

Identification of the Predominant Volatile Compounds Produced by *Aspergillus flavus*

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A culture of *Aspergillus flavus* grown on moistened wheat meal was homogenized with a blender, and the resulting slurry was vacuum-distilled at 5 mm of Hg and 35 C. The aqueous distillate was collected in traps cooled to -10 to -80 C. The culture volatiles were extracted from the distillate with CH₂Cl₂, and, after removal of the bulk of the solvent, the concentrated volatiles were examined by packed-column gas chromatography. Nineteen peaks were observed, and coupled gas chromatography-mass spectrometry was employed to identify the larger components. The compounds identified were: 3-methylbutanol, 3-octanone, 3-octanol, 1-octen-3-ol, 1-octanol, and *cis*-2-octen-1-ol. The two octenols were the predominant compounds, and sufficient sample was trapped from the gas chromatograph for infrared analyses; this confirmed the mass spectral identifications and permitted the assignment of the *cis* designation to 2-octen-1-ol. Both oct-1-en-3-ol and *cis*-2-octen-1-ol are thought to be responsible for the characteristic musty-fungal odor of certain fungi; the latter compound may be a useful chemical index of fungal growth.

Aspergillus is known as one of the most common representatives of the large group of molds called the *A. flavus-oryzae* group. This mold has been found capable of developing on many agricultural products over a wide range of temperatures and relative humidities (7). *A. flavus* exhibits a strong lipolytic activity and, under certain conditions, also proteolytic and amylolytic activities. *A. flavus* can cause discoloration of products high in fat (8) and has been stated to be the chief aflatoxin-producing microorganism (7). During storage of grain, *A. flavus* is capable of penetrating the bran, giving rise to interior microflora (13, 17).

It should be stressed that the odor defined as musty or musty-fungal is ascribed to *A. flavus*, and before the development of microbiological methods the sense of smell was the only tool available to detect this mold. The purpose of this study was the isolation and identification of the odorous volatiles produced by *A. flavus*.

MATERIALS AND METHODS

Microorganisms. *A. flavus* was isolated from wheat grain and maintained on Czapek-Dox agar slants at 3 C until used.

Culture media and growth conditions. The culture medium used was coarse, wheat meal sterilized

at 1 atm for 45 min. The wheat meal was moistened to 60% water content, placed on perforated aluminum plates (35 by 15 by 15 cm) and inoculated with conidia of *A. flavus* suspended in physiological saline, with 10 ml of suspension for 100 g of wheat meal. To prepare the suspension, the surface of a well-developed sporulating culture of *A. flavus* grown on 5% brewer's extract agar in a 750-ml flat-bottom Erlenmeyer flask was taken and suspended in physiological saline. After inoculation with the suspension, the wheat meal was mixed thoroughly, after which a 1- to 1.5-cm layer was formed. The mold was incubated at 28 to 30 C and 92 to 96% relative humidity. After 3 to 4 days, the conidia covered the whole area with mycelium.

For each use a 2-kg culture medium was prepared. The purity of the cultures was checked by microscope observation.

Isolation of volatiles from the medium. About 1,200 g of the coarse wheat meal incubated with *A. flavus* was cooled to -10 C and homogenized in a Waring Blendor with redistilled ice-cold water (in the ratio 1:3). The resulting slurry was transferred to a 10-liter round-bottom flask and subjected to vacuum distillation in an all-glass apparatus (14). The distillation step lasted for 4 hr and was done under nitrogen at 5 mm of Hg. The temperature of the water bath was 35 C, whereas that of the cold traps in which the distillate was collected ranged from -10 to -80 C. The extraction of volatiles with an organic solvent was carried out, with the distillate being collected in traps cooled to -40 and -80 C. Portions (350 ml) of the distillate were extracted with CH₂Cl₂ by liquid-liquid extraction in an all-glass apparatus. The con-

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centration step took about 8 hr. The resulting CH_2Cl_2 extracts were dried with anhydrous Na_2SO_4 and concentrated with a Vigreux distillation column down to a volume of 100 μl (14). The concentrate thus obtained was then transferred to a glass ampoule and sealed.

