fiedler *et al* [6] grew 12 different fungi species, 4 of which were different strains of *Aspergillus fumigatus*, on malt extract agar and chemically extracted with SPME and analyzed with GCMS. They identified many volatile components but the one of greatest abundance was *trans* β -farnesene, a sesquiterpene that is discussed in detail in section 3 of this paper. They also found that 2-ethylhexanol is present in abundance. Further, they found that the volatile profiles for different strains of *A. fumigatus* were very similar but the volatile signature differed substantially from other *Aspergillus* species.

Sybre *et al* [7] utilized SPME to sample headspace volatiles from Tedlar bags filled with lung air collected from patients with cystic fibrosis with and without *A. fumigatus* infection. They reported that the volatile chemical 2-pentylfuran was present in four out of four patients infected with *A. fumigatus*, three of seven patients with cystic fibrosis but no microbiological evidence of *A. fumigatus* infection, and zero of ten healthy patients. Chambers *et al* [8] conducted a follow-on *in vivo* study where breath samples were again collected in Tedlar bags from *A. fumigatus* infected individuals, and they also concluded that 2-pentylfuran was an indicator of *A. fumigatus* infection.

1.2.2. Stir bar sorptive extraction. Developed by Baltussen *et al* [9] and similar to SPME, stir bar sorptive extraction (SBSE) utilizes a PDMS coating on top of a glass layer that surrounds an inner, magnetized, metallic bar. An advantage of SBSE is that the mass of PDMS is greater than that with SPME; therefore, more volatiles are extracted improving detection limits for chemicals that have a high affinity for PDMS sorbent, such as terpenes and sesquiterpenes. A disadvantage of SBSE is that it does not utilize co-sorbents. SBSE usefulness has been proved to be diverse and is capable of extracting volatiles and semi-volatiles from aqueous media and solid media. A few of the many studies include the utilization of SBSE for extraction of aqueous media in chemical ecology and biological media, terpenoids in beer, wine, and semi-volatiles from water [10–12]. It was also successfully utilized to extract volatile components from saliva [13].

1.2.3. PDMS foam. Another means for making use of the unique properties of PDMS in volatile extraction is with a PDMS foam utilized as packing material inside thermal desorption tubes. A foam exhibits greater surface area but generally reduced thickness (compared to SBSE or SPME). Therefore, components with a greater affinity for adsorption onto PDMS surface may be extracted with greater effectiveness with foam.

1.2.4. Tenax. The sorbent chemical 2,6-diphenylene-oxide polymer, or Tenax, packed in thermal desorption tubes is another commonly utilized sorbent material in headspace extraction due to good thermal stability (upper limit is 350 °C) and hydrophobic properties. It has a propensity for extracting non-polar components and a lower affinity for polar compounds [14]. *Penicillium paneum*, a species of *Penicillium roqueforti* well known for their contributions in blue cheese flavor, was inoculated on yeast extract sucrose agar and volatile metabolites were collected by diffusive sampling onto sorbent tubes containing Tenax TA [15], and then thermally desorbed and analyzed by GCMS. Several sesquiterpenes were identified including α -bisabolene.

1.2.5. Activated carbon. Like Tenax, activated carbon is commonly used in headspace extraction and binds components by adsorption and by Van der Waals forces. Thermal desorption tubes packed with activated carbon can be exposed to higher thermal conditions (versus Tenax or PDMS) but may bind more components irreversibly.

1.3. Objectives

We sought to utilize different extraction methods and materials and further define volatile biomarkers produced by *A. fumigatus*. *In vitro* work would utilize growth media with and without the connective tissue protein elastin found in the lungs [16, 17]. Additionally, we would develop a model to simulate *in vivo* lung conditions. The *ex vivo* method would utilize actual infected and healthy lung cells to provide additional evidence of volatile compounds that are indicative of *A. fumigatus* infection. A final objective was to utilize previously identified *A. fumigatus* biomarkers and to determine if *A. fumigatus* infection was detected in nasal lavage collected from a patient diagnosed with chronic fungal sinusitis.

2. Experimental details

2.1. In vitro testing

A. fumigatus is considered a pathogen and experimentation was conducted with level 2 pathogen certification. Three strains of *A. fumigatus* were utilized (FGSCA1141; FGSCA1142 and FGSCA1163). Each strain was maintained on potato dextrose agar (Difco media) and cultured on Sabouraud's dextrose agar (SDA: 4% dextrose, 1% peptone, 2% agar, pH 5.8). One cm agar plugs from 5 day old cultures with relatively uniform growth were used to inoculate SDA plates and the plates incubated for further 5 days before extracting volatile compounds.

Baseline data were obtained by collecting the volatiles with SBSE (method described below) from SDA plates. Four types of media were prepared. Elastin protein (0.1%) was added to one media to test if this connective tissue protein, commonly found in the lungs, leads to novel volatiles. This step was incorporated to mimic growth conditions in the lungs. Another set of plates was infused with bovine serum albumin

