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Identification of Some Volatile Constituents of *Aspergillus clavatus*

The vacuum steam volatile concentrates from three strains of *Aspergillus clavatus* grown on standard media have been analyzed by capillary gas–liquid chromatogrphy–mass spectrometry. Fifty compounds (eight tentatively) were identified from all the strains. Aliphatic alcohols and ketones accounted for about half of the identified compounds while the rest were mainly aromatic compounds. The major compounds were oct-1-en-3-ol (30–52% in NRRL 2 and 11–21% in the other strains), 4-methylbenzaldehyde (34–40% in NRRL 5199), phenylacetaldehyde (10–27% in NRRL 5199 and NRRL 6320), and 2-methylphenol with lesser amounts of the other isomers (25–57% in NRRL 6320 and 13% in NRRL 5199). The chromatotographic pattern and known volatile composition were distinctive for each strain and could be used to characterize these fungi.

Recently gas–liquid chromatography (GLC) of fungal volatiles has been used to study the differentiate various fungi. Vincent and Kulik (1970, 1973) and Kulik and Vincent (1973) classified a number of common lower fungi using a mathematical analysis of their pyrolysis–gas–liquid chromatography (PGLC) patterns without identification of any volatiles. The method relies on exacting standard conditions and the assumption that the composition will always be indentical for the conditions used. Gunasekaran and Hughes (1980) used the GLC pattern of methylated fatty acids to distinguish several species of *Candida*.

Other researchers have identified fungal volatiles for characterization of fungi rather than relying completely on GLC pattern differences. Collins (1979) reviewing some of the literature of odor producing fungi discussed the identification of some major compounds and the techniques which were used for their isolation. A successful approach was the isolation of volatiles by vacuum steam distillation followed by GLC–mass spectrometry (MS). For example, Kaminski et al. (1972, 1974) identified the major volatiles from several fungal strains and Freeman et al. (1976) identified 22 compounds associated with spoilage of chicken using these techniques. However, other techniques have been used with assays for different areas of research. Halim et al. (1975) extracted odorous contituents of *Pencillium decumbens* directly and used column chromatography and GLC to separate major compounds for identification by their infrared spectra. Norrman (1977), applying headspace chromatography (HSGC) to one organism, suggested using it as a general method for following metabolic changes in fungi. Repke et al. (1978) extracted mushroom toxins with methanol and assayed the trimethylsilyl derivatives by GLC–MS.

Some microbial odorants are common pollutants of food and water. Gerber (1979) reviewed the most important odorants, particularly geosmin, which is produced by the bacterium *Streptomyces*, some algae, and a fungus.

Some of the best examples of comprehensive assays using a variety of successful techniques are the investigations of mushroom volatiles by Yajima et al. (1981, Thomas (1973), and Pyysalo (1976). Individually they used different but fairly mild techniques (headspace, extraction–vacuum distillation, continuous extraction ion–vacuum distillation) to obtain and identify 50–70 mushroom volatiles per species by GLC–MS. The characteristic and desirable aromas of edible mushroom were also investigated by Cronin and Ward (1971), Pyysalo and Suihko (1976), and Picardi and Issenberg (1973).

Some fungal volatiles may be unpleasant pollutants affecting food quality, or more seriously, the fungi themselves can contaminate food or crops, causing economic losses or even potential hazards to human health. The literature reviewed in this introduction documents some successes and exploration into identifying fungal volatiles and using volatile composition to detect and/or characterize fungi. However, few comprehensive studies of the lower fungi (comparable to the mushroom investigations) have been made. A knowledge of the volatile composition of fungi is important in dealing with the problem of food contamination.

The author's present report is on the volatile composition of some common odor-producing strains of *Aspergillus clavatus* grown on standard media. The object of this study was to differentiate and characterize closely related strains of fungi grown on a reference medium before investigating volatile composition on a more complex (volatile contributing or altering) agricultural crop or product.

EXPERIMENTAL SECTION

Materials and Methods. Reference strains of *A. clavatus* (NRRL 2, 5199, and 6320) maintained on 1% yeast extract Czapek's agar slants (Raper and Fennell, 1965) at 3 °C were used to inoculate 250 mL of Czapek medium (an inorganic fungal medium) supplemented with a 0.1% yeast extract (Raper and Fennell, 1965) in Fernbach flasks which were then incubated at 25 °C for 5–15 days. Typically, 30 flasks were prepared and harvested at maturity of the condidia (determined by microscopic examination). Maximum odor production (determined by sniffing) coincided with condidia maturity. Control samples (uninoculated medium) were treated similarly to inoculated samples. One control adjusted from neutrality with phosphate buffer to pH 8.2 was a second control for NRRL 2 which was alkaline at maturity.