The same technique was used for the isolation and concentration of the volatiles from noninoculated medium, i.e., a control consisting of the normal, coarse, wheat meal autoclaved at 1 atm for 90 min.

Gas chromatography. The separation of the volatile substances in the concentrated distillates was carried out with a Willy Giede model GCHF 18.3 gas chromatograph equipped with a flame ionization detector. The columns were stainless steel (3 m long; outer diameter, 3 mm) packed with 15% Carbowax 20 M terminated with terephthalic acid on 80 to 100-mesh, acid-washed, dimethyldichlorosilane (DMCS)-treated Chromosorb W. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min. Samples (2 μl) were applied to the column which was held isothermally at 120 C.

Trapping of pure compounds from the distillate for infrared analysis. The separation step was performed by means of the same gas chromatograph equipped with a katharometer. The columns were stainless steel (2 m long; outer diameter, 6 mm) packed with 5% free fatty acid phase (FFAP) on 80 to 100-mesh, acid-washed, DMCS-treated Chromosorb W. Nitrogen was used as the carrier gas at a flow rate of 40 ml/min. The column was operated isothermally at 100 C. The main peaks were trapped as they left the detector in small glass traps containing 1 ml of carbon tetrachloride cooled to -20 C. The concentration and purity of each compound in carbon tetrachloride were checked by means of the gas chromatograph equipped with a flame ionization detector.

Identification of the predominant volatiles by mass spectrometry. Coupled gas chromatography-mass spectrometry (GC-MS) was carried out at the Department of Food Science and Technology, Oregon

State University, Corvallis. The apparatus and operational parameters have been described (16), except for the addition of a single-stage Llewellyn helium separator to the GC-MS interface. Compounds were identified by comparing the mass spectra of the unknowns to a file of standard spectra (1) and to original literature (4). Tentative identifications were verified by GC-MS of the compounds identified and also by GC retention time.

IR spectrometry. Infrared (IR) spectra of the isolated fractions trapped from the distillate, as well as those of the standards, were examined by means of an IR 20 Zeiss apparatus. The spectra were taken in spectrally pure CCl_4 using cells 0.43 mm thick. The chart speed in the frequency range 700 to 4,000 cm^{-1} was 200 mm per min per 100 cm^{-1} .

RESULTS AND DISCUSSION

The gas chromatograms obtained from the concentrated distillate of *A. flavus* and the medium are presented in Fig. 1 and Fig. 2, respectively. The concentrated samples of *A. flavus* exhibiting a strong odor appeared complex in nature, yielding 19 gas chromatographic peaks, most of them homogeneous. A list of the volatiles identified in the concentrated distillate from *A. flavus* is presented in Table 1. These compounds were also detected in head space samples taken above the molds.

Of the six compounds that were identified, five contained eight carbons, and four of these five compounds were alcohols. Among the volatiles produced by *A. flavus*, 1-octen-3-ol and *cis*-2-octen-1-ol were most abundant and responsible for the characteristic odor of this mold. A comparison of IR spectra (Fig. 3 and Table 2) indicates that the 1-octen-3-ol isolated from *A. flavus* is identical with the synthetic preparation from Oregon State University. The

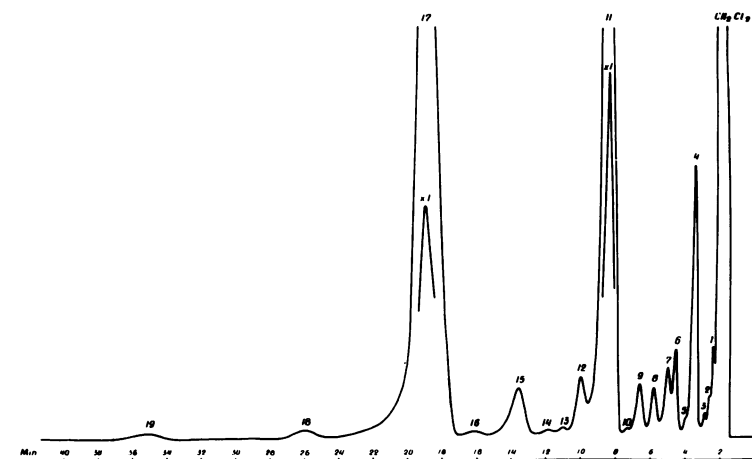


FIG. 1. Gas chromatogram of volatiles from concentrated distillates of *A. flavus*. (4) 3-Methylbutanol, (6) 3-octanone, (9) 3-octanol, (11) 1-octen-3-ol, (15) 1-octanol, (17) *cis*-2-octen-1-ol.

