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ORIGINAL PAPER

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Rapid identification of microbial VOCs from tobacco molds using closed-loop stripping and gas chromatography/time-of-flight mass spectrometry

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Abstract Several microbial volatile organic compounds (MVOCs) that can serve as potential chemical markers for microbial contamination in tobacco have been identified. Four different fungal species, *Aspergillus niger* (AN), *A. ornatus* (AO), *Pencillium chrysogenum* (PC) and *Rhizopus stolonifer* (RS), commonly reported in moldy tobacco were cultured and screened for MVOCs. Because the MVOCs emitted by a microbial species are substrate specific, the fungal strains were separately grown on potato dextrose agar (PDA) and tobacco products. MVOCs from the mold cultures grown on PDA and tobacco products were extracted using closed-loop stripping analysis (CLSA) and identified by gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS). Some of the prominent tobacco mold markers identified by this method include: 1-octen-3-ol; 2-octen-1-ol; 2-methyl-1-butanol; 3-methyl-1-butanol; 1-octene and 2-pentanone. In particular, 1-octen-3-ol was detected in all the mold cultures and moldy tobacco samples analyzed. Olfactory evaluation of 1-octen-3-ol indicated a characteristic musty odor and the odor threshold was determined to be approximately 200 ng/ml. The limits of detection for 1-octen-3-ol using GC/TOF-MS and GC/mass selective detector (MSD) in the full-scan mode and selected ion monitoring (SIM) mode were investigated. The CLSA-GC/TOF-MS demonstrates a fast, sensitive and semi-quantitative analytical technique for screening tobacco materials for the presence of mold via chemical markers of microbial contamination.

Keywords Microbial volatile organic compounds · MVOC · Mold · Tobacco · Closed-loop stripping analysis · GC/TOF-MS

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Introduction

Unpleasant mold-like odors in finished products are a potential source of consumer complaints and present a significant challenge to the manufacturer. Furthermore, complaints of mold-like odors are quite common from compost facilities with unsatisfactory indoor air quality. These off-odors are often caused by bacterial and fungal metabolites known as microbial volatile organic compounds (MVOCs) and result from microbial growth. Many MVOCs have odor thresholds in the low nanogram per liter (parts per trillion) concentration range. A complex mixture of MVOCs, including alkenes, alcohols, ketones, aldehydes, ethers, esters, and terpenes, have been identified from microbial metabolism [1–3]. MVOCs have been detected in damp buildings where occupants often complain about irritation to the eyes, throat and skin, symptoms commonly referred to as "sick building syndrome" [4, 5]. Therefore, it is important to locate and monitor MVOCs in our consumables and living environment in order to sanitize sites of microbial contamination. Also, there is a need to develop rapid analytical methods to reduce the overall time for identification of mold contamination by traditional microbiological techniques (e.g., growing cultures).

MVOCs produced by strains of *Aspergillus* and *Pencillium* mold have been extensively studied by Kaminski et al. [6]. Whitfield and Tindale [7] have reported a threshold for microbial metabolites commonly found in fresh, processed and packaged foods. Fiedler et al. [8] screened cultures of twelve fungal species grown on

various substrate materials for MVOCs using headspace solid-phase microextraction. Using this method, more than 150 volatile compounds were identified, and 1-octen-3-ol, 3-octanone, 2-methyl-1-butanol and 3-methyl-1-butanol were observed in a large majority of examined species. Anderson et al. [9] isolated molds of *Pencillium-1* sp., *Pencillium-2* sp., *Cladosporium* sp. and *Geotrichum* from a packaging area and identified the musty off-flavor compounds in defective beer cans as 2-methylisoborneol, 2,4,6-trichloroanisole and 2,3,4-trichloroanisole. The musty aroma compounds produced by cultures of *Aspergillus* and *Pencillium* when grown on agar and whole-wheat bread were attributed to the presence of geosmin and 2-methylisoborneol [10, 11]. Wilkins and Larsen [12] studied the variation of VOCs from *Aspergillus* and *Pencillium* species that are dominant in damp buildings. Santos et al. [13] identified the compounds resulting in off-flavor in fishes as 3-methyl-1-butanol, pentanone, pentanal, hexanal, 1-octen-3-ol, octanal and some sulfur compounds. In this study, we have identified MVOCs from tobacco mold. Tobacco is a natural substrate for microorganisms (bacteria and fungi) from the time the seed germinates until the commercial product reaches the consumer. Because moldy or damaged tobacco is a loss to the farmer, manufacturer and the buyer, it is important to keep the tobacco dry and free of mold. Various environmental conditions, such as humidity, temperature, rainfall, wind, sunlight and dust, are known to influence the development of MVOCs in tobacco [14].

