

Pyrolysis-Gas-Liquid Chromatography of Fungi: Differentiation of Species and Strains of Several Members of the *Aspergillus* *flavus* Group

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Four fungi of the *Aspergillus flavus* group were differentiated to the species level and strain level by pyrolysis-gas-liquid chromatography. Comparisons of pyrochromatograms revealed more similarities than dissimilarities among both species and strains in the pyrolytic elution patterns. Quantitative analysis was made by comparing the number of peaks in which two strains or reference species agreed or disagreed, the degree of superimposability between the pyrolytic elution patterns of strains and reference species, and the presence or absence of peaks for strain pairs within each species. The accuracy and precision of these techniques suggest that pyrolysis-gas-liquid chromatography may have wide application in the detection, enumeration, and identification of fungi by nonmycologically trained personnel.

Methods which have been used to characterize microorganisms include analysis of volatile products in head space (6), fatty acid composition (3, 8, 12), deoxyribonucleic acid base composition (5, 11), infrared spectroscopy (20), electrophoresis (19), and gas chromatography (1, 2). However, results from studies with these methods give an incomplete picture of the genotypic expression of microorganisms and are usually too tedious and time consuming for routine analysis.

A comparatively new method for identifying and characterizing microorganisms combines pyrolytic degradation of cells or cell fragments with gas-liquid chromatographic analysis (15). Resulting pyrochromatograms show elution patterns characteristic of genus, species, and, in some instances, strains (16). Pyrolysis-gas-liquid chromatography (PGLC) has previously been used to characterize bacteria (14), fungi (13), and nucleotides or nucleosides (22).

Differentiation of species or strains of fungi by PGLC has not been comprehensively described, although Oyama and Carle (13) examined one taxon from each of several fungal genera. Moreover, no workers, with the exception of Cone and Lechowich (4) who were unsuccessful in differentiating strains of *Clostridium botulinum*, have used the PGLC method to differentiate strains of microorganisms based on analysis of their spores.

This study evaluates the FGLC method for use in routine laboratory identification of economically important spoilage fungi by nonmycologically trained personnel. If the FGLC method is linked to electronic data-processing systems, fungal identification by nonmycologists could consist of placing a sample of an unknown in a PGLC instrument. The pyrolytic elution pattern (PEP) of the unknown could then be compared with the PEP of most spoilage species to correctly identify the organism. The potential of PGLC for electronic data processing has been discussed by Reiner and Ewing (17), who reported that subspecies among several gram-negative bacteria could be differentiated by their unique pyrolysis profiles. They point out that pyrolysis profiles are directly related to the chemical composition of the microorganism and reflect actual differences among the closely related forms rather than possible inherent variations in the PGLC technique. In a later paper, Reiner and Kubica (18) showed that certain constant peaks within the PEP range could aid in differentiating, classifying, and possibly identifying mycobacteria based on the precision and accuracy of the PGLC technique. The predictive nature of PGLC may aid workers engaged in studies of spoilage and adulteration of meats, seeds, and grains by *Aspergillus flavus* group members, especially in connection with

afatoxin production. This paper reports studies on four members of the *A. flavus* group.

MATERIALS AND METHODS

Microorganisms. Table 1 shows the source of fungi of the *A. flavus* group that were used in this study. These fungi were maintained on Czapek-Dox agar slants at 3 C until used.

Culture media and growth conditions. Conidia were transferred from slants to Czapek-Dox agar medium in plastic petri dishes (15 by 100 mm) and incubated in darkness at 25 C for 7 days. Six plate cultures were prepared for each strain for analysis at two different times.

Harvesting of conidia. Conidia were washed from mycelial mats with methanol, concentrated twofold over nitrogen, and lyophilized. Lyophilized conidia were transferred to 1-ml tared-glass vials and weighed. Methanol (1 ml) was added to the vials which were sealed with Teflon-lined screw caps. Sample vials were stored at -20 C.

Controls. Control samples (noninoculated Czapek-Dox agar medium) were treated in the manner used for harvesting conidia.