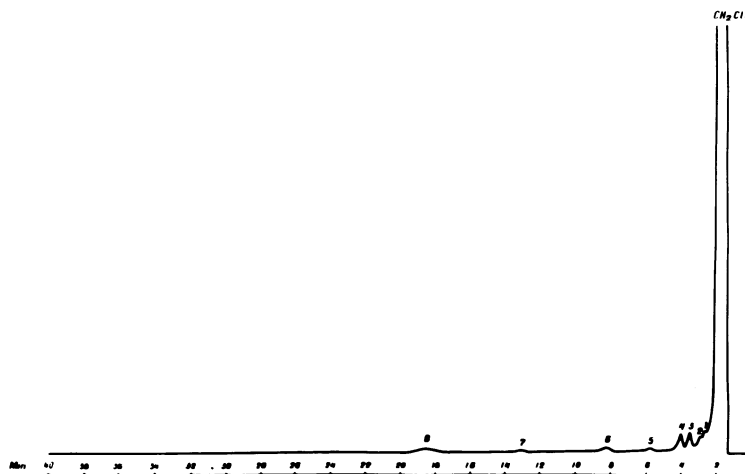


FIG. 2. Gas chromatogram of volatiles from concentrated distillates of coarse wheat meal. (3) 3-Methylbutanol, (4) pentanol, (5) hexanol, (6) 1-octen-3-ol, (7) 1-octanol, (8) 2-octen-1-ol.

TABLE 1. Volatile compounds occurring in the concentrated distillate from cultures of *A. flavus*

No. of peak	Compound	Presence identified by ^a		
		GLC	MS	IR
4	3-methylbutanol	+	+	
6	3-octanone	+	+	
9	3-octanol	+	+	
11	1-octen-3-ol	+	+	+
15	1-octanol	+	+	
17	<i>cis</i> -2-octen-1-ol	+	+	+

^a Abbreviations: GLC, gas-liquid chromatography; MS, mass spectrometry; IR, infrared spectrometry.

2-octen-1-ol isolated from the mold was found to differ from the synthetic preparation (Compagnie Parento, Inc.) within the IR range at 700 to 1,050 cm^{-1} and at 1,650 to 1,670 cm^{-1} (Fig. 4 and Table 3). According to the data obtained, the synthetic preparation is in the *trans* form, and that isolated from the mold is of the *cis* form. The IR spectra of these compounds at wave numbers 2,700 cm^{-1} to 3,030 cm^{-1} gave a very intense band; therefore to obtain an on-scale spectrum at these wave lengths, a 0.1-mm cell was used instead of 0.43 mm.

Pure 1-octen-3-ol yields a strong characteristic fungal-resinous odor. In concentrations close to the threshold value, 0.01 $\mu\text{g}/1$ ml of water, the odor resembled that of mushroom. 2-octen-1-ol exhibits a characteristic and strong musty-oily odor. The recognition threshold of this compound in water is 0.1 $\mu\text{g}/1$ ml. According to results obtained in this laboratory, these two compounds, i.e., 1-octen-3-ol and 2-octen-1-ol, are produced by molds other than *A. flavus*;

TABLE 2. IR band interpretations for authentic oct-1-en-3-ol (A) and the compound trapped from concentrated distillates of *A. flavus* (B)

