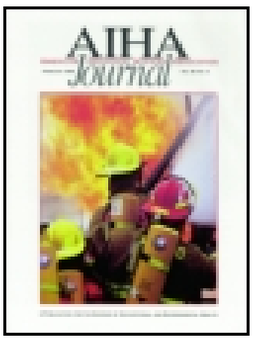

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Determination of Unique Microbial Volatile Organic Compounds Produced by Five *Aspergillus* Species Commonly Found in Problem Buildings

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This study identified unique microbial volatile organic compounds (UMVOCs) produced by five *Aspergillus* species (*A. fumigatus*, *A. versicolor*, *A. sydowi*, *A. flavus*, and *A. niger*) cultivated on malt extract agar and gypsum board. The hypothesis was that UMVOCs can be used to predict the presence of *Aspergillus* species. During the cultivation humidified air was continually supplied and evenly distributed through each of the culture flasks. Volatile metabolites were collected using Tenax TA tubes on Days 8, 16, and 30 after inoculation. The volatile metabolites were determined by gas chromatography/mass spectroscopy after thermal desorption. Nine compounds recognized as UMVOCs—3-methyl-1-butanol; 2-methyl-1-propanol; terpineol; 2-heptanone; 1-octen-3-ol; dimethyl disulfide; 2-hexanone; 3-octanone; and 2-pentylfuran—were found on the cultures in detectable amounts. The first two compounds were detected at the highest frequency when combining both media. The first four compounds were found to be the dominant UMVOCs on gypsum board, which could be used as chemical markers of the common *Aspergillus* species grown indoors.

Keywords: *Aspergillus*, cultures, fungal contamination, indoor air quality, problem buildings, unique MVOCs

The number of complaints about the quality of indoor air in workplaces has grown considerably in recent years. Both fungi and bacteria are often thought to be the causes of the problem. Currently, industrial hygienists investigate problem buildings by determining total or culturable fungi and bacteria in air or settled dust samples. These methods, however, usually do not give an accurate measure of exposure because microorganisms may grow within building structures or underneath carpets, avoiding detection. The measurements have been performed without showing any correlation between level of airborne spores and disease.[1–4]

As an alternative, industrial hygienists are beginning to use volatile organic compound analysis to search for microbial volatile organic compounds (MVOCs) as markers of microorganism overgrowth indoors.[5] To date, researchers have identified a number of volatile organic compounds that are unique to fungi and bacteria,[6,7] that is, unique MVOCs (UMVOCs). Just as the name implies, UMVOCs are substances produced by fungi or bacteria, but not other sources in common indoor environments. For instance, benzene could be produced by certain fungi and thus classed as an MVOC, but it is not a UMVOC because it could be released from some building materials.

Wessén and Schoeps[7] listed a total of 23 compounds as UMVOCs and grouped them into Class A and Class B, as shown in Table I. UMVOC Class A contains a total of 14 compounds that were selected as markers of excessive microbial growth in investigation of damp Swedish houses by Stöm et al.[6] Three of the Class A

TABLE I. A List of Unique Microbial Volatile Organic Compounds

Class A		Class B
2-Hexanone	2-heptanone	2-pentylfuran
3-Methyl-1-butanol	3-methylfuran	2-nonanone
1-Butanol	2-methyl-2-butanol	4-methyl-3-heptanone
2-Methyl-1-propanol	3-octanone	fenchone
3-Octanol	1-octen-3-ol	endoborneol
2-Octen-1-ol		ethyl isobutyrate
Dimethyl disulfide		karveol
2-Pentanol		thujopsene
Geosmin		terpineol

Source: Reference 7.