The MVOCs from tobacco mold samples were concentrated by closed-loop stripping analysis (CLSA) and analyzed using gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS). CLSA is a widely used method for preconcentration of taste and odor compounds from aqueous samples [15, 16]. The CLSA method developed by Grob et al. [17] uses activated carbon as an adsorbent for preconcentration and analysis of purgeable organics at the parts per trillion level. The organic substances are eluted from the charcoal using methylene chloride and the CLSA extract is subjected to chromatographic analysis. The results from GC/TOF-MS are compared to more conventional GC/mass selective detectors (MSD). The high sensitivity of a TOF-MS detector is critical for analysis of trace chemical components, such as MVOCs, that exist in the low parts per trillion concentration range.

This report describes a semi-quantitative analytical method for identification of MVOCs from tobacco mold. Four different fungal strains, *Aspergillus niger* (AN), *A. ornatus* (AO), *Pencillium chrysogenum* (PC) and *Rhizopus stolonifer* (RS), commonly identified in decomposing tobacco, were grown on potato dextrose agar (PDA) and tobacco products and screened for MVOCs. Cigarettes were kept under humid conditions to artificially develop mold and MVOCs emitted from the mold growth were studied. The odor threshold and instrumental limits of detection for the key mold marker 1-octen-3-ol were determined.

Materials and methods

Chemicals

The chromatographic conditions were optimized using an in-house prepared mold standard, a mixture of common MVOCs reported in the literature and air-quality inspection reports from tobacco handling facilities. All substances used were of analytical grade and included 2-pentanol; 3-methyl-2-butanol; 3-methyl-1-butanol; 2-hexanone; 2-heptanone; 2-pentanone-4-hydroxy-4-methyl; 3-octanone; 1-octen-3-ol; 3-octanol; 2-octen-1-ol; alpha terpenieol; 2,6-dichloroanisole, 2,4-dichloroanisole; 2,4,6-trichloroanisole and geosmin. All the above chemicals, with the exception of 2,4,6-trichloroanisole, were purchased from Acros Organics (Fair Lawn, N.J., USA). 2,4,6-Trichloroanisole was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Decanophenone purchased from Acros Organics was used as the internal standard. For CLSA analysis, the MVOCs trapped on the carbon trap were eluted using methylene chloride solvent (Fisher Scientific, Fair Lawn, N.J., USA) containing 40 μg/ml decanophenone (ISTD).

Preparation of mold cultures

American Type Culture Collection (ATCC, Manassas, Va., USA) fungal strains of *A. niger* (AN,1004), *A. ornatus* (AO,16921), *P. chrysogenum* (PC,10002) and *R. stolonifer* (RS,14037) were grown on PDA with antibiotics and cut-tobacco filler that had been sterilized prior to inoculation. For the samples grown on PDA medium (Culture Media and Supplies Inc., Oswego, Ill., USA), lypholized mold cultures were first suspended in 0.3 ml of in-house prepared sterile water. This suspension was then placed into a test tube containing 5 ml of sterile water and was allowed to sit at room temperature (25°C) for 5 h. One hundred μl of each culture was plated on PDA plates with antibiotics (five plates per culture) and then incubated at 25°C prior to CLSA extraction. The same inoculation procedure was followed for the tobacco. Both PDA- and tobacco-derived cultures were incubated for at least 7 days before being prepared by CLSA for GC/TOF-MS analysis. A minimum of three plates per culture were analyzed for each fungal strain to evaluate the sample variability.