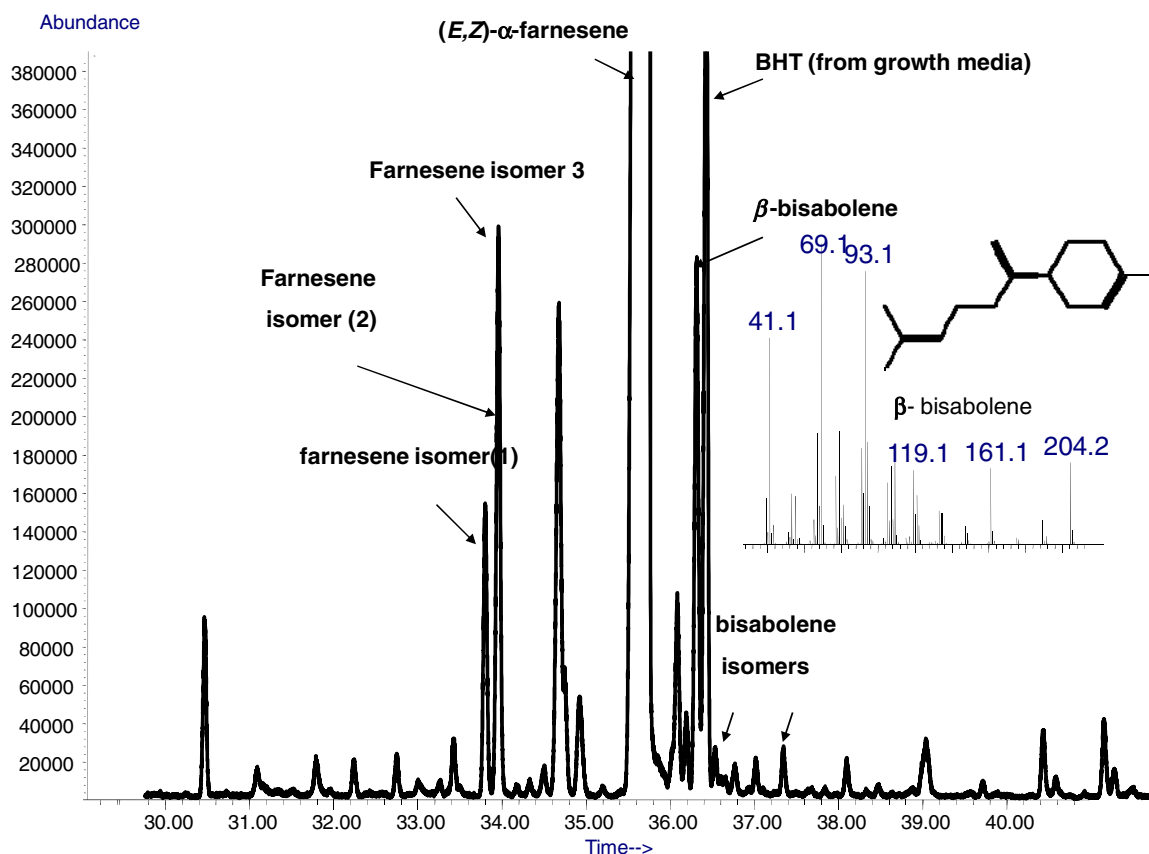


Figure 1. Results from 60 min headspace SBSE of *A. fumigatus* strain FGSCA1141 grown on cultured plates. Note the four farnesene and three bisabolene components detected.

(BSA; 0.1%), a protein commonly utilized in many biological testing applications to serve as a control. Plates that had not been inoculated and contained elastin or BSA were used as negative controls.

2.1.1. Growth plate preparation. Three replicate plates were prepared for each strain of *A. fumigatus* tested on media containing elastin. All plates were inoculated with equal fungal mass at the same time to facilitate maturity congruence. BSA plates were prepared in a similar manner. Un-inoculated plates (elastin and BSA) served as controls.

Plates were inoculated on day 1 and then incubated at 30 °C for 5 days. A cellulose filter was placed over the fungal culture to prevent dispersal of spores. On day 5, when visible growth was apparent, a round, flat, magnet disk, 3 cm diameter, 0.5 cm in thickness, was placed on top of each Petri dish lid. One SBSE device was placed on the underside of the lid and was held in place by the exterior mounted magnet (SBSE contain inner magnetized core). The cellulose filter prevented spores from coming in contact with the SBSE.

2.1.2. Stir bar sorptive extraction. Headspace volatiles were extracted by exposure to SBSE devices (Gerstel, Mülheim an der Ruhr Germany). Three plates were prepared for each strain of *A. fumigatus* tested on media containing elastin (plate 1 was extracted for 3 h, plate 2 for 6 h and plate 3 for 8 h;

9 plates total). BSA plates were extracted one time for 8 h. Blanks (media before inoculation) for elastin and BSA were also extracted.

2.1.3. Gas chromatography—mass spectrometry analysis. An Agilent GCMS, 5975MSD equipped with Gerstel components was utilized. Desorption liners (cylindrical glass tubes) containing SBSE device (one device per tube) were placed in a MPS2 storage tray. The MPS2 autosampler transferred liners from the storage tray to the thermal desorption unit (TDU) where tubes were heated to 300 °C for 4 min operated in splitless mode with purge time 1.00 min. Thermally desorbed volatiles passed from the TDU at 300 °C and were trapped in a cooled injection system (CIS) glass liner cryo-cooled with liquid N₂ to −80 °C (CIS4 liner in split mode 30:1). The liner contained glass beads for increased adsorption. The liner was heated to 300 °C for 3 min holding time and volatiles were transferred to the analytical DB-5 MACH column with dimensions 30 m × 0.25 mm I.D. × 0.25 μm film thickness. Column heating conditions employed two ramp rates: 5 min at 35 °C holding time and then 4 °C min^{−1} to 220 °C for 0 min holding time and 7 °C min^{−1} to 300 °C for 1 min holding time. The total ion chromatogram time was 63.68 min. The pressure ramped with initial pressure 46.9 kPa for 5 min then 1.6 kPa min^{−1} to 120.0 kPa for