compounds—3-methyl-1-butanol, 2-hexanone, and 2-heptanone—were reported by Miller et al.[8] as microbial markers in a study of Canadian houses. A significant increase was observed by Stöm et al.[6] in the concentration of MVOC in houses with microbial odor problems compared with unaffected houses and outdoor air samples. Of the 52 houses investigated by Miller et al.,[8] 44% contained 3-methyl-1-butanol, 89% contained 2-hexanone, and 89% contained 2-heptanone, with an average ratio of relative concentrations of 1:2:4, respectively. On the other hand, UMVOC Class B contains the other 9 compounds that were selected after Class A, including thujopsene, 2-pentylfuran, and terpineol. Although almost all the UMVOCs having odors were categorized in Class A, such as geosmin (earthy), 3-octanol (nutty), and 1-octen-3-ol and 2-methyl-1-propanol (musty), the distinction of these two classes was mainly based on the frequencies at which they could be found in problem buildings, rather than the odor threshold. Usually, the frequency of Class A is higher than Class B. However, it has been shown that the inclusion of UMVOC Class B was of great use in explaining the cause of problem buildings, whereas for some cases, using the MVOC Class A alone, it was not possible to correlate microbial impact with the indoor environment as the MVOC concentration was too low.[7]

In this study volatile metabolites produced by five *Aspergillus* species, that is, *A. fumigatus*, *A. versicolor*, *A. sydowi*, *A. flavus*, and *A. niger*, were studied. These species are frequently found in problem buildings and have been associated with adverse health effects.[9,10] The main objective was to identify any UMVOCs produced by these *Aspergillus* species, which may be used by industrial hygienists as markers of potential microbial overgrowth. As with the other studies of this type in the literature, the current study does not include duplicate experiments, so statistical comparison is not addressed.

MATERIALS AND METHODS

Cultivation Media

Malt extract agar (MEA) and gypsum board were chosen because MEA is appropriate for the sampling of saprotrophic fungi and gypsum board (dry wall) is one of the most common building materials. The formulation for MEA was 20 g malt extract, 1 g peptone, 20 g glucose, and 15 g agar per liter of purified water. Gypsum boards (1.1 cm thick, United States Gypsum Co., Chicago, Ill.) were sawed into small pieces with dimensions of 1.5 cm × 10 cm. The boards were plaster sheets wrapped with paper that provided fungi with the necessary carbon source for growing. Plaster sheets were gypsum (calcium sulfate) plus sand and water.

The cultivation flask, as shown in Figure 1, had a volume of 2.8 L with glass adapter tubes for inlet and outlet airflow. Six flasks were used for cultivation on each medium, one for each species and one for the control. Thus, a total of 12 cultivation flasks was used for the study. Each flask was either filled with 600 mL MEA or 10 pieces of gypsum board to provide similar surface areas between the cultivation media where fungi would grow. The flasks had a headspace volume of about 2.2 and 2.6 L for the cultures on MEA and gypsum board, respectively. Media, flasks, tubing, and all connectors used in the experimental setup were autoclaved before use.

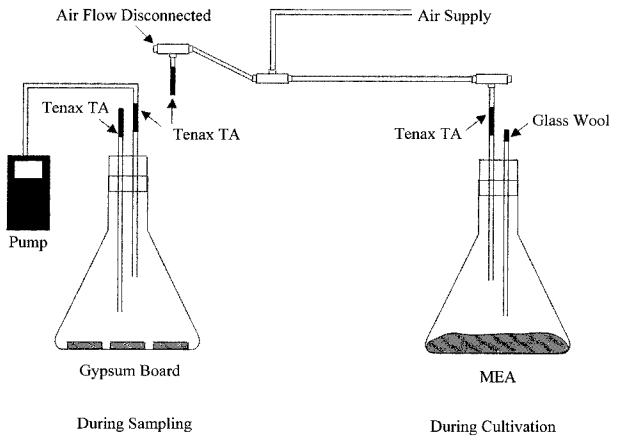

FIGURE 1. Schematic diagram of the equipment used for cultivating fungi and sampling volatiles

Preparation of Spore Suspension and Inoculation

Except for *A. niger* which was provided by another laboratory at the authors' facility, the other species were purchased from the American Type Culture Collection (Manassas, Va.). Freeze-dried samples of the five *Aspergillus* species were revived and cultured on agar slants for 5 days. Spore suspensions of each species, at a concentration of 4×10^6 spores/mL, were prepared. The preparations were carried out in a Biological Safety Cabinet Class II to avoid possible contaminations by other microbes.