Pyrolysis-gas-liquid chromatographic analysis. A dual-column gas-liquid chromatograph (series 5000; Barber-Colman Co., Rockford, Ill.), fitted with a hydrogen-flame ionization detector, and a pyrolyzer (model 5180) were used. The general procedure for quantitative analysis of conidia was to reduce the temperature of the pyrolysis ribbon to -50 C with liquid air before addition of 500 µg of conidia in methanol. Oxygen and volatiles (boiling point of 175 C or less) were removed from the sealed chamber by purging for 5 min with helium. Pyrolysis was carried out at 900 ± 10 C for 10 sec in a continuous stream of carrier within closely defined instrumental parameters for quantitative reproducibility. Samples were analyzed in duplicate. Pyrochromatograms recorded as "fingerprints" or PEP represent detection of thermal fragmentation products.

Operating conditions for PGLC. "U"-shaped glass columns, 6 feet (1.8 m) by 4 mm inner diameter, were packed with Gas-Chrom Q (80 to 100 mesh) coated with 10% Carbowax 20 M. The packed column was conditioned for 2 weeks at 180 C in a forced stream of helium before use. Operating parameters were as follows.

We used a hydrogen-flame ionization detector with a sensitivity of 2×10^{-10} amp (signal attenuated). The gases were: air, 15 psi-gauge, hydrogen, 35 psi-gauge, helium 25 psi-gauge. The temperatures (C) were: pyrolyser body heat, 175, injection port, 300, detector, 320; pyrolyser filament (nickel), 900 ± 10 C for 10 sec. Temperature programs were: initial isothermal period and temperature, 5 min at 40 C; linear programmed-temperature increase rate, 7.5 C per min to a final temperature of 200 C; isothermal at 200 C until completion of analysis. Chart speed was 20 inches per hr.

The distance (millimeters) from emergence of the initial recorder tracing to a peak appearing in all samples evaluated was used for reference. The average distance of the reference peak was 137 mm. Other

TABLE 1. Sources of aspergilli used in pyrolysis-gas-liquid chromatographic analysis

Species	Strain or isolate no.	Source ^a
<i>A. parasiticus</i> Speare.....	HS-5	H. Schroeder, MQ-ARS
<i>A. parasiticus</i> Speare.....	MK-72/64	M. Kulik, MQ-ARS
<i>A. parasiticus</i> Speare.....	HS-64R8	H. Schroeder, MQ-ARS
<i>A. flavus</i> Link.	M-338	A. Schindler, FDA
<i>A. flavus</i> Link.	HS-54	H. Schroeder, MQ-ARS
<i>A. flavus</i> Link.	QM-3480	R. Roegner, FDA
<i>A. tamarii</i> Kita.....	MK-35/64	M. Kulik, MQ-ARS
<i>A. tamarii</i> Kita.....	MK-4/67	M. Kulik, MQ-ARS
<i>A. tamarii</i> Kita.....	MK-65/64	M. Kulik, MQ-ARS
<i>A. oryzae</i> (Ahlburg) Cohn.....	NRRL A-9822 ^b	J. Ellis, NURDD
<i>A. oryzae</i> (Ahlburg) Cohn.....	NRRL A-9824	J. Ellis, NURDD
<i>A. oryzae</i> (Ahlburg) Cohn.....	NRRL A-9825 ^b	J. Ellis, NURDD

^a Abbreviations: FDA, Food & Drug Administration; MQ-ARS, Market Quality Research Division, Agricultural Research Service, U. S. Department of Agriculture; NURDD, Northern Utilization Research & Development Division, U. S. Department of Agriculture.

^b Culture numbers NRRL A-9822 and NRRL A-9825 have been changed to NRRL 3487 and NRRL 3488, respectively (C. W. Hesseltine, *personal communication*).

peaks common to all tracings of pyrochromatograms were also used for reference.

Criteria used in comparing strains or species were as follows: (i) determination of the number of peaks in the PEP in which two strains or reference species agree or disagree; (ii) visual examination of pairs of pyrochromatograms of strains for superimposability of the PEP; (iii) bar graph composite averages of PEP for the three strains of each of the four species; (iv) occurrence of peaks common to two or four reference

species; (v) the presence or absence of peaks at a given retention time for pairs of strains within each species.