Isolation of the Volatile Oil. Contents of control or uninoculated flasks (7.5 L) were transferred to a sterile odor free 12-L round-bottom flask fitted with a Likens-Nickerson steam distillation continuous extraction head cooled by 5 °C water. An additional dry ice cooled reflux condenser was attached to the outlet of the head. The side arm of the head was fitted with a 250-mL flask containing distilled hexane (100 mL) with a trace of Ionox 330 antioxidant. The distillation and extraction were carried out at 98 mmHg, ca. 50 °C, for 3.5 h. After extraction the hexane, dried by freezing out water, was concentrated on a steam bath through a Vigreaux column to ca. 1 mL and then further concentrated in a small tube to 5–10 μL by using a stream of argon.

Estimation of Yields. The dry weight of the fungal mat after distillation and extraction was obtained by filtering the mat under vacuum and then drying to a constant weight at 105 °C. A 0.3-μL aliquot from the known volume of concentrated fungal volatiles was injected onto the capillary column housed in a 5880 Hewlett-Packard gas chromatograph equipped with a automatic integrator. The precentage of hexane solvent was determined from the integration. The yield could then be calculated from the dry fungal weight and the volume of the volatile oil.

Capillary GLC–Mass Spectral Analysis. Analysis of the whole oil was made on two 150 m long by 0.64 mm i.d. Pyrex glass capillary columns coated with Carbowax 20-M. Temperature programming conditions were to hold at 50 °C for 10 min after injection and then program at 1 °C/min from 50 to 170 °C, holding at the upper limit for 60 min. The column inlet pressure was 16 psi helium. One of the columns was coupled to a modified Consolidated 21-620 cycloidal-type mass spectrometer (electron ionization at 70 eV) by using a single-state Lewellyn-Littlejohn silicone rubber membrane molecular separator. The other column housed in a Hewlett-Packard 5880 gas chromatograph with automatic integrator was used to obtain relative percents of the GLC peaks.

Kovat's indices were determined for the GLC peaks by using a standard series of normal hydrocarbons run under the same conditions as fungal volatiles. The Kovat's GLC index is given to the nearest 10 units with a few exceptions.

RESULTS AND DISCUSSION

A total of 50 compounds were identified (8 tentatively) from all *A. clavatus* samples. A large number of minor compounds could not be identified. NRRL 5199, the least chemically complex strain, contained about 30 compounds while the other strains studied had double that number of compounds. Four compounds were identified in the pH 7 control and nine in the pH 8.2 control, which contained a high percent of pyrazines not present in the unaltered control.

The results of the analysis of volatiles from three strains of *A. clavatus* and their controls are listed in Table I. There are two controls for the assays of fungal volatiles. The unaltered media at pH 7 is identical with the media used for all fungal samples. Strain NRRL 2 was alkaline at maturity, exhibiting a strong ammonia odor and pH change from neutrality after distillation of the volatiles. A sample of media was adjusted to pH 8.2 to serve as a second control for the NRRL 2 sample.

The assays were begun with NRRL 2 and that assay is listed as "NRRL 2 (early)". Subsequent assays of the three strains were fairly consistent with their replicates, but the NRRL 2 assays (listed as "late") were different from the earlier NRRL 2 samples. Both early and late NRRL 2 assay results are listed.

There are some variation in the Kovat's index value between the two columns and in the same column with different samples. The most consistent value is listed in Table I. Multiple compounds within one GLC peak are shown by the repeated Kovat's index with compounds either named or designated as "unidentified".

The relative percent figure is obtained from the automatic integrator value of the GLC peak area with the solvent peaks eliminated from the calculation. The bracketed relative percent figures are used to indicate multicomponent GLC peaks which integrated as one value.

It shoud be noted that 2,5-dimethylpyrazine identified in control pH 8.2 at a Kovat's index of 1320 was also tentatively identified by its mass spectra in NRRL 2 at Kovat's index 1390. The mass spectra of the two compounds appear identical with reference spectra. The discrepancy in the Kovat's index value for the NRRL 2 compound required it to be listed as tentatively identified.