Each cultivation flask, except for the two controls, was inoculated with 2.5 mL of a species specific spore solution, resulting in 1×10^7 spores. To the gypsum boards, 25 mL of autoclaved distilled water was added to each flask to provide an adequate amount of initial moisture. As shown in Figure 1 (during cultivation), humidified air through a water bottle was continually supplied and evenly distributed through each culture flask at a flow rate of 40 mL/min during cultivation, resulting in approximately one air change per hour for each flask. This flow rate was based on other investigations to simulate air circulation indoors.[11] Although not measured, humidity inside the flasks was estimated to be greater than 90% because water condensation on the internal walls of the flasks was observed over the experiment duration. The preliminary study showed that CO_2 levels could elevate to about 10% (v/v) without supplying air, which could greatly influence volatile profiles according to another study.[11] Also shown in Figure 1, a Tenax TA adsorbent tube (SKC, Eighty Four, Pa.) was connected to the supplied air line for each flask to trap any volatiles in the air supply and to avoid the possible diffusion of a volatile from one flask to another. The adsorbent tube was replaced with a clean one during the air sampling. A piece of clean glass wool was inserted in the end of the other glass tube to avoid possible

TABLE II. UMVOCs Produced by the Five *Aspergillus* Species on Gypsum Board (GB) and MEA (μg/m³)

		Species[A]															
		A. fumigatus			*A. flavus*			*A. niger*			*A. sydowi*			*A. versicolor*			Average
Compound	Day	8	16	30	8	16	30	8	16	30	8	16	30	8	16	30	μg/m³
3-Methyl-1-butanol	GB	—	0.5	—	0.8	3.3	0.7	—	—	—	—	2.2	3.3	0.3	0.8	1.1	1.4
	MEA	—	1.3	0.41	61.9	62.6	19.6	—	—	0.1	—	4.0	2.1	—	—	—	19.0
1-Octen-3-ol	GB	—	—	—	—	—	—	1.0	1.6	1.3	0.7	—	—	—	—	—	1.2
	MEA	—	—	—	—	—	—	—	—	—	42.5	21.0	—	—	—	—	31.8
2-Methyl-1-propanol	GB	—	—	—	—	—	0.1	—	—	—	0.7	0.9	0.3	0.4	0.6	—	0.5
	MEA	—	—	—	47.6	10.3	29.0	0.9	—	—	—	0.8	2.97	—	—	0.25	13.1
2-Pentylfuran	GB	0.5	0.5	—	—	—	—	—	—	—	—	—	—	—	—	—	0.5
	MEA	—	1.1	—	—	—	—	—	—	—	—	—	—	—	—	—	1.1
2-Heptanone	GB	2.0	—	1.3	—	0.1	—	—	—	—	—	—	—	0.6	1.1	0.5	0.9
2-Hexanone	GB	—	0.1	—	—	—	—	—	—	—	—	—	0.2	—	0.2	—	0.2
3-Octanone	MEA	0.1	9.9	2.2	—	—	—	—	—	—	—	—	—	—	—	—	4.1
Dimethyl disulfide	GB	—	—	—	—	0.1	0.6	—	0.1	—	—	—	0.6	—	—	—	0.4
Terpineol	GB	—	—	—	2.2	2.7	—	—	2.0	1.4	2.7	1.9	—	—	7.4	4.0	3.0

[A] A dashed cell (—) indicates that the compound was not produced by the species on the specific medium and the given sampling occasion, or was present below the detectable level.

gravitational settling of any microorganisms. The flasks were kept in a reach-in incubator (Forma Scientific, Inc., Marietta, Ohio) at 21°C, that is, 1°C below room temperature, to maintain a higher relative humidity inside the flasks.