RESULTS AND DISCUSSION

A common reference peak appeared at 134.5 to 139.5 mm from the initial peak (ordinate) on all pyrochromatograms. Tracings of pyrochromatograms were adjusted by using 137 mm as the corrected distance of the reference peak from the origin. Two peaks were considered similar if their retention times (R_t) were within 1% of each other.

Figures 1 to 4 show tracings of pyrochromatograms of each strain of the four *A. flavus* group species studied. Tracings in each figure are aligned to show representative pyrochromatograms of three strains of each species.

Reference species for each species studied were constructed by recording every peak common or uncommon for PEP of all strains. Similarities and differences between all six pairs of reference species, based on the number of peaks having the same R_t in each pair, are shown in Table 2. The many similar peaks (i.e., 23) shared by the four reference species suggest that they may form a natural group. Similarity values (S), expressed as % S , were calculated from the total number of peaks (i.e., 123) present in all possible reference species PEP pairs from the following modified formula of Sokal and Sneath (21): % $S = N_s / (N_s + N_d) \times 100/1$, where N_s is the number of similar peaks present in pairs of reference species, and N_d is the number of peaks present in the first reference species but not in the second, plus the number present in the second reference species but not in the first.

If this value (i.e., S) for the *A. flavus*-*A. tamarii* combination (0.58) is made equal to 100, then the adjusted per cent similarity values are as follows: *A. oryzae*-*A. tamarii*, 95%; *A. flavus*-*A. oryzae*, 82%; *A. flavus*-*A. parasiticus*, 59%; *A. oryzae*-*A. parasiticus*, 52%; and *A. parasiticus*-*A. tamarii*, 51%. These data suggest that *A. tamarii* is more similar to *A. flavus* (the type species) than are the other group members, and *A. parasiticus* shows the least degree of similarity to the other reference species studied.

Tracings for each of the three strains of each species studied have been combined into composite bar graphs to show the number of strains with common, atypical, or dissimilar peaks, the average height for each peak, and the R_t for each peak (Fig. 5 to 8). Differences among strains are shown in these figures by the presence of dotted or cross-hatched bars indicating the absence of one or two peaks, respectively. These differences among strains may also be demonstrated by

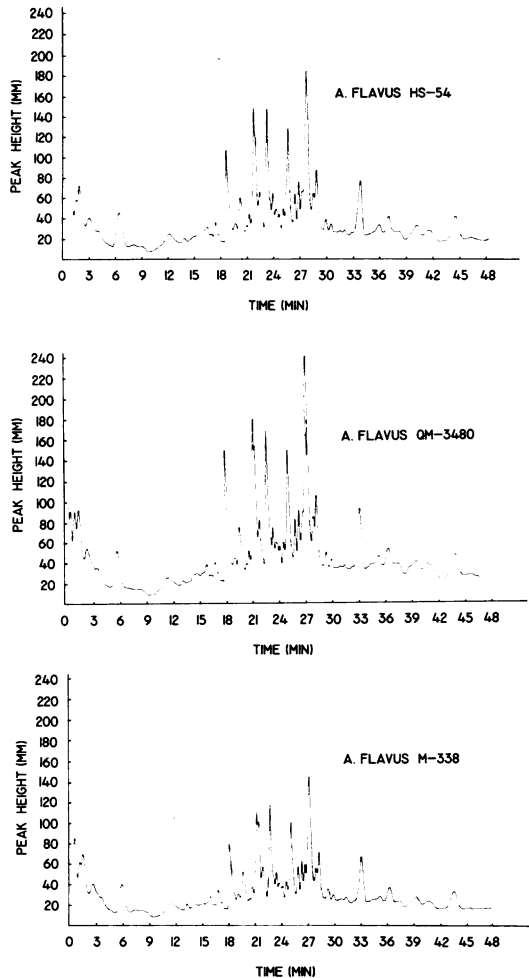


FIG. 1. Tracings of pyrochromatograms of *Aspergillus flavus* strains HS-54, QM-3480, and M-338.

constructing a grid in which the presence or absence of peaks for each strain is shown. We have used *A. oryzae* (Fig. 9) as an example of this method of presentation.