Preparation of mold growth on cigarettes

Mold was artificially grown on new-production Philip Morris USA cigarettes by storing the cigarettes in a closed container under 80% relative humidity (RH). In a separate experiment, cigarettes were placed in a closed container under 20% RH. In each of the two RH experiments, the cigarettes were not inoculated with any

mold species. After a 2-week period, a green mold was present on the cigarettes placed under 80% RH while no mold growth was observed on the cigarettes placed under dry conditions. The two cigarettes with visual mold growth were analyzed by CLSA-GC/TOF-MS.

Closed-loop stripping analysis

The MVOCs from mold cultures were extracted using a CLSA apparatus (Fig. 1) purchased from Brechbühler (Spring, Tex., USA). The sample compartment of the CLSA apparatus was modified from its original design, for efficient purging of volatiles from tobacco samples. Approximately 2 g of the mold grown on either PDA or the tobacco products was transferred into the CLSA sample container and then placed in a thermostated water bath at 40°C. The condenser heater block assembly was heated to 95°C to avoid water condensation on the glass tubing or carbon trap. The volatile components from the sample were continuously stripped by air and adsorbed onto 5 mg activated carbon trap (Brechbühler), with a stripping time of 20 min. After the sampling period, the VOCs trapped on the activated carbon were eluted twice with 50 µl methylene chloride directly into a GC autosampler microvial.

Olfactory evaluation of 1-octen-3-ol

The odor characteristic and threshold of 1-octen-3-ol were characterized by a five member sensory panel. A range of standard solutions (200 µg/ml, 20 µg/ml, 2 µg/ml, 200 ng/ml, 20 ng/ml and 2 ng/ml) of 1-octen-3-ol were prepared in deionized water. Deionized water was selected as the solvent to reduce the solvent interference required for sensory evaluation and also because 1-octen-3-ol is soluble in water.

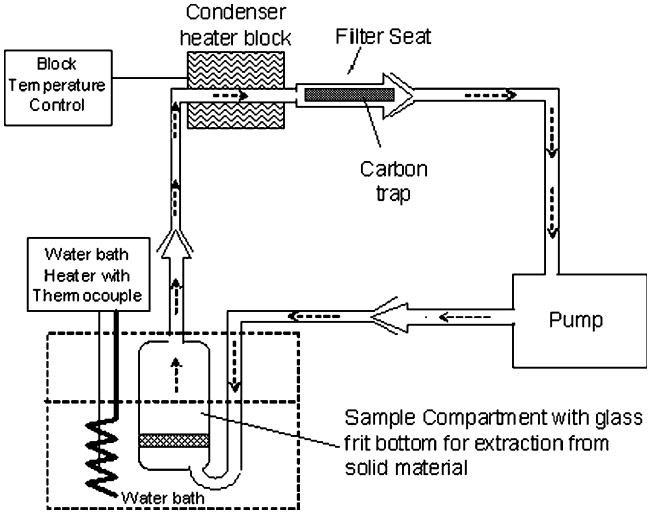

Fig. 1 Closed-loop stripping apparatus. Sample flow direction is shown with sample compartment modification for extraction of volatile organic compounds (VOCs) from liquid and solid samples (Published with permission of Brechbühle R)

Chromatographic conditions

GC/TOF-MS

An Agilent Technologies (Palo Alto, Calif., USA) 6890 Fast GC equipped with a Pegasus III TOF-MS (LECO,, St. Joseph, Mich., USA) was used for separation and identification of MVOCs. The compounds were separated using a 30 m×0.32 mm inner diameter Rtx-1701 (Restek, Bellefonte, Penn., USA) fused silica capillary column with a 0.25-µm film thickness. The column oven was held at 40°C for 1 min, programmed to 280°C at 60°C/min, and held for 2 min. The injector and transfer line temperatures were held at 270°C and the MS ion source was set at 225°C. One µl CLSA extract was injected into the CIS-4 GC inlet (Gerstel, Baltimore, Md.,USA) under splitless conditions using Gerstel's MPS 2 Multi Purpose Sampler. Helium was used as the carrier gas with a constant flow rate of 3 ml/min. The GC analysis time was 7 min and MS data acquisition was started after a 1.67-min solvent delay. The mass spectral data were acquired at 30 spectra/s over a mass range of 35–350 amu.