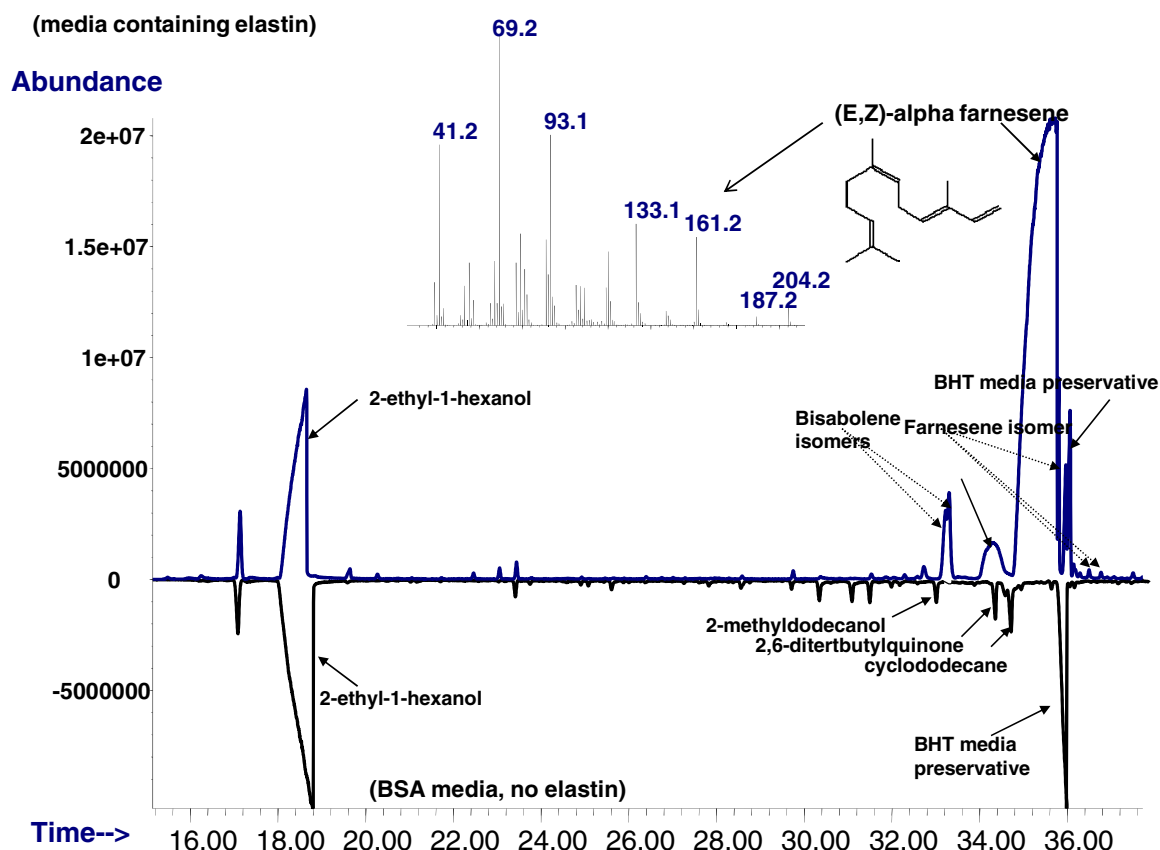


Figure 2. Results from the GC/MS analysis of SBSE exposed 8 h to headspace of *A. fumigatus* strain FGSCA1141 grown on plates with elastin (top) and BSA (no elastin; bottom).

0 min and 2.8 kPa min^{-1} to 151.4 kPa for 1 min. MSD transfer line temperature was 280°C . Scan parameters were 35–350 amu. Components were identified by matching spectra with a Wiley 2008 library. Data analysis was conducted by integrating total ion chromatograms with Agilent ChemStation and copying selected peak areas to an Excel spreadsheet. Graphs and figures were generated utilizing Microsoft Excel and PowerPoint programs.

2.1.4. Time of exposure. To determine at what period of time growth plate headspace must be extracted to detect any volatile compounds, and how much fungal growth must exist in the growth plate before an identifiable volatile signature is obtained, replicate growth plates for the same three strains of *A. fumigatus* were prepared as before on media containing elastin. Initial plate headspace extraction was conducted in the manner previously described for 5 min, 10 min and 30 min after 14 h of growth plate inoculation. As before, headspace extractions were conducted with SBSE and thermal desorption with GC/MS.

2.2. Quantitative analysis of farnesene produced by *A. fumigatus* colonies grown on human lung cells (*ex vivo*)

Human lung cells were grown *ex vivo* in the manner described by Ehrhardt *et al* [18]. Lung fibroblast cell line (ATCC CCL151) was purchased from American Type Culture

Collection (ATCC). Stock cell line was first transferred into a vented 25 cm^2 culture flask (Denville Scientific) filled with 5 mL of Ham's F12K growth medium containing 15% of fetal bovine serum. The flask was cultured in a CO_2 incubator with 5% CO_2 and 95% humidity for 1 week until a monolayer of cells was formed in the flask. Cells in the flask were transferred to a new 150 cm^2 culture flask filled with the same growth medium using fresh 0.25% trypsin and 0.03% EDTA as a detaching reagent. The culture was maintained under 5% CO_2 and 95% humidity for additional 2 weeks.

2.2.1. Model lung system. After the *ex vivo* grown cells formed a monolayer, they were detached using 0.25% trypsin, 0.03% EDTA and separated into additional three flasks filled with the same culture medium. After culturing for 1 week, the three flasks were used for interaction experiments with *Aspergillus fumigatus* culture. For inoculation, 10^4 spores from the fungus were introduced into each flask and incubated in the CO_2 incubator for 24 h until the fungal spores germinated and formed hyphal threads. The growth of the fungus was verified via microscopy. After waiting 24 h following inoculation, the headspace inside the vessel was extracted by drilling two small holes in the vessel cap (5 mm diameter), positioned at the top and bottom of the vessel cap. Sterile Pasteur pipettes packed with glass wool were inserted through holes into the vessel interior, above the growth media and colonies. To the upper pipette a thermal desorption tube

Table 1. Volatiles from *A. fumigatus* extracted with SBSE from the headspace of Petri dishes with growth media containing elastin or bovine serum albumin. For negative controls, volatiles were extracted with SBSE from the headspace of media plates containing elastin or BSA that were not inoculated.

	Strain: FGSCA1141				Strain: FGSCA1142				Strain: FGSCA1163				Elastin blank 8 h	BSA blank 8 h
	Elastin			BSA 8 h	Elastin			BSA 8 h	Elastin			BSA 8 h		
	3 h	6 h	8 h		3 h	6 h	8 h		3 h	6 h	8 h			
Acetic acid	—	—	—	—	—	—	—	—	—	—	+	—	—	—
4-heptanone	—	—	—	—	+	+	+	—	+	+	+	—	—	—
2-heptanone	—	—	—	—	—	—	—	+	+	+	+	+	—	—
2-heptanal	—	—	—	—	—	—	—	—	—	+	+	—	—	—
3-methyl-4-heptanone	—	—	—	—	+	+	+	—	—	—	+	—	—	—
2-ethyl hexanal	+	+	+	+	+	+	+	+	+	+	+	+	—	—
3-octanone	+	+	+	—	+	+	+	—	+	+	+	—	—	—
2-octanone	—	—	—	+	—	—	—	+	—	—	—	+	—	—
p-mentha-1,3,8-triene	+	+	+	—	+	+	+	—	+	+	+	—	—	—
2-ethylhexanol	+	+	+	+	+	+	+	+	+	+	+	+	—	+
methyl-2-ethylhexanoate	—	—	—	u ^a	+	+	+	u ^a	+	+	+	u ^a	—	—
Propylcyclopropane	+	+	+	—	+	+	+	—	+	+	+	—	—	—
3-isopropyl-6-methylene-1-cyclohexene	+	+	+	—	+	+	+	—	+	+	+	—	—	—
2-ethyl-1-hexylacetate	+	+	+	+	+	+	+	—	+	+	+	+	—	—
Farnesene 1 ^b	+	+	+	—	+	+	+	—	+	+	+	—	—	—
Farnesene 2	+	+	+	—	+	+	+	—	+	+	+	—	—	—
Farnesene3 ^b	+	+	+	—	+	+	+	—	+	+	+	—	—	—
Farnesene4 ^b	+	+	+	+	+	+	+	+	+	+	+	+	—	—
Bisabolene1 ^c	+	+	+	—	+	+	+	—	+	+	+	—	—	—
Bisabolene2 ^c	+	+	+	—	+	+	+	—	+	+	+	—	—	—
Octyladipate	+	+	+	+	+	+	+	+	+	+	+	+	—	—

+ Volatile component detected in headspace.

— Component not detected in headspace.