The volatile oils obtained from each of the three strains (NRRL 2, 5199, and 6320) of *A. clavatus* were in the range of 5–15 parts per million (ppm) of the dry weight of the fungal mat. Dry weights of the fungal mat varied ca. 20% between the same strains to ca. 50% between different strains and averaged 30 g for the 30 flasks. The yield of volatile oil from the pH 7 control (medium only) was too low to measure. The higher yield of oil obtained from the adjusted pH 8.2 control was significantly lower than the yield from fungal samples. Therefore, the media did not contribute measurably to the fungal volatiles.

Major Components: GLC Patterns and Composition Compared. Figure 1 is a line graph representation of the major (over 1%) GLC components of the three *A. clavatus* strains, NRRL 2 (early and late), 5199, and 6320, plotted as relative percent vs. Kovat's index. The broken portion of a line represents sample variation. Components

Table I. Volatile Compounds Identified in *A. clavatus* and Control Samples

Kovat's GLC index[a]	compounds[b]	relative %[c]					
		control pH 7[d]	control pH 8.2	NRRL 2 (early)	NRRL 2 (late)	NRRL 6320	NRRL 5199
1050	hexanal			[0.4]	[1.5]	[0.6]	[0.9]
1080	hexan-2-one	X	4.2			0.2 (0.2-1.2)	
1085	pentan-2-ol			0.2	0.3		
1180	heptan-2-one[e]			0.1	2.0	[1.7]	[1.0]
1200	hexan-3-ol		3.6				
1200	pentan-2,4-dione			[5.0]	[3.8]	[5.9]	[2.2]
1210	2-methylbutanol						
1210	3-methylbutanol				[1.4]		1.0 (0.5-1.0)
1220	hexan-2-ol[f]						
1220	2-methylpyridine						
1220	dimethoxyethane		1.2				
1220	1-penten-4-ol			[5.0]			
1230	2-pentylfuran[f]				2.7	0.3	
1250	octan-3-one			6.2 (6.2-14.7)	8.4 (5.4-9.4)	3.2 (2.6-3.9)	1.6
1280	octan-2-one			1.0 (0.3-1.3)	1.0 (0.3-1.0)		
1290	1-octen-3-one			0.3		0.1	
1320	2,5-dimethylpyrazine		27.0				
1320	5-methyl-4-hexen-3-one[e]			0.3	0.4	0.2	0.5
1350	branched hydrocarbon[e]					0.1	
1355	hexanol[f]			[2.5]	[10.5 (9-13)]	0.3	0.2
1355	a thiazole (mol ion 127)[e]						
1355	nonan-3-one				0.7		
1390	2-ethyl-5-methylpyridine						
1390	2-ethyl-5-methylpyrazine		1.6				
1390	nonan-4-one[e]			1.3	0.9		
1390	2,5-dimethylpyrazine					0.4	
1400	2,3,5-trimethylpyrazine		7.7				
1400	octan-3-ol[f]			[4.3 (1.4-16.0)]	[3.5 (1.7-3.5)]	[3.0 (2.1-4.3)]	[2.3 (1.9-2.6)]
1400	unidentified			0.5			
1420	C_8 alkenone[e]				0.9	0.9	
1430	1-methoxy-3-methylbenzene						
1450	1-octen-3-ol[f]			[30.0 (30-52)]	[34.0 (34-42)]	[16.0 (11-21)]	[17.0 (16-21)]
1450	unidentified						
1485	1-decen-3-one			1.1	1.3	0.9	0.6
1505	benzaldehyde	X	9.4			0.3	0.6
1560	alkylpyrazine[e]			0.2			
1570	3,5-dimethyl-1,2,4-trithiolane						
1570	unidentified				[0.9 (0.9-2.8)]		
1570	α-bergamotene					2.0	1.0
1580	heptanol					0.2	0.3
1590	undecan-2-one			0.2	0.2		
1610	2-octen-1-ol			2.3 (0.4-2.3)	0.5		
1615	acetophenone				0.2		
1620	1-(1-cyclohexen-1-yl)-1-propanone[e]			0.4	0.2	0.4	