The chromatographic conditions were optimized by analyzing a mold standard containing common MVOCs. Figure 2 shows the total ion chromatogram (TIC) with the separation between the chemical components of a 10 µg/ml mold standard. Fifteen MVOCs were identified in a 7-min GC analysis. As seen from the inset of Fig. 2, the high spectral acquisition rate of a TOF-MS detector results in adequate data points across closely eluting chromatographic components such that accurate chromatographic and mass spectral data are provided. All the samples in this study were analyzed using the above chromatographic and MS setpoints. Peak identities were confirmed by matching the retention time and mass spectra of the component peak to the data observed for the pure compound (Fig. 2) or by matching the component mass spectra to the NIST/EPA/NIH Mass Spectral Database (Gaithersburg, Md., USA).

GC/MSD

For application of this method to more conventional GC/MS systems, the sensitivity of 1-octen-3-ol was compared using an Agilent Technologies 6890 GC equipped with an Agilent 5973 N MSD. Compounds were separated using a Rtx-1701 column (30 m×0.32 mm inner diameter × 0.25-µm film thickness). The initial oven temperature of 40°C was held for 3 min and then increased to a final temperature of 280°C at a rate of 35°C/min and held for 2 min. Mass spectral data were collected in both the full-scan and the selected

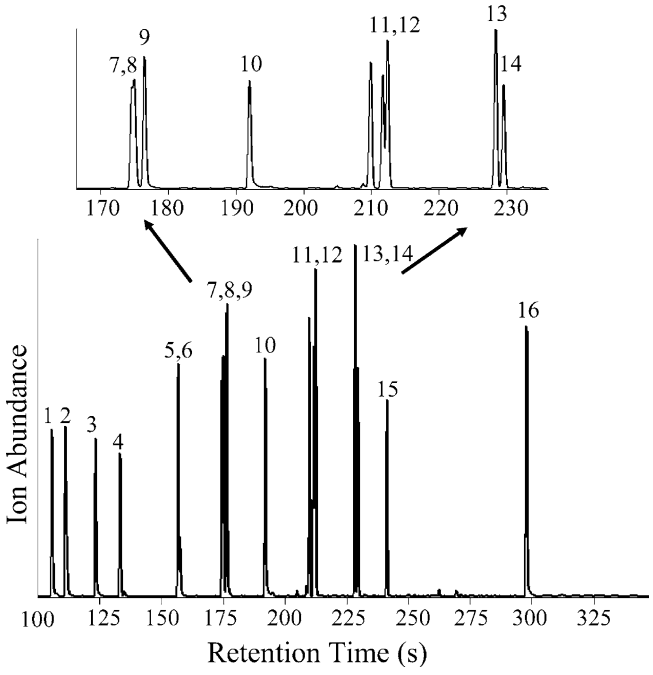

Fig. 2 Total ion chromatogram of 10 μg/ml standard of common mold metabolites. Peak identification: *1* 3-methyl-2-butanol; *2* 2-pentanol; *3* 3-methyl-1-butanol; *4* 2-hexanone; *5* 2-heptanone; *6* 2-pentanone; 4-hydroxy-4-methyl; *7* 3-octanone; *8* 1-octen-3-ol; *9* 3-octanol; *10* 2-octen-1-ol; *11* alpha terpenieol; *12* 2,6-dichloroanisole; *13* 2,4-dichloroanisole; *14* 2,4,6-trichloroanisole; *15* geosmin; *16* decanophenone (ISTD).