^a Peak obscured by 2-ethylhexanol; components detected by matching library spectra with confidence factor > 80.^b Farnesene isomers also match the retention time of the racemic mixture of authentic standard.^c Bisabolene isomers also match the retention time of the racemic mixture of authentic standard.

packed with sorbent material was attached with a 1 cm segment of Tygon tubing. To the output side of the thermal desorption tube was attached a portable sampling pump (model 224-PCXRB; SKC Inc. Eighty Four, PA, USA). The pump drew headspace from the interior of the *ex vivo* culture and into the sorbent packed thermal desorption tube. As headspace was drawn into the thermal desorption tube, exterior lab air was drawn into the *ex vivo* culture through the other pipette. This flow of air into and out of the vessel containing infected lung cells was similar to the flow of air in lungs.

Volatiles from the *ex vivo* cultures were sampled for 60 min at a flow rate of 50 mL min⁻¹. Each of the two *ex vivo* cultures (blank was identical cell media not inoculated with *A. fumigatus*) inoculated with *A. fumigatus* was sampled separately with each of four different sorbent materials. Materials included activated carbon (CarboPakB), Tenax TA, a PDMS foam and an identical empty glass thermal desorption tube utilized with the other sorbents; only this one contained a single PDMS-coated stir bar (SBSE). All tubes were purchased from Gerstel (Mülheim an der Ruhr, Germany). Following extraction, tubes were thermally desorbed and analyzed by GCMS in the manner described above.

2.2.2. Quantification. A five-point standard curve was prepared for farnesene quantification by direct injection

of different farnesene concentrations. This permitted an approximation of farnesene mass extracted from the model lung system.

2.3. Preliminary *in vivo* testing: analysis of volatiles from nasal swab

As previously discussed, *A. fumigatus* is frequently responsible for chronic fungal sinusitis, although it is not the only pathogenic fungus that causes this condition. In an attempt to determine if volatile profiles could be successfully utilized to detect *A. fumigatus in vivo*, nasal swabs from two patients clinically diagnosed with chronic fungal sinusitis were obtained from a local pulmonary specialist physician.

Swab-extracted lavage was cultured on elastin media to isolate fungi into pure culture. Isolated cultures were transferred to new elastin plates and grown in the manner previously described in section 2.1. Headspace extracts' collection and GCMS analyses of the volatiles generated from isolated fungi were also carried out in the manner previously described with SBSE.

2.3.1. DNA fingerprinting to identify the fungal species. In order to validate the volatile signature data with a specific fungal strain, a rapid DNA-based identification procedure was utilized that permitted identification of the fungus in

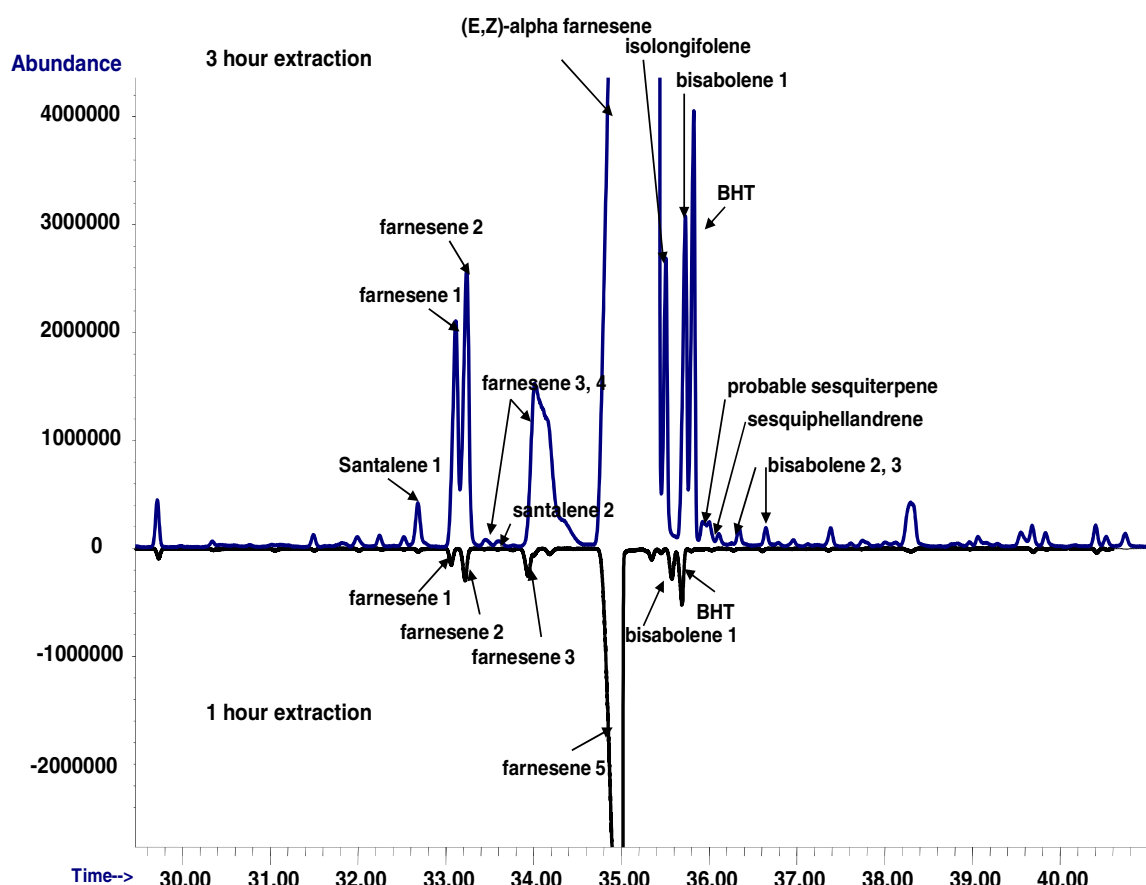


Figure 3. Comparison of volatile abundance when SBSE conducted for 3 h (top) versus 1 h (bottom). *A. fumigatus* strain 41 utilized in both extractions.

less than 24 h. The procedure utilized rapid DNA isolation with a microwave technique, followed by PCR using universal rRNA gene primers for ITS1-ITS4 and direct sequencing PCR products on an ABI310 DNA analyzer and database searching. This provided sufficient information for species-level ID of unknown fungi.

3. Results and discussion

3.1. *In vitro* identification of volatiles and effect of growth media

The most striking observation was the effect on *A. fumigatus* metabolism (and volatile production) when 0.1% connective tissue protein (elastin) was added to Sabouraud's dextrose agar media. There was a very large (*E,Z*)- α -farnesene abundance when the connective tissue protein found in lungs (elastin) was added but none when BSA protein was added (no elastin). There were also sizeable quantities of other sesquiterpenes, including bisabolene isomers, present in elastin containing media headspace (figures 1 and 2).