Sampling of Volatile Metabolites

On Days 8, 16, and 30 after inoculation, headspace air in each flask was collected using an air sampling pump (Model LFS-113, Gillian Instrument Corp., W. Caldwell, N.J.) for 5 hours at a flow rate of 40 mL/min. The same flow rates between air sampling and air supplying were needed to keep a constant dilution factor inside the flasks. As shown in Figure 1 (during sampling), the supply air line was disconnected and the SKC Tenax TA tube in the line was replaced with a SIS clean adsorbent tube (Scientific Instrument Services, Ringoes, N.J.), which was packed with 100 mg of Tenax TA. The glass wool used during the culture was replaced by an SKC Tenax TA tube to trap any volatiles from the incoming air during the sampling. The control flasks, one for each medium, were sampled in the same fashion as the cultivation flasks.

All adsorbent tubes were conditioned at 300°C with 20–30 mL/min of helium for 4 hours before use. The main difference between the Tenax TA tubes and the SKC and SIS is that the latter has screw threads in both sides which can be easily attached to a syringe needle and the connector tube on the desorption unit.

Sample Analysis and Compound Identification

The adsorbent tube was attached to a Short Path Thermal Desorption System (TD-4, Scientific Instrument Services) and fitted with a syringe needle. The tube was first flushed with helium carrier gas at 128 mL/min at room temperature for 4 min to remove methanol, moisture, and carbon dioxide. The sample was then injected into the gas chromatograph (GC) injection port. The desorption system heating blocks were closed around the desorption tube and the system rapidly heated to 250°C. This released the trapped volatiles to a cryo trap placed at the front of the GC column. The cryo trap was previously cooled to −120°C by a liquid nitrogen cooling system to condense the volatiles. The cryo trap was then rapidly heated to 220°C, evaporating the volatiles to the GC column for subsequent analysis. The outlined procedure was processed automatically via a program.

The gas chromatographic analyses were carried out on a Hewlett Packard Model 5890 GC with a fused silica column (HP Ultra 2, 50 m × 0.2 mm i.d.) and an HP Model 5972 mass selective detector (Hewlett Packard, Palo Alto, Calif.). The GC injection port was maintained at 250°C, and the temperature of the transfer line was set at 280°C. The temperature program for the GC oven was 35°C for 5 min followed by a ramp of 10°C/min to 220°C, and a further ramp of 30°C/min to 280°C to clean the column of high-temperature boiling compounds. A scan mode with a range of 40 to 260 atomic mass units was selected. The analyzed volatiles were quantified as equivalents of toluene, as performed by other investigators in similar studies.[12,13]

The NIST98 reference standard library was used to aid in the identification of compounds. Retention times by GC/mass spectrometry were confirmed by matching to standards for 18 of the 23 UMVOCs based on Wessén and Schoeps.[7] Among the five compounds that are currently unavailable, only one (i.e., oct-2-en-1-ol) belongs to Class A. The reference UMVOCs are as follows: 3-methyl-1-butanol, 1-butanol, 2-heptanone, 2-pentanol, 2-hexanone, 1-octen-3-ol, 3-octanol, dimethyl disulfide, ethyl isobutyrate, and 3-methyl-2-butanol purchased from Supelco (Bellefonte, Pa.) at a concentration range of 1000 ± 5 μg/mL in methanol; 2-nonanone (99+%), 3-octanone (99%), 2-methyl-1-propanol (99%), and terpineol (98%) from Fisher Scientific (Pittsburgh, Pa.); geosmin (100.0 μg/mL), thujopsen (100.5 μg/mL) in methanol, and endoborneol (100.2 μg/mL) from Accu-Standard (New Haven, Conn.); and 3-methylfuran (100 μg/mL) from Protocol Analytical Supplies (Middlesex, N.J.), in methanol.