ion monitoring (SIM) mode. The injector, transfer line and ion source temperatures were set at 250, 280 and 230°C, respectively. A sample volume of 1 μl was injected into the GC inlet using splitless mode. Helium was used as the carrier gas with a constant column flow rate of 1.5 ml/min. For MS analysis, an electron impact ionization (70 eV) was used to generate ions and a mass range of 35–350 amu was scanned at 4.5 spectra/s. For SIM-MS analysis, the sum of three mass spectral ions (m/z = 43, 57 and 72) was used for peak identification. The sensitivity for detection of 1-octen-3-ol was determined by analyzing a series of standard solutions (20 μg/ml, 2 μg/ml, 200 ng/ml and 20 ng/ml) of 1-octen-3-ol prepared in methylene chloride using GC/TOF-MS and GC/MSD (SIM mode and full scan mode).

Results and discussion

Identification of MVOCs from fungal strains

In order to determine the chemical markers (MVOCs) specific to a strain, fungal species were grown on PDA cultures and tobacco products. Both of these were analyzed for background VOCs that may be observed in mold cultures. Individual mold (2 g) was scraped from the culture and transferred to the CLSA sample container with minimum transfer of the nutrient medium. Because it is difficult to separate the mold from its medium, some background VOCs from PDA and tobacco will appear in the chromatograms of the extract from mold cultures. Table 1 lists the MVOCs identified from the fungal strains grown on PDA and tobacco products. As seen from the table, 1-octen-3-ol was detected in all the four strains grown on PDA and tobacco products. Both 3-methyl-1-butanol and 2-methyl-1-butanol were produced in the cultures of *A. ornatus*, *P. chrysogenum*, *and R. stolinifera* grown on PDA, while 2-methyl-1-butanol was predominant for the same strains grown on tobacco products. 1-Octene and 2-pentanone were detected in strains grown on tobacco products and were not observed in PDA cultures. In short, some common microbial VOC markers (1-octen-3-ol and 2-methyl-1-butanol) and markers specific to tobacco products (1-octene and 2-pentanone) were identified from the analysis of PDA cultures and tobacco products.

The TICs of the CLSA extract from *A. ornatus* grown on PDA and tobacco products are shown in Fig. 3a, b respectively. MVOC markers 1-octen-3-ol and 2-methyl-1-butanol were detected in both PDA cultures and tobacco products. A higher amount of 1-octen-3-ol was detected in PDA cultures of *A. ornatus* than in cultures grown on tobacco products. For similarly aged samples ($n = 3$), the relative standard deviation of the peak area of 1-octen-3-ol relative to the internal standard was approximately 20%. However, the concentration of 1-octen-3-ol in both PDA and tobacco products was observed to increase with aging of the culture, indicating additional microbial growth.

Identification of MVOCs from cigarette mold

Figure 4 shows the chromatogram of VOCs detected from two moldy cigarettes artificially prepared in the laboratory. Three potential MVOC markers, 1-octen-3-ol, 2-methyl-1-butanol and 2-octen-1-ol, were identified from the CLSA extract of cigarette mold. The concen-

Table 1 Microbial volatile organic compounds (MVOC) markers identified for the fungal strains *Aspergillus niger* (AN), *Aspergillus ornatus* (AO), *Pencillium chrysogenum* (PC), *Rhizopus stolonifer* (RS), grown in PDA and cut tobacco filler. MVOC markers are shown in bold letters; *NI* not identified

MVOC markers	Fungal species identified in PDA	Fungal species identified in tobacco
2-Methyl-1-butanol	PC, AO, RS	PC, AO, RS
3-Methyl-1-butanol	PC, AO, RS	RS
1-Octen-3-ol	PC, AO, RS, AN	PC, AO, RS, AN
1-Octen	NI	PC, AO, RS, AN
3-Methyl-1-buten-1-ol	PC	NI
2-Hexen-1-ol	PC	NI
2-Octen-1-ol	AN	NI
2-Pentanol	NI	AN
2-Pentanone	NI	PC, AO, AN
3-Methyl-3-buten-1-ol	AO, RS	RS
3-Octanol	PC, AO	NI
3-Octanone	PC	NI