Overall there were 21 components found only from plates inoculated with *A. fumigatus* (includes plates with elastin and with BSA). Volatiles specific to *A. fumigatus* grown in elastin media (no BSA) included 13 components. Volatiles from *A.*

fumigatus grown on BSA media (no elastin) uniquely included only one component, 2-octanone (table 1, figure 1).

As previously pointed out, of compounds detected, the one present in far greatest abundance was the sesquiterpene farnesene. Farnesene exists as both an α (α) form or 3,7,11-trimethyl-1,3,6,10-dodecatetraene and a β (β) form or 7,11-dimethyl-3-methylene-1,6,10-dodecatriene (isomers differ by the location of one double bond). Further, the α form can exist as four stereoisomers and the β form can exist as two stereoisomers. Four separate farnesene peaks were detected, and of these (*E,Z*)- α -farnesene was the largest. Confirmation of farnesene was conducted by analyzing an authentic farnesene standard (SAFC, St Louis, MO, USA).

The chemical present in greatest abundance when fungi were grown with BSA was 2-ethylhexanol followed by butylated hydroxytoluene (BHT), a chemical preservative used in growth media. Both of these components were also detected in headspace from elastin containing media.

Results from this study strongly indicate that *A. fumigatus* utilized elastin protein, along with other components present in SBA media to produce 13 unique volatiles (unique from those produced when *A. fumigatus* grew on identical media with BSA exchanged for elastin). Of the unique volatiles, farnesene isomers were present in greater abundance by far than any other volatile compounds. As previously discussed, Fiedler *et al* also reported abundant *trans* β -farnesene (and 2-ethylhexanol)

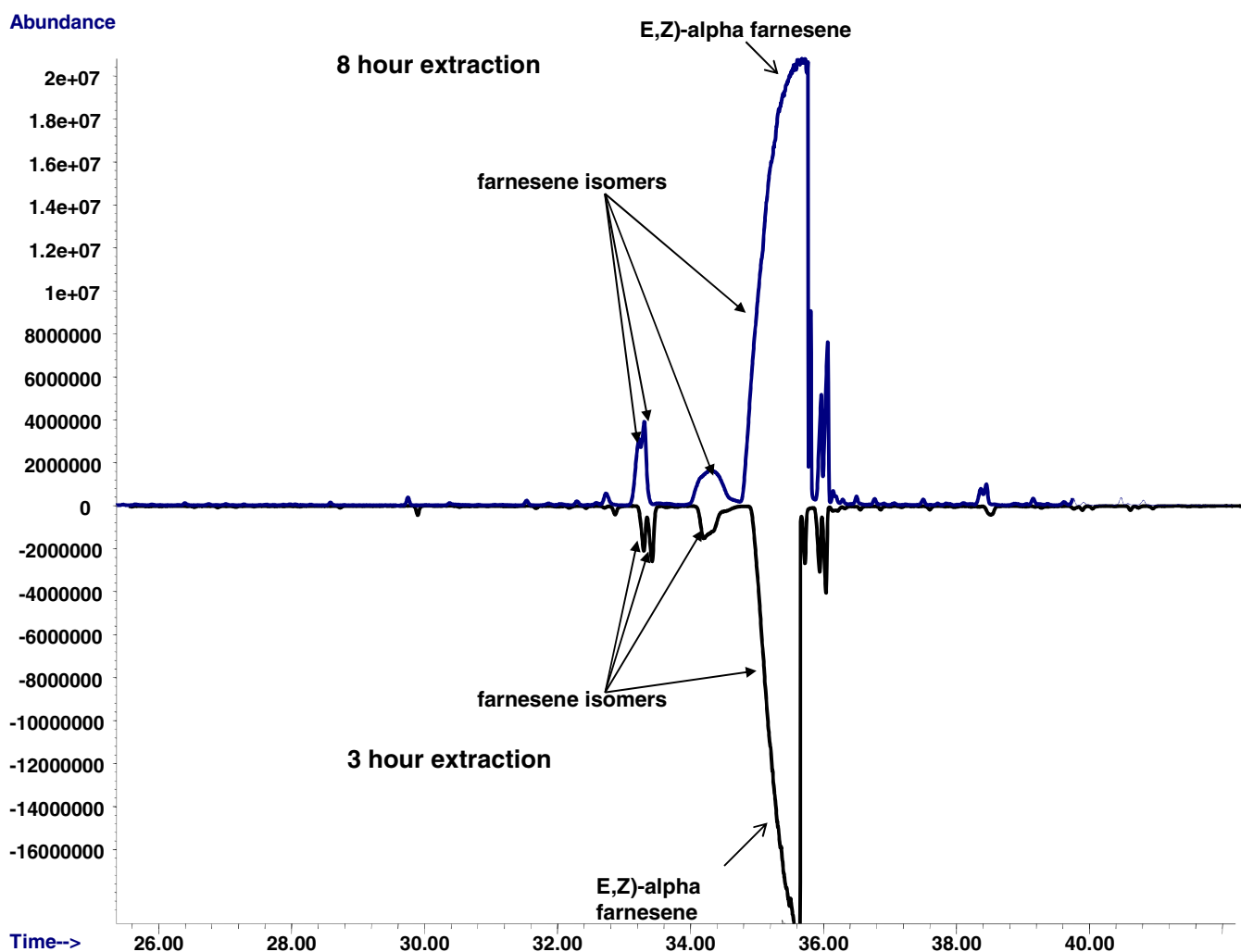


Figure 4. Comparison of volatile abundance when SBSE conducted for 8 h (top) versus 3 h (bottom). Large farnesene abundance resulted in poor separation and peak shape especially in the 8 h extract.

when *A. fumigatus* was grown *in vitro* and extracted by SPME. Farnesene and bisabolene are sesquiterpenes produced by many plant and fungi species via an isoprenoid biosynthesis pathway [19]. Although it is well known that different volatiles are produced based on differences in growth media, the striking difference between the media containing elastin and BSA was unexpected.