Band	Wave length (cm^{-1}) ^a	
	Authentic compound (A)	Trapped compound (B)
=CH ₂	927 s	927 m
=CH	995 s	996 m
C—O stretch	1,020 m	1,020 w
O—H deformation	1,260 w	1,260 vw
C—H symmetric deformation in CH ₃	1,380 m	1,380 vw
=CH ₂ in plane deformation	1,427 m	1,430 w
C—H asymmetric deformation in CH ₃	1,470 m	1,470 m
C=C stretch	1,647 vw	1,650 vw
Overtone	1,745 vw	
Overtone	1,850 vw	
C—H asymmetric stretch in CH ₂	2,860 s	2,860 s
C—H asymmetric stretch in CH ₃	2,875 s	2,860 s
C—H asymmetric stretch in CH ₂	2,935 s	2,930 s
C—H asymmetric stretch in CH ₃	2,962 s	2,950 s
C—H stretch in CH=CH ₂	3,085 w	3,070 w
O—H stretch frequency	3,620 m	3,610 w

^a Abbreviations: s, strong; m, medium; w, weak; v, variable.

however, the quantities and their relative abundances are different.

A. flavus was also cultivated on corn, barley, oats, rice, soybeans, and rape. In the head space samples, in all the cases there were more and

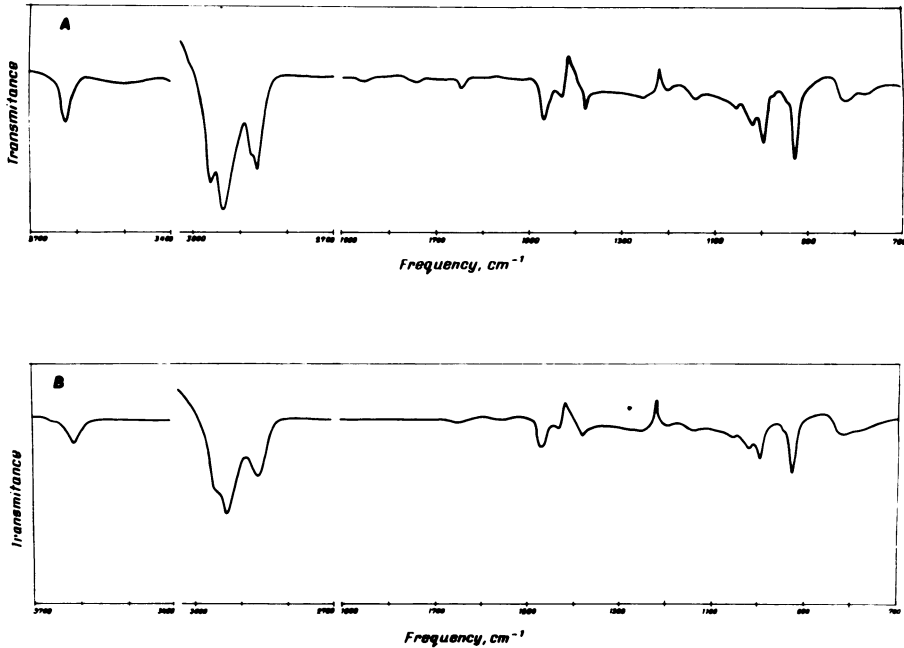


FIG. 3. Infrared spectra of carbon tetrachloride solutions of: (A) authentic 1-octen-3-ol and (B) compound isolated from *A. flavus* culture.