1630	4-methylbenzaldehyde							34.0 (34–40)
1630	phenylacetaldehyde	X	10.0			[17.0 (10–26)]		[12.0 (12–27)]
1650	unidentified							
1650	ethyl benzoate							
1665	thialdin				1.0 (0.8–1.4)			
1720	1,2-dimethoxybenzene			0.4			2.4	
1730	decalin				0.2			
1790	2-methoxyphenol[e]					0.1		
1890	phenylethanol			0.9		1.9	0.4	
1930	4-phenyl-2-buten-1-al[e]		0.8					
1950	benzothiazole					0.4	0.4	
1980	*p*-methoxybenzaldehyde			0.1				
2000	1,5-di(*tert*-butyl)-3,3-dimethylbicyclo-[3.1.0]hexan-2-one[e]	X		1.3	0.6			
2070	2-methylphenol[f]					[57.0 (25–57)]	[13.0]	
2090	3-methylphenol and/or 4-methylphenol					0.5		
2150	2-methoxyacetophenone					0.4		
2160	*p*-ethylphenol					0.1		
2200	pentylbenzene[e]					0.4		
2350	indole							

[a] Obtained by using a Carbowax 20-M capillary column as described under Experimental Section. [b] Compounds listed are consistant with Kovat's GLC indices and mass spectra of authentic compounds. [c] Values listed are those most frequently found. Ranges are given in parentheses. Brackets indicate one value for several compounds. [d] "X" means the percent was too low to measure. [e] Tentative indentication. [f] Major component in a multicomponent area or peak.

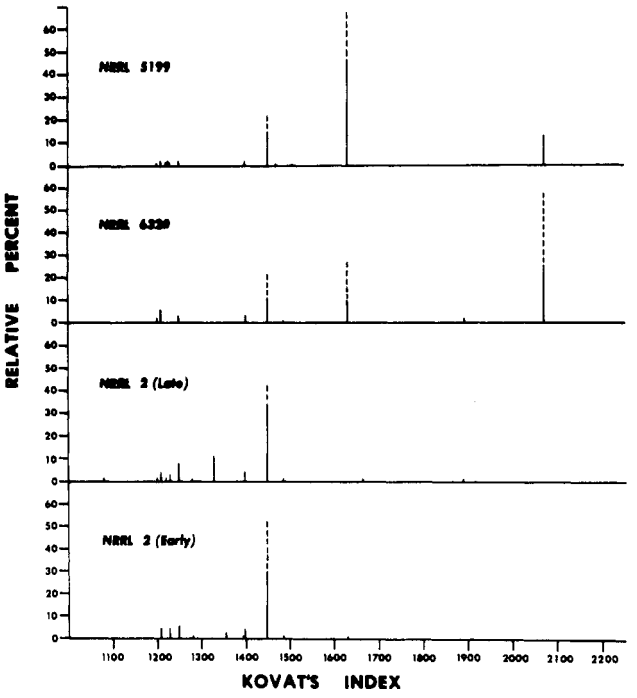

Figure 1. Major volatile component patterns of *A. clavatus* NRRL 2 (early and late), 5199, and 6320.

with close retention times are represented by one line. The general patterns of the two NRRL 2 samples although not identical show similarities common to that strain. The patterns of the three strains are different from each other; however, patterns alone should not be considered adequate for fungal identification because different compounds can occur at the same place and in similar concentrations in a chromatogram. For example, α-bergamotene in NRRL 5199 and 6320 and 3,5-dimethyl-1,2,4-trithiolane in NRRL 2 both occur in similar concentration at Kovat's index 1570.

The major pattern differences distinguishing the three strains occurred at Kovat's indices 1450, 1630, and 2070. Strain NRRL 2 had a high (30–52%) concentration of 1-octen-3-ol compared to lower levels (11–22%) in the other strains at 1450. Strain NRRL 5199 had a high (46–67%) concentration of components in the 1630–1650 range, i.e., 4-methylbenzaldehyde (34–40%) and phenylacetaldehyde mixed with some ethyl benzoate (12–27%). At 1630 NRRL 6320 had only one component, phenylacetaldehyde (11–23%). In the 2050–2090 range (shown as a line at 2070 in Figure 1) the major component, 2-methylphenol with lessor amounts of 3- and/or 4-methylphenol, ranged from 25 to 57% in NRRL 6320 while NRRL 5199 had 13%. NRRL 2 did not have significant levels of any components at 1660 and 2070.