For electronic data processing, other methods of data treatment must be used. Although a two-state test (presence or absence of peaks) can be used to define a standard to record characters (i.e., differences or absence of peaks among strains) on which identification of the various strains and reference species may be made, closely related species and especially strains are usually difficult to differentiate. This problem may be partly resolved, for example, by constructing multiple-state tests in which this information [presence or absence of peaks (x, y)] is plotted against a third coordinate (z) such as peak height

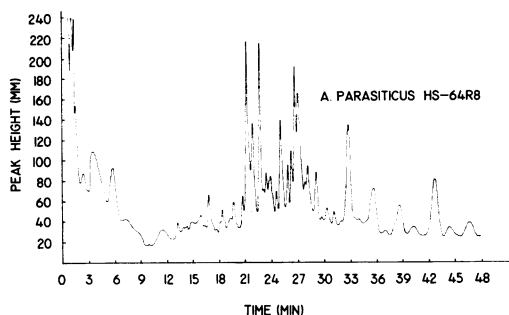
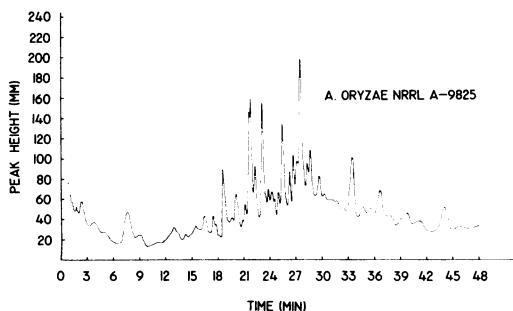
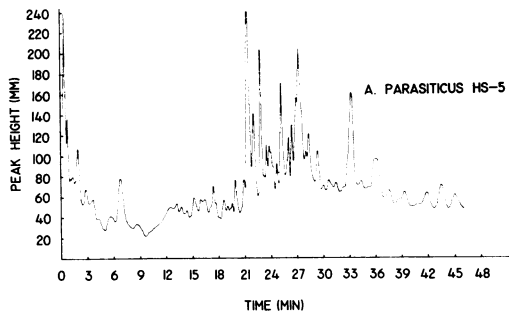
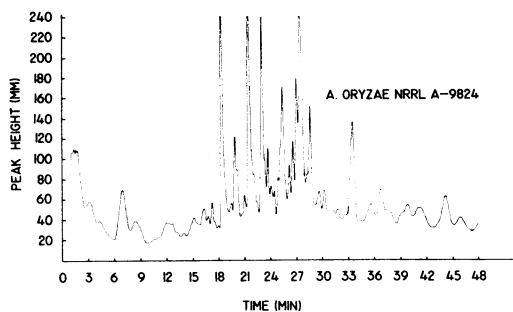
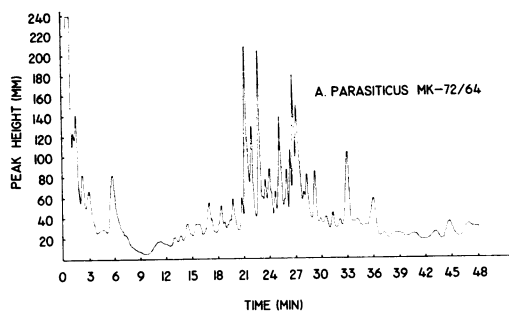
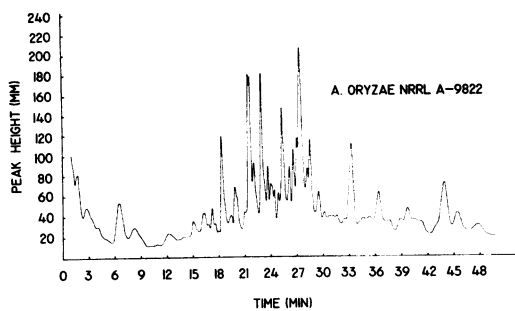


FIG. 2. Tracings of pyrochromatograms of *Aspergillus oryzae* strains NRRL A-9822, NRRL A-9824, and NRRL A-9825.