The component 2-pentylfuran, reported as a possible *A. fumigatus* biomarker by Shyre *et al* [8] and Chamber *et al* [9], was not detected from any of the samples analyzed in the present study, and that was also true when searching spectra using ion extraction mode. This difference (we did not detect 2-pentylfuran) may be due to some unique condition or unique tissue found in the human body, although the *ex vivo* conditions we utilized should yield similar results provided lung cells harbored the infection. Another major difference involved analytical extraction methods and GCMS analysis. As discussed previously, in [8] and [9], the component 2-pentylfuran was collected in Tedlar bags and then an SPME fiber was inserted into the bag. Volatiles were extracted by the SPME fiber, and then the fiber was thermally desorbed

in the GC injection port. Arguably by extracting directly from a culture with SBSE, or extracting from infected cells in our model lung system with thermal desorption tubes packed with a variety of sorbent materials, a greater quantity of volatile components would be collected. Further, thermal desorption and cryo-focusing effluent that had been collected onto a greater mass of sorbent material (the amount of sorbent material in SBSE and thermal desorption tubes was greater than that present on an SPME fiber) onto a capillary column may produce different results than thermal desorption in a heated GC injection port. Tedlar bags are known to scalp volatile compounds, especially non-polar terpenoids. However, this would decrease sensitivity for 2-pentylfuran, and it was readily detected despite using them. Farnesene was not mentioned in [8] and [9], and this may have been due to scalping by the Tedlar bags.

3.1.1. Strain differentiation. Volatile profiles for *A. fumigatus* strains were similar; however, there were some differences. All strains produced abundant (*E, Z*)- α -farnesene when grown in media containing elastin; however, strain

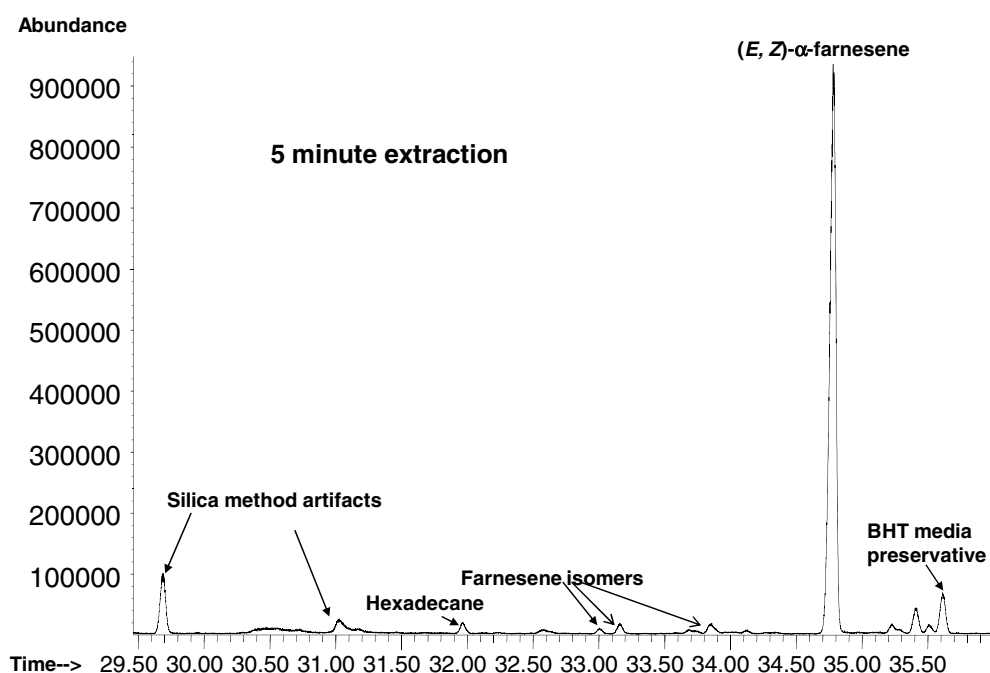


Figure 5. Volatile abundance when sample headspace extracted with SBSE for 5 min. A relatively large abundance of (*E, Z*)- α farnesene was observed as well as smaller abundances of farnesene isomers.

63 consistently produced less abundant volatiles compared with the others. Strain 41 appeared to produce the greatest abundance of farnesene followed closely by strain 42. This difference/similarity was also observed with farnesene isomers and sesquiterpenes. Strain 63 uniquely produced acetic acid and 2-heptanal. It also produced 2-heptanone in elastin and BSA, whereas strain 42 produced 2-heptanone in BSA only and strain 41 did not produce 2-heptanone in either media. Strains 42 and 63 produced 4-heptanone, 3-methyl-2-heptanone and methyl-2-ethylhexanoate, whereas strain 41 did not.

3.1.2. Time of SBSE exposure. Volatile diversity and peak abundance increased with time of exposure (figure 3). However, extraction times greater than 3 h exhibited poor peak separation (figure 4). Subsequent testings revealed that farnesene and bisabolene peaks were readily extracted and identifiable after 5 min SBSE exposure (figure 5) for strains 41 and 42, and after 10 min for strain 63.

3.2. Ex vivo analyses

Headspace extraction of the 150 mL vessel that contained lung cells infected with *A. fumigatus* resembled the action inside a lung where air passes out and in, as it is exhaled and inhaled. Air was pumped from the vessel at 50 mL min⁻¹; therefore, over the 60 min sampling period, all air was swept from the vessel 20 times. As air was pumped from the vessel, external air was drawn in.

Results from extraction of headspace components present in the model lung system indicated that one large farnesene isomer tentatively identified as (*E, Z*)- α -farnesene was detected

Table 2. Quantitative analysis of (*E, Z*)- α -farnesene present in 150 mL vessel headspace. Vessel extracted for 60 min at 50 mL min⁻¹ and volatiles trapped with sorbent materials.

Sorbent material	(<i>E, Z</i>)- α -farnesene mass (μ g)
Activated carbon	0.43
Tenax TA	0.20
SBSE	0.25
PDMS foam	0.00 (none detected)

from SBSE, activated carbon and Tenax TA thermal desorption tubes. It was not detected in PDMS foam effluent (figure 6). The mass of farnesene, calculated by construction of a five-concentration standard curve with farnesene standard, was determined and listed in table 2.

In addition to farnesene, large abundances of 2-ethylhexanol and styrene were also detected from all sorbents. 2-ethylhexanol was also detected when growing *A. fumigatus* *in vitro* and also reported by Fiedler *et al* [6] as one of the most abundant volatiles present in *A. fumigatus* headspace. However, this chemical was detected *in vitro* only when SBSE was exposed to media (both BSA and elastin) for 3 h or more. It was hypothesized that this chemical was formed by *A. fumigatus* during a more mature stage of growth and this could be tested by extracting *A. fumigatus* colonies of different ages.