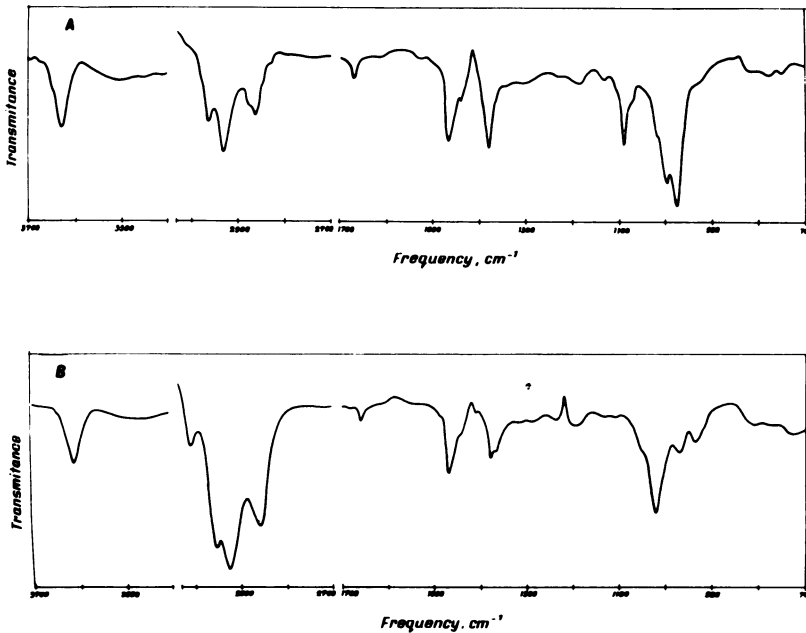


FIG. 4. Infrared spectra of carbon tetrachloride solutions of: (A) authentic 2-octen-1-ol and (B) compound isolated from *A. flavus* culture.

less volatile substances. The cereal grains were found to be richer in volatiles than the oil seeds. In all cases, 1-octene-3-ol and 2-octen-1-ol were found, only in different amounts.

In wheat, some authors stress the absence of such compounds (12, 15). In our experiments, however, in which strongly concentrated distillates were used, these two compounds were

TABLE 3. IR band interpretations for authentic 2-octen-1-ol (A) and the compound trapped from concentrated distillates of *A. flavus* (B)

Band	Wave length (cm ⁻¹) ^a	
	Authentic compound (A)	Trapped compound (B)
C—H deformation in <i>cis</i> HC=CH		730 vw
=C—H deformation		935 w
CH deformation in <i>trans</i> HC=CH	975 s	968 w
C—O stretch	1,020 vw	1,020 s
C—O stretch	1,000 s	
C—H symmetric deformation in CH ₃	1,380 s	1,378 m
C—H asymmetric deformation	1,468 m	1,468 m
C=C stretch		1,658 w
/cis/		
/trans/	1,670 w	
C—H symmetric stretch in CH ₂	2,863 s	2,855 s
C—H symmetric stretch in CH ₃	2,875 s	2,870 p
C—H asymmetric stretch in CH ₂	2,930 s	2,925 s
C—H asymmetric stretch in CH ₃	2,960 s	2,955 s
C—H stretch in HC=CH		3,005 m
O—H stretch frequency	3,625 m	3,610 m

^a Abbreviations: s, strong; m, medium; w, weak; v, variable; p, shoulder.

found in the control samples (Fig. 2). In the sample from the *A. flavus* culture, however, the quantity of these compounds was about 1,000 times higher. The occurrence of these two compounds even in sound wheat grain may be due to the infection of the grain by interior microflora (17). Several papers on 1-octene-3-ol are in the literature; it has been detected in milk and milk products (10), soybean and its products (18, 19), and potatoes, in which it occurs as the main component of the volatile fraction (5). 1-Octen-3-ol was also found in clover, in which it also comprises the main component of the volatiles (11), and it is also found in cranberries (3) and black currants (2). 1-Octen-3-ol was first isolated from a Japanese mushroom by Murahashi (Chem. Abstr., 32:3755), and it has also very recently been identified in another mushroom, *Agaricus bisporus* (6).

There is scant information available on the occurrence of 2-octen-1-ol in foods, although it has recently been reported in *A. bisporus*.

The occurrence of these two compounds in food products is due, first of all, to the development of microflora. The molds, as well as their spores, are ubiquitous on foodstuffs, and under

conditions favoring their development they produce these compounds.

The possibility cannot be completely eliminated that these compounds are produced in a purely chemical way through oxidation of linoleic acid (9).

In certain foodstuffs, these compounds may come from animal feed. According to Honkanen and Moio (11), 1-octen-3-ol entered the milk via the bloodstream and originated from the fodder of the cows.

Generally speaking, the occurrence of 1-octen-3-ol and especially 2-octen-1-ol in agricultural products can indicate their contamination by molds, although in certain products the origin of 1-octen-3-ol may be quite different.

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