FIG. 3. Tracings of pyrochromatograms of *Aspergillus parasiticus* strains MK-72/64, HS-5, and HS-64R8.

in a three-dimensional mode. Each character corresponds to a given dimension and thus would disperse hyperellipsoidally. The dispersion of vectors x , y , and z would occur in decreasing order, i.e., the longest first, followed by the second orthogonal to the first, etc. A computer would be required to consolidate the original data on each reference species and strain into three coordinates to locate the points of reference in their final identification spaces.

Thus, unknown strains and species may be properly identified, provided these organisms match with all or with a predetermined number of points representative of the reference species or strains in the three-dimensional identification space. The computer would compare the PEP of

the unidentified organism with each PEP of the reference organisms. When reference groups share overlapping coordinates, the unknown organism would then be placed in the reference group with which it has the greater affinity. The computer would then indicate the points on which the unidentified organism differs from the principal reference group pattern.

Quantitative differentiation of strains also may be achieved by comparing the ratio of any given peak height to the reference-peak height or, where peak-height inversions appear, the ratio of inverted peak-heights relative to the reference-peak height. This may be shown by comparing the PEP of our two strains of *A. tamaritii* (Fig. 10). Eleven peaks with similar R_t have been numbered

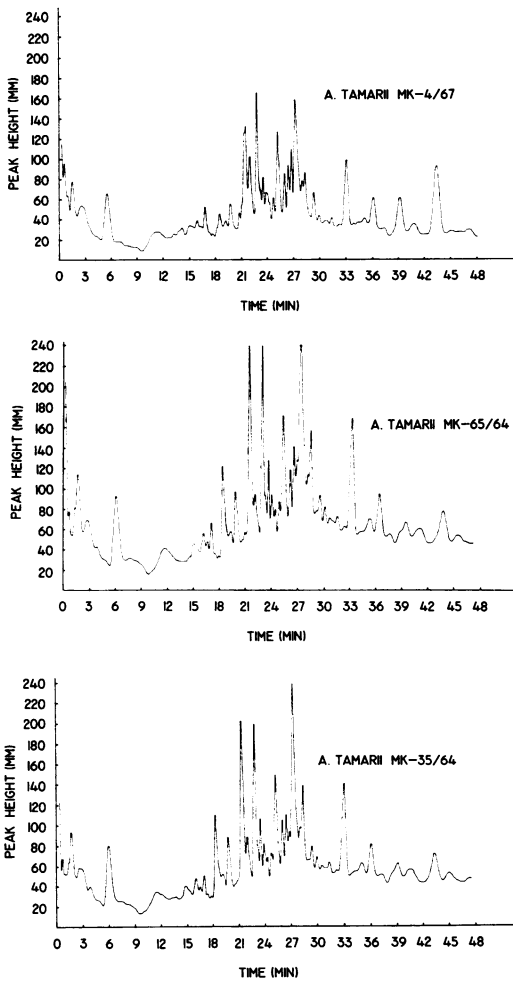


FIG. 4. Tracings of pyrochromatograms of *Aspergillus tamarii* strains MK-4/67, MK-65/64, and MK-35/64.

in the two pyrochromatogram tracings. Prominent peak inversions are observed between strains MK-4/67 and MK-35/64 in the heights of peaks 1 and 2, 3 and 5, 5 and 8, and 4 and 6. Also present are differences in the intensities of two peak heights relative to one another as may be seen in peaks 3 and 4, 4 and 5, 7 and 8, 9 and 10, and 10 and 11. The foregoing illustrates the nonseparability of two strains based only on a two-state test, thus necessitating the introduction of at least a third parameter for differentiation. In a multi-state test scheme for computer identification of unknown strains compared to reference strains, the addition of further axes, i.e., *x*, *y*, *z*, *q*, may be necessary (thus creating a four-dimensional mode). This could determine the affinity figure

TABLE 2. Comparison of similarity among pairs of reference species of four *Aspergillus flavus* group members

Pairs of reference species	No. of similar peaks	No. of dissimilar peaks ^a	Similarity values between pairs of reference species
<i>A. flavus</i> + <i>A. tamarii</i>	40	29	.58
<i>A. oryzae</i> + <i>A. tamarii</i>	43	35	.55
<i>A. flavus</i> + <i>A. oryzae</i>	36	40	.47
<i>A. flavus</i> + <i>A. parasiticus</i>	34	65	.34
<i>A. oryzae</i> + <i>A. parasiticus</i>	34	79	.30
<i>A. parasiticus</i> + <i>A. tamarii</i>	33	78	.30

^a Number of peaks present in the first reference species (left) but not in the second, plus the number of peaks present in the second reference species (right) but not in the first.