To further complicate matters, 2-ethylhexanol was detected from the un-inoculated model lung system in abundances similar to those present after the cell-*A. fumigatus* interaction. It was hypothesized that 2-ethylhexanol is also a metabolic product of lung cells. Styrene was present in headspace extracted from un-inoculated *in vitro* media and

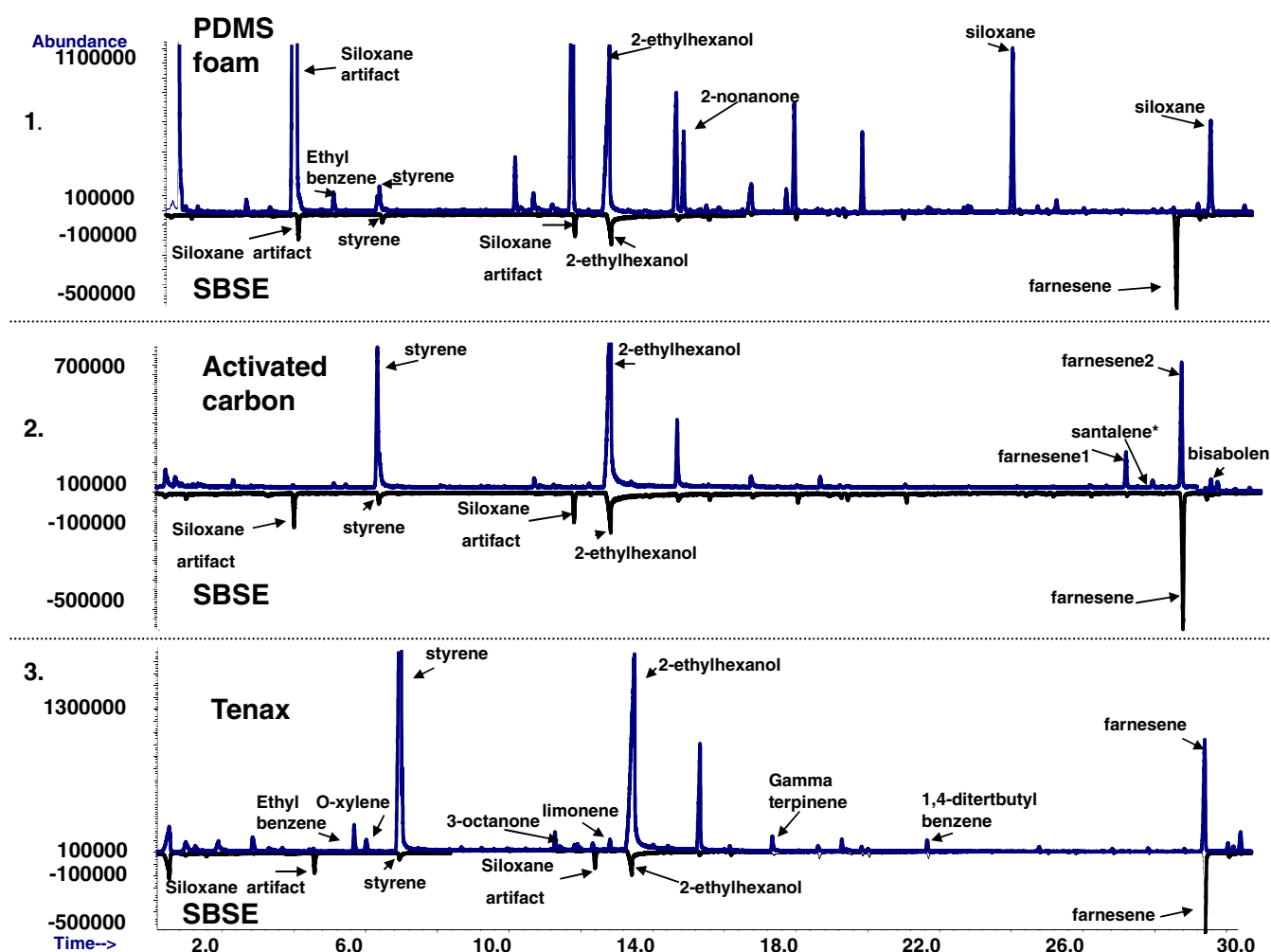


Figure 6. Results from extraction and GCMS analysis of the model lung system with (1) PDMS foam (top) and SBSE (bottom), (2) activated carbon (top) and SBSE (bottom), (3) Tenax (top) and SBSE (bottom). Note that the peaks labeled ‘siloxane’ were silicon artifacts from PDMS.

ex vivo media; therefore, the source was attributed to media and not *A. fumigatus*.

Utilizing SBSE in this dynamic headspace extraction method worked in that a single farnesene component produced by *A. fumigatus* was readily extracted from the artificial lung. The abundance of farnesene was greater than the abundance of 2-ethylhexanol when extracted by SBSE, a trend not indicated by results with activated carbon or Tenax, but this correlated with previous *in vitro* SBSE data. An undesirable result of utilizing SBSE was the siloxane artifacts that originated from PDMS material.

Activated carbon arguably worked the best (figure 6) because in addition to the one farnesene component, a second farnesene isomer was extracted as well as santalene and bisabolene, and abundances were sufficient to match MS library spectra. Other components were extracted but did not match library spectra, or were present at low concentration and spectra were poor. Tenax also extracted other components, notably 3-octanone, a chemical that was detected in all three *A. fumigatus* strains grown *in vitro* with elastin (table 1, figure 6). PDMS foam did extract 2-ethylhexanol but not farnesene,

and there were numerous siloxane artifacts that may have obscured the presence of other volatiles. It was hypothesized that the larger components such as farnesene were extracted by absorption into liquid PDMS. With PDMS foam, surface thickness is less compared to that present on SBSE, and for this reason it was not effective for the present application.

The component 2-pentylfuran, identified as a possible *A. fumigatus* biomarker by Syhre *et al*, was not detected in *ex vivo* samples.

3.3. Chronic fungal sinusitis

DNA fingerprinting identified the fungal species present in nasal lavage collected by swab from a chronic fungal sinusitis patient as *Epicoccum nigrum*. While growing the isolated fungus, it was obvious that the species was not *A. fumigatus* (figure 7). However, for the purpose of demonstrating volatile profile differences between the two species, the volatiles were collected and revealed a very dissimilar profile (figure 8). Farnesene and all sesquiterpenes were completely absent from the headspace of *Epicoccum nigrum*,

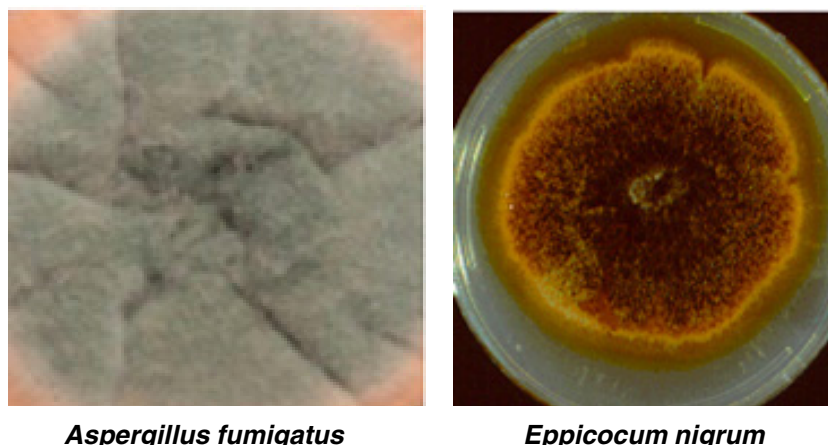
*Aspergillus fumigatus**Eppicocum nigrum*

Figure 7. Photographs of mature colonies of *A. fumigatus* and the species present in nasal lavage, identified as *E. nigrum*, revealed that the species were clearly not the same. However, a volatile profile was collected for comparison purposes.