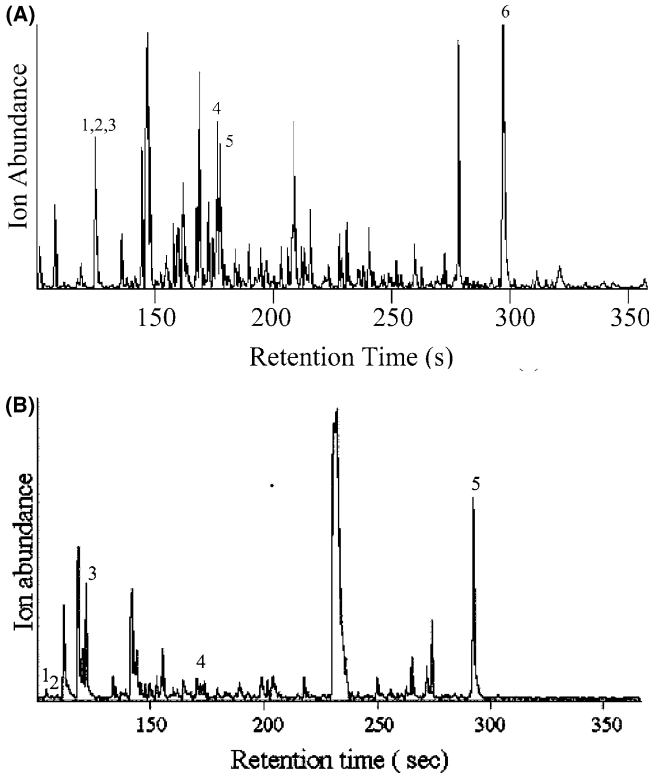

Fig. 3 Chromatogram representing the MVOCs from *A. ornatus* grown in **a** PDA. Peak identification: *1* 3-methyl-3-buten-1-ol; *2* 3-methyl-1-butanol; *3* 2-methyl-1-butanol; *4* 1-octen-3-ol; *5* 3-octanol; *6* decanophenone (ISTD). Chromatogram representing the MVOCs from *A. ornatus* grown in **b** tobacco cut filler. Peak identification: *1* 2-pentanone; *2* 1-octene; *3* 2-methyl-1-butanol; *4* 1-octen-3-ol; *5* decanophenone (ISTD)

tration of the chemical marker 1-octen-3-ol in the moldy cigarette was estimated to be 40 μg/ml based on the response factor from the internal standard.

Olfactory evaluation of 1-octen-3-ol

Among the MVOC markers identified from the analysis of mold cultures and cigarette mold samples (Table 1), 1-octen-3-ol was produced by all the samples studied. As 1-octen-3-ol is not a known tobacco constituent, it can serve as a potential chemical marker for microbial contamination of tobacco. The odor of 1-octen-3-ol was characterized as earthy, musty and wet leaves or wet wood. The odor threshold of 1-octen-3-ol determined by sensory analysis is approximately 200 ng/ml.

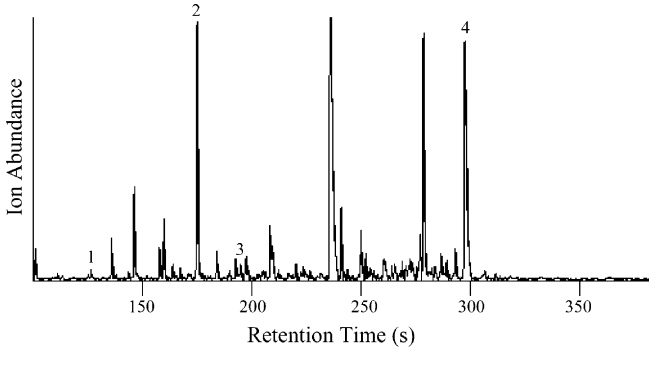

Fig. 4 Total ion chromatogram of CLSA extract from moldy cigarettes artificially prepared in the laboratory. Peak identification: *1* 2-methyl-1-butanol; *2)* 1-octen-3-ol; *3* 2-octen-1-ol; *4* decanophenone (ISTD)