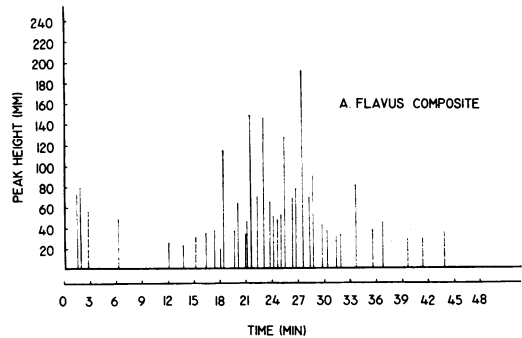


FIG. 5. Bar graph composite of pyrochromatograms for three strains of *Aspergillus flavus*. Solid lines refer to the presence of a peak common to the three strains; dotted lines indicate two of the three strains have a common peak; and cross-hatched lines denote the presence of a peak unique to only one of the three strains present at the indicated retention times.

which could best describe the group in which an unknown strain would fit in terms of identification space for each characteristic point relative to characteristic points assigned to reference strains.

Because of the great importance of members of the *A. flavus* group in toxic and nontoxic spoilage of animal and plant products, it is very desirable to have a clear understanding of these taxa. Unfortunately, their inherent variation makes identification by nonmycologically trained personnel by using morphological characteristics quite difficult.

Hesseltine et al. (7) attempted to distinguish species of the *A. flavus* group based on carbon-assimilation patterns and other biochemical

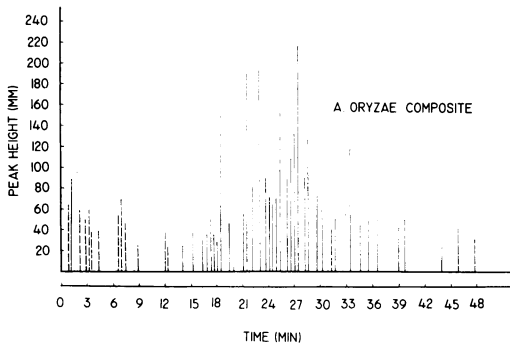


FIG. 6. Bar graph composite of pyrochromatograms for three strains of *Aspergillus oryzae*. Solid lines refer to the presence of a peak common to the three strains; dotted lines indicate two of the three strains have a common peak; and cross-hatched lines denote the presence of a peak unique to only one of the three strains present at the indicated retention times.

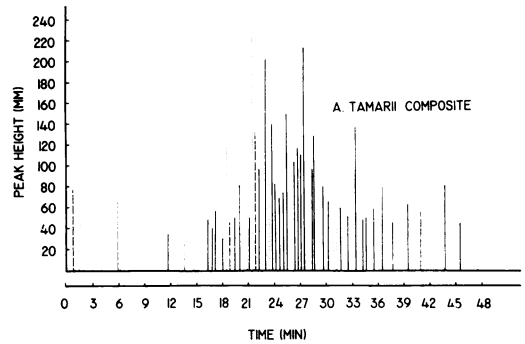


FIG. 8. Bar graph composite of pyrochromatograms for three strains of *Aspergillus tamarii*. Solid lines refer to the presence of a peak common to the three strains; dotted lines indicate two of the three strains have a common peak; and cross-hatched lines denote the presence of a peak unique to only one of the three strains present at the indicated retention times.