RESULTS AND DISCUSSION

UMVOCs Produced by the *Aspergillus* Species

As shown in Table II, 9 of the 23 UMVOCs described by Wessén and Schoeps[7] were produced in detectable levels by the five *Aspergillus* species. Except for two UMVOC Class B compounds (i.e., terpineol and 2-pentylfuran), all the others belong to UMVOC Class A.[7] The significance that nearly all the UMVOCs belong to Class A indicated that it might be sufficient to concentrate only on the Class A UMVOCs, rather than all 23, if *Aspergillus* were predominant.

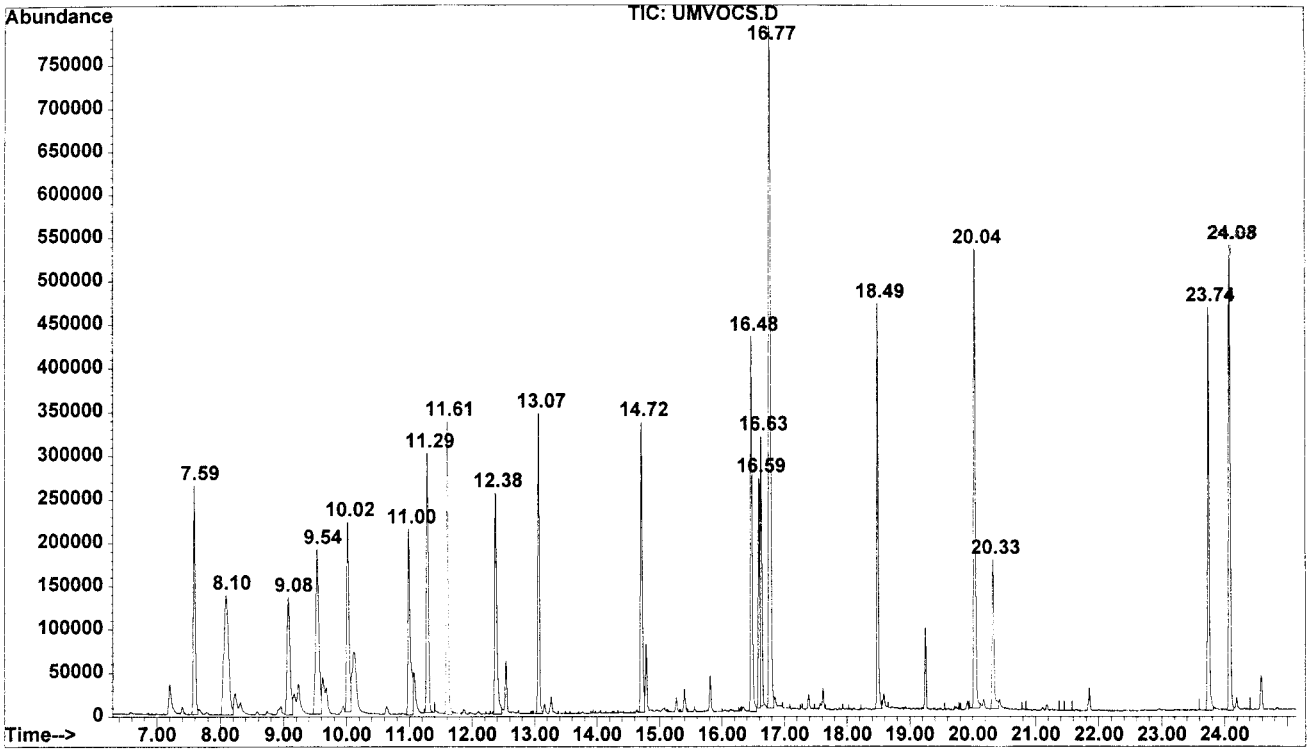

FIGURE 2. Total ion chromatogram of the 18 reference UMVOCs. 3-methylfuran (retention time [RT] = 7.59 min), 2-methyl-1-propanol (RT = 8.10 min), 1-butanol (RT = 9.08 min), 3-methyl-2-butanol (RT = 9.54 min), 2-pentanol (RT = 10.02 min), 3-methyl-1-butanol(RT = 11.00 min), dimethyl disulfide (RT = 11.29 min), ethyl isobutyrate (RT = 11.61 min), 2-hexanone (RT = 12.38 min), 2-heptanone (RT = 14.72 min), 1-octen-3-ol (RT = 16.48 min), 3-octanone (RT = 16.63 min), 3-octanol (RT = 16.77 min), 2-nonanone (RT = 18.49 min), endoborneol (RT = 20.04 min), terpineol (RT = 20.33 min), geosmin (RT = 23.74 min), and thujopsen (RT = 24.08 min). Peaks with RTs of 13.07 and 16.59 min were the results of silicone background bled from the system.

Identifications of the UMVOCs were confirmed by using the reference chemicals previously described. A total ion chromatogram for the reference UMVOCs is shown in Figure 2. Among the peaks integrated on the chromatogram, two with retention times of 13.07 and 16.59 min were silicone background. Extensive work on the silicone background by the manufacturer of the thermal desorption system used in this study has shown that the silicone background was the result of collecting septum bleed on the column during cryofocusing, and/or came from the silanized glass wool that was used for plugging the ends of sorbent tubes.[14] Although not included in the chromatogram, the internal standard toluene had a retention time of 11.86 min under the GC conditions.