Sensitivity of 1-octen-3-ol using GC/TOF-MS and GC/MSD

As shown in figure 5, the detection limits for 1-octen-3-ol using GC/TOF-MS, GC/MSD in the SIM and full scan mode was calculated to be 20 ng/ml, 200 ng/ml and 2 μg/ml, respectively. The differences in sensitivity using TOF-MS and MSD were mainly due to differences in data acquisition principles and the chromatographic separation speed. The GC/MSD method [12-min GC run time (RT)] in the full-scan mode acquires complete mass-range (35–350 amu) spectra at 4.5 spectra/s but results in less sensitivity than the SIM–MSD method, in which three characteristic ions (m/z = 43, 57 and 72) are monitored at 20 spectra/s. The higher scan rate using SIM–MSD increases the dwell time (the time taken by the detector to look at each mass unit) of the detector, increasing the signal-to-noise ratio and improving sensitivity. In contrast, the TOF-MS method (7-min GC RT) acquires complete mass spectral data at 30 spectra/s and simultaneously monitors all the ions in the mass range (35–350 amu) resulting in higher sensitivity than obtained with SIM–MSD. The peak tailing observed for the 1-octen-3-ol peak using the GC/MSD method (flow rate = 1.5 ml/min) was possibly due to slow volatilization and slow transfer of the sample onto the GC column. The GC/TOF-MS method uses a higher carrier-

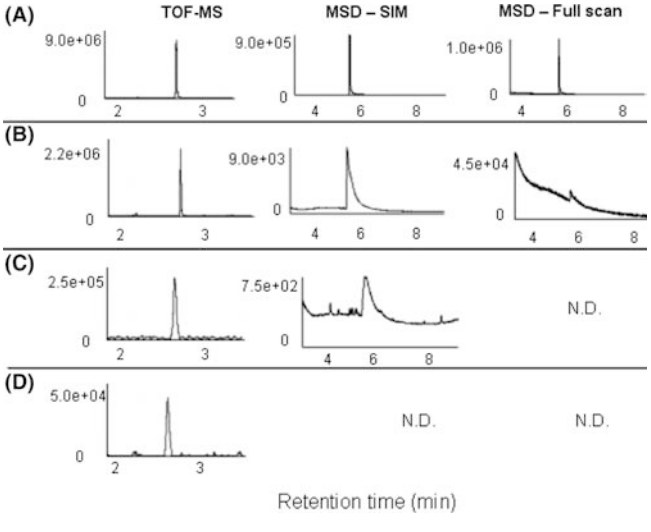

Fig. 5 Comparison of sensitivity for detection of 1-octen-3-ol using GC/TOF-MS (RT = 2.7 min) and GC/MSD (RT = 5.8 min) in the SIM TIC (m/z = 43, 57 and 72) and full-scan mode. **a** 20 μg/ml, **b** 2 μg/ml, **c** 200 ng/ml and **d** 20 ng/ml. *ND* Not detected