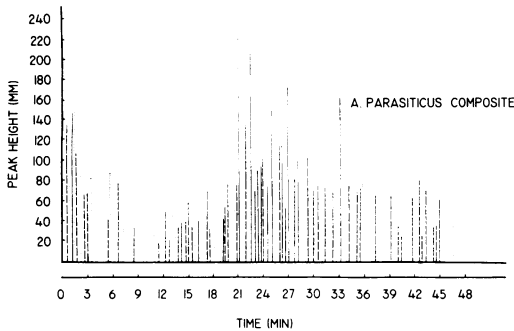


FIG. 7. Bar graph composite of pyrochromatograms for three strains of *Aspergillus parasiticus*. Solid lines refer to the presence of a peak common to the three strains; dotted lines indicate two of the three strains have a common peak; and cross-hatched lines denote the presence of a peak unique to only one of the three strains present at the indicated retention times.

methods. Twenty-eight representative strains of the *A. flavus* group were evaluated by these workers in an attempt to separate species on the basis of (i) utilization of various carbon sources, (ii) production of specific fungal metabolites (i.e., kojic acid and aflatoxins), (iii) production of amylase, (iv) presence or absence of growth at various temperatures, and (v) production of sclerotia under various concentrations of NaNO_3 and sucrose. The authors did not find any physiological or biochemical tests that could be used to distinguish *A. flavus* group species, although aflatoxin production in *A. flavus* and *A. parasiticus* may be useful.

The results of our study support the findings of Kulik and Brooks (9), who demonstrated that, on the basis of the migration of soluble proteins during electrophoresis, *A. flavus* was more closely associated with *A. oryzae* than with *A. parasiticus*. Unfortunately, electrophoresis is generally too

ASPERGILLUS
ORYZAE
ISOLATE

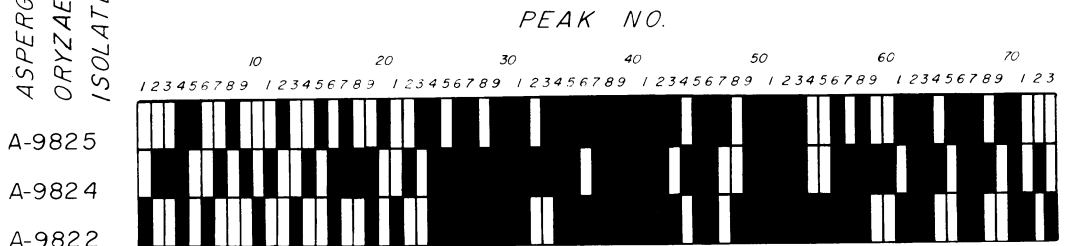


FIG. 9. Degree of resemblance of pyrochromatograms from three strains of *Aspergillus oryzae*. Presence and absence of peaks are denoted by scored and blank blocks, respectively.

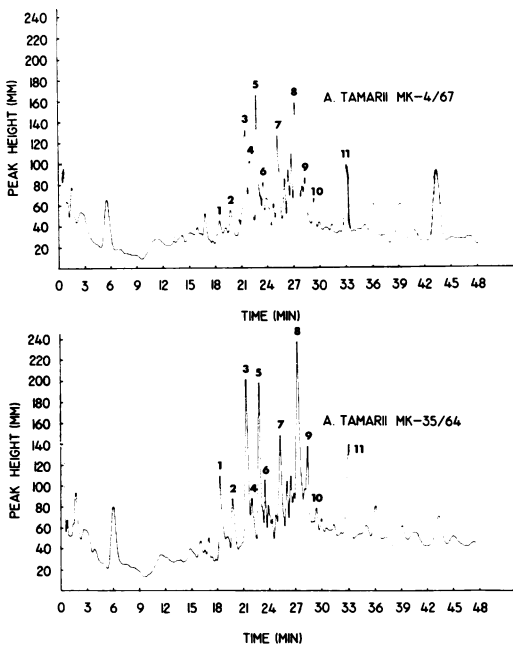


FIG. 10. Tracings of pyrochromatograms of *Aspergillus tamarii* strains MK-4/67 and MK-35/64 showing interstrain variation based on relative peak heights and peak height-inversions.

tedious and involved for routine analysis. However, data presented in the current study suggest that the PGLC technique may eventually be used by nonmycologically trained personnel either for routine work or for more specific problems involving characterization of microorganisms and their metabolites. It is recognized that before PGLC can be practically employed for specific detection and identification of *Aspergillus* species, a large number of strains should be evaluated by using double-blind tests (coded specimens). Members of the *A. glaucus* group are currently under evaluation.

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