1-butanol, as a UMVOC Class A compound described by Wessén and Schoeps,[7] was detected from all the controls with only one exception (MEA control on Day 30). No other UMVOCs were detected from the controls. Average concentrations of 1-butanol were detected to be 4.4 and 0.9 $\mu g/m^3$ from the controls of MEA and gypsum board, respectively. This compound, however, was not found in any cultures at detectable levels. Most likely, adsorption competition between 1-butanol and other volatile metabolites occurred. One study has shown that Tenax TA has a relatively low breakthrough volume for 1-butanol, that is, 56 L/g compared with 1800 L/g for 1-hexanol.[15] However, the selection of Tenax TA as the sorbent used in this study was based on other investigators' recommendations. For instance, Sunesson et al.[12] compared the eight most commonly used sorbents, including Anasorb 727, Anasorb 747, and Tenax TA, and concluded that Tenax TA was the sorbent with the overall best properties for sampling complex mixtures of volatiles. A sampling volume up to 48 L using Anasorb 727[6] or Anasorb 747[7] has been used without notable breakthroughs. Thus, it is assumed that a sampling volume of 12 L in this study would not cause any significant breakthrough for most of the volatiles.

Results presented in Table II indicate that UMVOCs were different with different media, species, and experiment duration. For instance, 2-hexanone, 2-heptanone, terpineol, and dimethyl disulfide were detected from the cultures on gypsum board but not on MEA, whereas 3-octanone was not detected from any cultures on gypsum board. Detectable amounts of 3-octanone and 2-pentylfuan were produced only by *A. fumigatus.* 1-octen-3-ol was produced by *A. sydowi* on both media and by *A. niger* on gypsum board only. Dimethyl disulfide and 2-hexanone were not produced by any species until 16 days after inoculations.

A. versicolor produced many more different types of UMVOCs on gypsum board than on MEA—five on gypsum board but only one (i.e., 2-methyl-1-propanol) on MEA. This was also the case in other studies. Sunesson et al.[13] even found no detectable UMVOCs released by *A. versicolor* on MEA. In general, cultures on gypsum board produced a greater variety of UMVOCs than cultures on MEA, that is, only one to three UMVOCs from each species on MEA, whereas there were two to seven UMVOCs for those from gypsum board. The fact that *A. versicolor* produced multiple UMVOCs on gypsum board is helpful in exposure assessment, because gypsum board is a common building material