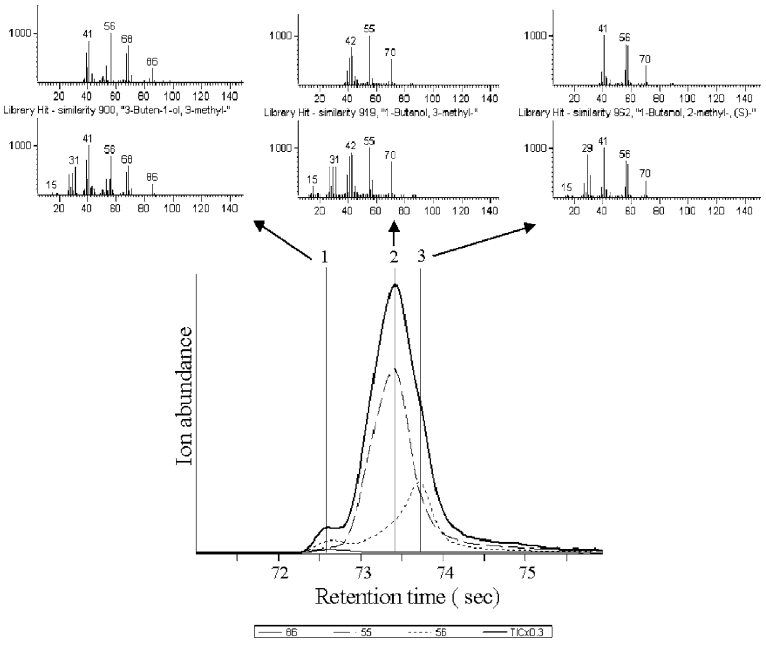

Fig. 6 Identification of overlapping chromatographic components using deconvolution algorithm and unique ion masses from the component spectra. The three components were identified as *1* 3-buten-1-ol, $m/z = 66$;*2* 3-methyl-1-butanol, $m/z = 55$; *3* 2-methyl-1-butanol, $m/z = 56$

gas flow rate (3 ml/min), which reduces the residence time of the analyte in the hot injection port and results in improved peak shape.

Identification of chemically equivalent MVOC markers by TOF-MS detection

As seen from Table 1, the MVOC markers identified by the CLSA-GC/TOF-MS method are highly volatile compounds containing four- to eight-carbon atoms, and show a great deal of similarity in their chemical structure. For example, 3-methy-l-butanol and 2-methyl-1-butanol and, similarly, 1-octen-3-ol and 2-octen-1-ol are structural isomers. Also, the chemical and physical properties of five-carbon compounds (2-pentanone, 3-penten-2-ol and 2-pentanol) or the eight-carbon compounds (3-octanone, 1-octen-3-ol and 3-octanol) are very similar in their respective groups. As a result of this similarity in their chemical makeup, the MVOC markers tended to co-elute during chromatography. Such closely eluting chromatographic components can be accurately identified using the high data-acquisition rates of a TOF-MS. Figure 6 shows a 2-s TIC peak representing three different components that are chemically similar: 3-methyl-3-buten-1-ol; 3-methyl-1-butanol and 2-methyl-1-butanol. Although the three component peaks are not apparent in the combined TIC, the peaks can be resolved by plotting the unique ion masses and identified by matching the deconvoluted component mass spectra to the mass spectral library. The deconvolution was performed using Leco's ''Peak Find Algorithm'' . The similarity match factors between the extracted component mass spectra and library spectra for the three peaks were 900, 919 and 962 on a scale of 0–1,000. This ability to separate and identify closely eluting components using TOF-MS decreases the need for highly resolved peaks and permits faster analysis.

In conclusion, several MVOCs specific to fungal species known to damage tobacco have been identified using CLSA and GC/TOF-MS. The prominent markers identified by this method include 1-octen-3-ol; 2-octen-1-ol; 2-methyl-1-butanol; 3-methyl-1-butanol; 1-octene and 2-pentanone. In particular, 1-octen-3-ol was detected in all the mold cultures regardless of growth media and has been identified as a potential chemical marker for microbial growth in tobacco. Larsen and Frisvad [3] suggested that 1-octen-3-ol, 3-octanol and 3-octanone are produced by the enzymatic oxidative breakdown of linoleic acid and linolenic acids. The odors of these compounds were perceived as earthy/metallic/raw mushroom. Larsen and Frisvad [3] also indicated the low odor threshold for these eight-carbon compounds and low sensitivity using MSD but no mention as to the detection limits of 1-octen-3-ol was made. In this work, the odor threshold of 1-octen-3-ol was determined to be 200 ng/ml by sensory analysis and its odor was characterized as earthy, musty and wet leaves or wet wood. The limits of detection for 1-octen-3-ol using GC/TOF-MS, GC/MSD in the SIM and full-scan mode were determined to be 20 ng/ml, 200 ng/ml and 2 μg/ml, respectively. Using the CLSA-GC/TOF-MS method, the MVOCs in tobacco raw materials and finished products can be identified in less than 60 min. These MVOCs can therefore serve as chemical markers to screen tobacco raw materials, finished products and consumer complaint samples for the presence of microbial contamination.

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