It is interesting that the lowest yield of 1-octen-3-ol occurred in NRRL 5199 and 6320, which contained a high phenolic content. It has generally been accepted that 1-octen-3-ol is formed by oxidation of linoleic acid (Yajima et al., 1981) via a free radical reaction (Forss, 1967). The methylphenols may be acting as antioxidants, inhibiting formation of 1-octen-3-ol. The presence of methylphenols especially in such high levels appears to be unique for these fungal volatiles.

Kaminski et al. (1974) reported the major volatiles from several strains of *Aspergillus*, *Penicillum*, and *Fungi imperfecti* were 3-methylbutanol, octan-3-one, octan-3-ol, octanol, 2-octen-1-ol, and 1-octen-3-ol (which alone accounted for 35–92%). These compounds except for octanol were also found in the *A. clavatus* strains reported in Table I. However, 2-octen-1-ol was found only in NRRL 2 and

1-octen-3-one was found only in NRRL 2 and 6320. Pyysalo (1976) also reported finding these volatiles in mushrooms where 1-octen-3-ol in particular was an important contributor to mushroom aroma (Pyysalo and Suihko, 1976).

The majority of the compounds in Table I are aliphatic alcohols and ketones followed by aromatic compounds. The only terpenoid, α-bergamotene, which has not been previously reported in fungi volatiles before, was found in NRRL 5199 and 6320. Fair amounts of benzothiazole and indole were also found in these two strains. The unusual phenolic content of these strains has already been discussed.

Unusual Compounds. Two sulfur compounds, not previously found in fungi volatiles, were thialdin and 3,5-dimethyl-1,2,4-trithiolane in NRRL 2 (late) samples. These sulfur heterocyclic compounds were found in beef broth (Brinkman et al., 1972) and in cooked red beans (Buttery et al., 1975). These compounds can be readily formed from hydrogen sulfide, ammonia, and acetaldehyde (Brinkman et al., 1972). It is possible that these sulfur heterocycles in fungi could be formed by a similar reaction. However, the intermediate very volatile compounds needed for this reaction have not been identified. The authors' subjective observation of ammonia and hydrogen sulfide-like odors from NRRL 2 are the only evidence for these compounds. A disulfide (molecular ion 166) was also found in NRRL 2 during some preliminary assays, but the results of that assay could not be repeated.

The most unusual compound in the fungal volatiles was found in NRRL 2 and also in the pH 7 control (at a much lower concentration) and was tentatively identified as 1,5-di(*tert*-butyl)-3,3-dimethylbicyclo[3.1.0]hexan-2-one from its close mass spectral similarity with the EPA/NIH mass spectral data base spectra (Heller and Milne, 1978) for that compound. Coincidently, Buttery (1981) in our laboratory also obtained an identical mass spectra from an unidentified compound isolated in a hexane extract of a vacuum steam distillate of "off-flavor" sugar from sugar beets. The Kovat's index of the compound from sugar beet sugar and the NRRL 2 fungal and pH 7 control samples matched. The identification of this compound is only tentative, since an authentic compound was not available for comparison. This bicyclic compound has been synthesized by photolysis of 2,3,4-tri(*tert*-butyl)furan (van Tamelen and Whitesides, 1971), but as far as the authors know it has not been found before in nature. The exact origin of this compound is unknown, although sucrose and possible processing contaminates were common to the three samples containing this compound.

Odor Descriptions. Raper and Fennell (1965) describe *A. clavatus* as having a "strongly fetid odor in some strains, not pronounced in others". The author's odor description for the mature *A. clavatus* strains studied are "strong, unpleasant, fetid-decaying fish" for NRRL 2, "weak, moldy, rubbery" for NRRL 6320, and "weak, rotten, fetid" for NRRL 5199. The rotten odor seems to be associated with the increase in pH of the fungal samples. Interestingly, before development of mature condidia, the rotton-smelling samples had a pleasant ester-like aroma. None of the identified compounds had odors individually that could be called characteristic of their fungal parents.

Conclusions. The results of this study show that closely related fungi have different volatile compositions which can be used to distinguish them from each other. The ability to distinguish fungi grown on crops or other food sources has yet to be explored. Elution patterns are not sufficient for fungal identifications, but the technique of fingerprinting fungi by their volatile composition could be useful in detecting unwanted fungi in foods before extensive damage could occur.

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