TABLE III. Frequency of UMVOCs Produced by the *Aspergillus* Species

Compound	Gypsum Board	MEA	Subtotal
3-Methyl-1-butanol	9/15	8/15	17/30
2-Metnyl-1-propanol	6/15	7/15	13/30
Terpineol	8/15	0/15	8/30
2-Heptanone	6/15	0/15	6/30
1-Octen-3-ol	4/15	2/15	6/30
Dimethyl disulfide	4/15	0/15	4/30
2-Hexanone	3/15	0/15	3/30
3-Octanone	0/15	3/15	3/30
2-Pentylfuran	2/15	1/15	3/30
Total	42/15	21/15	63/30

and *A. versicolor* (together with *Stachybotrys chartarum*) has been found to be the predominant fungi in water-damaged buildings.[16]

Table III shows the frequency of UMVOCs produced by these *Aspergillus* species on both media. The most common UMVOCs were 3-methyl-1-butanol and 2-methyl-1-propanol when both media were combined. This corresponds to a previous study investigating MVOCs produced by *Penicillium spp*. on pine wood and gypsum board.[17] These two compounds, together with terpineol and 2-heptanone, were found to be the four predominant UMVOCs detected on gypsum board. Three of these compounds (terpineol was the exception) were used by Stöm et al.[6] as microbial markers when investigating problem buildings in Sweden. Miller et al.,[8] who first used three MVOCs as markers for excessive fungal growth in buildings, selected 3-methyl-1-butanol and 2-heptanone.

Levels and Time Dependence of the UMVOCs

Levels of UMVOCs were quantified as equivalents of toluene. Generally, identified compounds were found in small quantities. The limit of detection for the method was estimated to be 0.1 $\mu g/m^3$ equivalent to toluene. As shown in Table II, MEA produced significantly higher levels of UMVOCs than gypsum board did. This was expected because MEA is a nutritionally rich medium, whereas gypsum board is not. As can be seen, different species on gypsum board produced approximately consistent amounts of UMVOCs within a compound type.

3-Methyl-1-butanol, produced by *A. flavus* on MEA, had the highest concentration, with levels of 61.9, 62.6, and 19.6 $\mu g/m^3$ on Days 8, 16, and 30, respectively. Similarly, levels of quite a few other UMVOCs, including 3-octanone by *A. fumigatus* and 2-heptanone by *A. versicolor*, were the highest on Day 16. In contrast, 2-methyl-1-propanol produced by *A. flavus* had the lowest level on Day 16, whereas 3-methyl-1-butanol produced by *A. versicolor* on gypsum board had monotonously increasing levels during the study, that is, 0.3, 0.8, and 1.1 $\mu g/m^3$ on Days 8, 16, and 30, respectively. The latter may imply that the compound would be produced in a later period during the life cycle. It is assumed that MVOCs with fluctuating levels may have more limited potential to be used as microbial markers when compared to those that are produced in relatively constant levels during the growth period. In reality, however, different generations of fungi usually coexist; thus, levels of individual compounds are assumed to be less fluctuating.

SUMMARY AND CONCLUSIONS

The following summary and conclusions are made based on this study.

(1) Nine of the 23 UMVOCs were detected in this study and most of them belonged to UMVOC Class A. MVOCs produced by different *Aspergillus* species were different. For a given species, the MVOCs were different with various cultivation media and exhibited experiment duration dependency.

(2) The most common UMVOCs on gypsum board (in descending order of frequency) were 3-methyl-1-butanol, 2-methyl-1-propanol, terpineol, and 2-heptanone. These could be used as markers of *Aspergillus spp*. growth indoors.

(3) Because 1-butanol has a relatively low breakthrough volume on the sorbent and is sometimes present as a solvent in paints, it is not a good compound to be described as a UMVOC.

(4) Additional studies using other building materials as cultivation media and using other species and mixed cultures in laboratory studies are needed so that information on volatile metabolites would be more meaningful for investigation of indoor air quality problems.

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