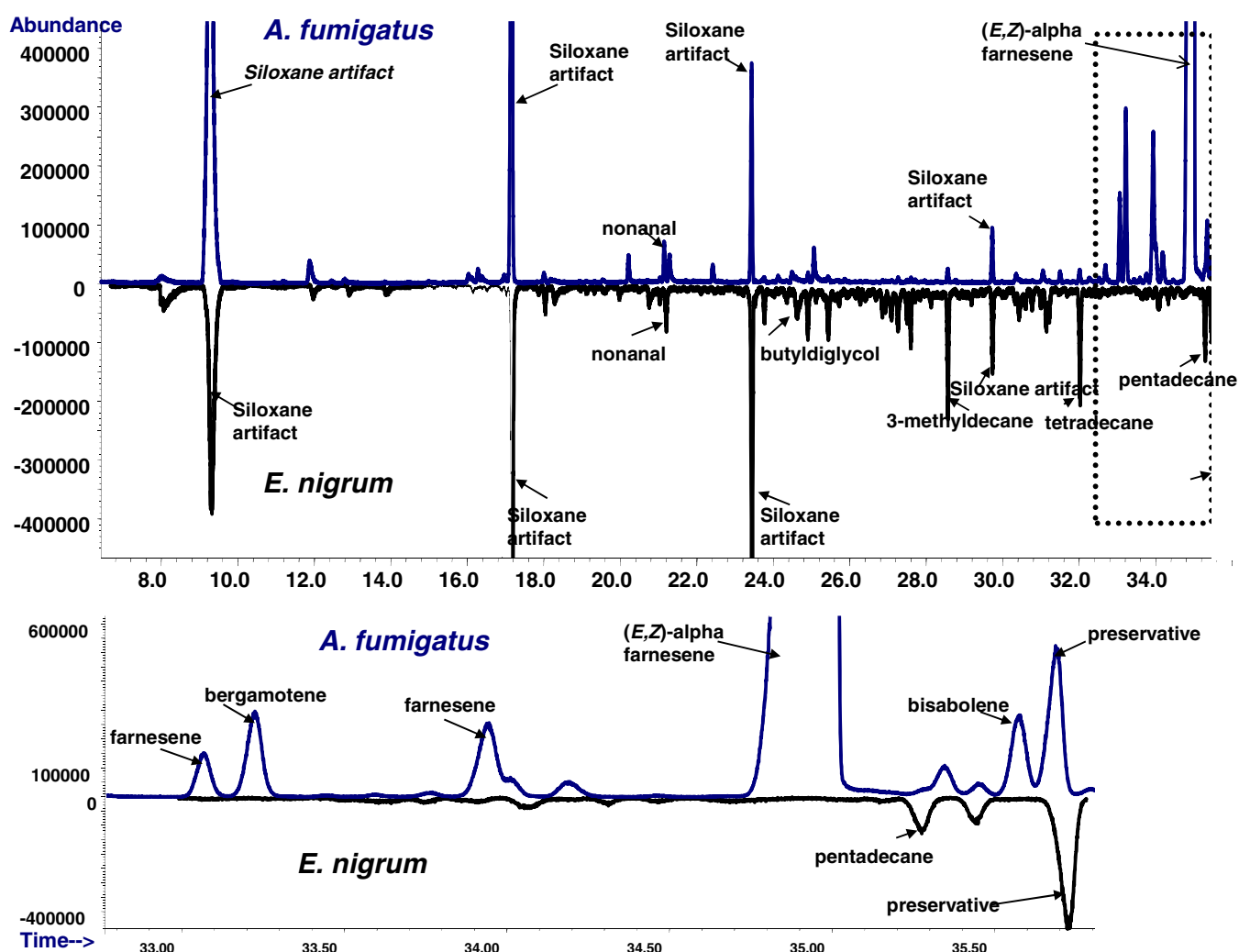


Figure 8. Comparison of *A. fumigatus* volatile profile with that of *Epicoccum nigrum* (fungi isolated from nasal lavage and responsible for chronic fungal sinusitis condition). The key components such as farnesene and other sesquiterpenes were not present in *E. nigrum*, whereas saturated hydrocarbons from *E. nigrum* headspace were not detected in *A. fumigatus* headspace. The area inside the box is shown in greater detail in the bottom chromatogram.

whereas saturated hydrocarbons 3-methylundecane, tetradecane and pentadecane were detected only from *E. nigrum* headspace.

4. Summary and conclusions

Although different media and sorbent materials were utilized, many of the volatile compounds reported by Fiedler *et al* were also detected in our studies, particularly an abundance of sesquiterpenes. We did not detect the component 2-pentyl furan. Volatile chemical profiles collected from the headspace of *A. fumigatus* fungi grown on media with the connective tissue protein elastin, found in lung tissue, contained a large abundance of the sesquiterpene farnesene and, depending on extraction time and sorbent material, other farnesene isomers and sesquiterpenes such as bisabolene. These components were not present in headspace when BSA was substituted for elastin; therefore, elastin appears to be important in the metabolism of farnesene.

When human lung cells were cultured externally and infected with *A. fumigatus*, farnesene was generated in each model lung system as determined by headspace extraction/GCMS analysis with three of four sorbents (PDMS foam did not work for reasons discussed previously). One sorbent, activated carbon, also revealed the presence of another farnesene isomer and two additional sesquiterpenes.

Lastly, nasal lavage collected from a patient diagnosed with chronic fungal sinusitis, a condition frequently caused by *A. fumigatus*, revealed the presence of another pathogenic fungi less frequently responsible for sinusitis, identified as *E. nigrum*. The volatile profile of *E. nigrum* differed markedly from that of *A. fumigatus* with no sesquiterpenes detected.

Although a final clinical study is planned to test lung air from patients diagnosed with aspergillosis, it is clear that a rapid means for detecting volatile chemicals previously identified as biomarkers in breath, such as farnesene, would provide at a minimum an effective screening tool currently lacking for fungal disease.

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