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# PYROLYSIS GAS CHROMATOGRAPHY AS AN AID TO THE IDENTIFICATION OF *PENICILLIUM* SPECIES

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## SUMMARY

Using pyrolysis gas chromatography it was possible to identify each of a series of eleven *Penicillium* species and the related species *Aspergillus niger* CMI 31821 and *Neurospora crassa* CMI 75723, based on relative peak heights and retention times of the most reproducible peaks in each pyrogram.

## INTRODUCTION

The methods currently available for the detection and classification of microorganisms are time consuming and non-quantitative. There have been several attempts to apply physical methods to the identification of bacteria, in particular infrared spectrophotometric methods have been examined by Randall *et al.*[1] and Levine *et al.*[2,3], with limited success in obtaining reproducible results[4]. Partial identification is possible in the case of certain bacteria[5]. The related technique of attenuated total reflection has been examined and the differences between normal and diseased tissues can be demonstrated[6], however, as these spectra provide essentially the same information as transmittance spectra, it is unlikely to be any more successful for identification purposes.

Pyrolysis gas–liquid chromatography (GLC) gives characteristic and reproducible fragment patterns which can be used as fingerprints of the original samples. Reiner[7] applied this technique to bacteria, attempting to exploit the possible chemical differences between bacterial strains of similar antigenic or pathogenic character. He showed that each organism gave a unique pyrogram, although the profiles obtained for *Escherichia coli* and *Shigella boydii* were similar[8]. Subsequently it was shown that such programs could provide a basis for classification at subspecies level[9], in particular the *Salmonella*[10]. Oyama[11] and Oyama and Carle[12] agreed with these observations but showed that the media should be identical when considering different organisms.

The technique of pyrolysis GLC has also been used by Myers and Watson[13] in the diagnosis of viral and fungal diseases in plants and by Hall and Bennett[14] in the identification of cockroaches at species level. The coupling of a mass spectrometer

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to the pyrolysis GLC system has permitted an examination of the chemical structure of pyrolysis products and the potential of the technique for the detection of extra-terrestrial life has been examined by Simmonds[15] and Simmonds *et al.*[16].

Prior pyrolysis followed by silylation of the less volatile polar products allows separation and identification of algae and *E. coli* in sea-water[17]. Combined pyrolysis–mass spectroscopy has been applied by Anhalt and Fenselau for the characterisation of certain pathogenic bacteria[18].

The *Penicillia* are ubiquitous and can grow on a variety of organic substrates[19] and can be responsible for spoilage and economic loss. Several species are important

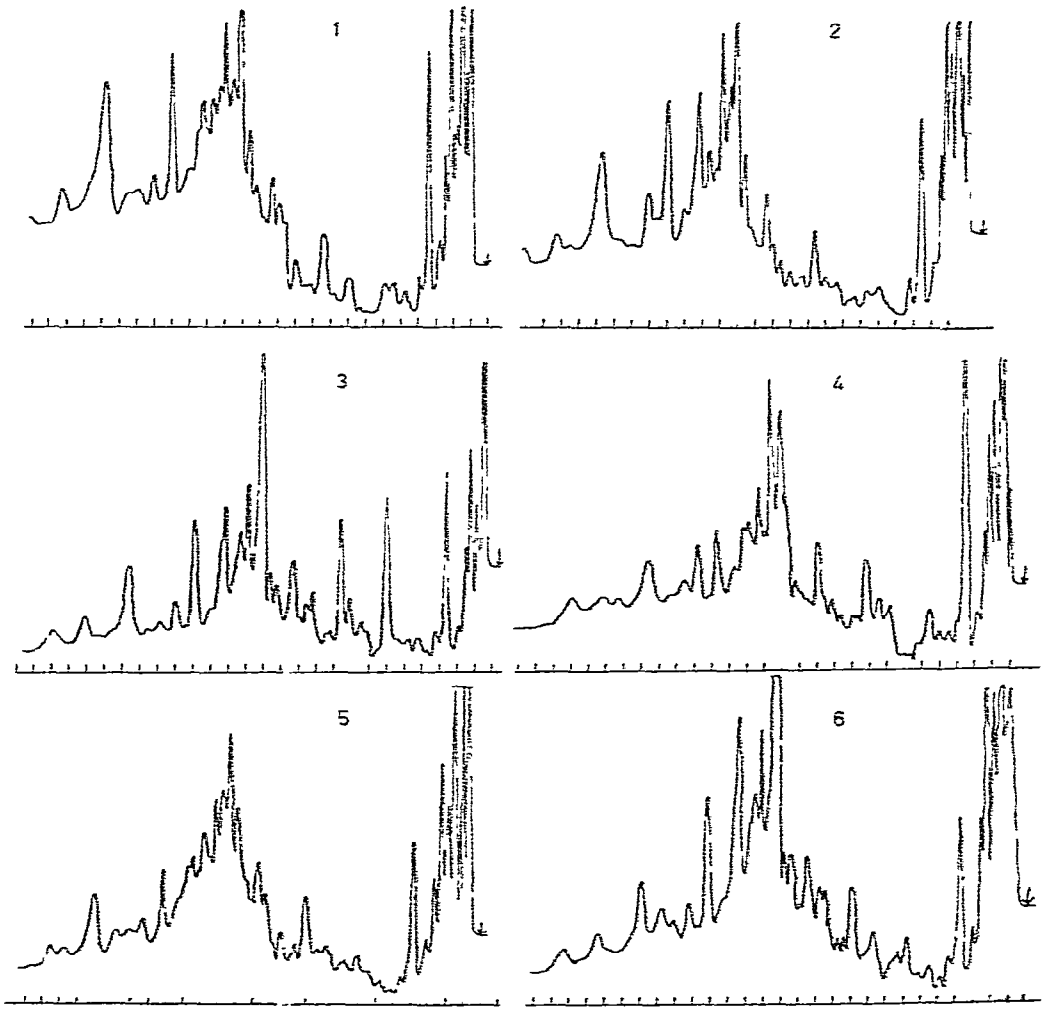


Fig. 1. Pyrograms for *Penicillium* and related species: (1) *P. chrysogenum*, CMI 26211; (2) *P. luteum*, A40; (3) *P. griseofulvum*, A41; (4) *P. lanosoviridae*, CMI 39818; (5) *P. charlesii*, CMI 40232; (6) *P. lavendulum*, CMI 40570; (7) *P. parvum*, CMI 40587; (8) *P. thomii*, CMI 40027; (9) *P. rubrum*, CMI 40036, (10) *P. patulum*, CMI 28808; (11) *P. pulvillorum*, CMI 29215, (12) *Aspergillus niger*, CMI 31821; (13) *Neurospora crassa*, CMI 75723.

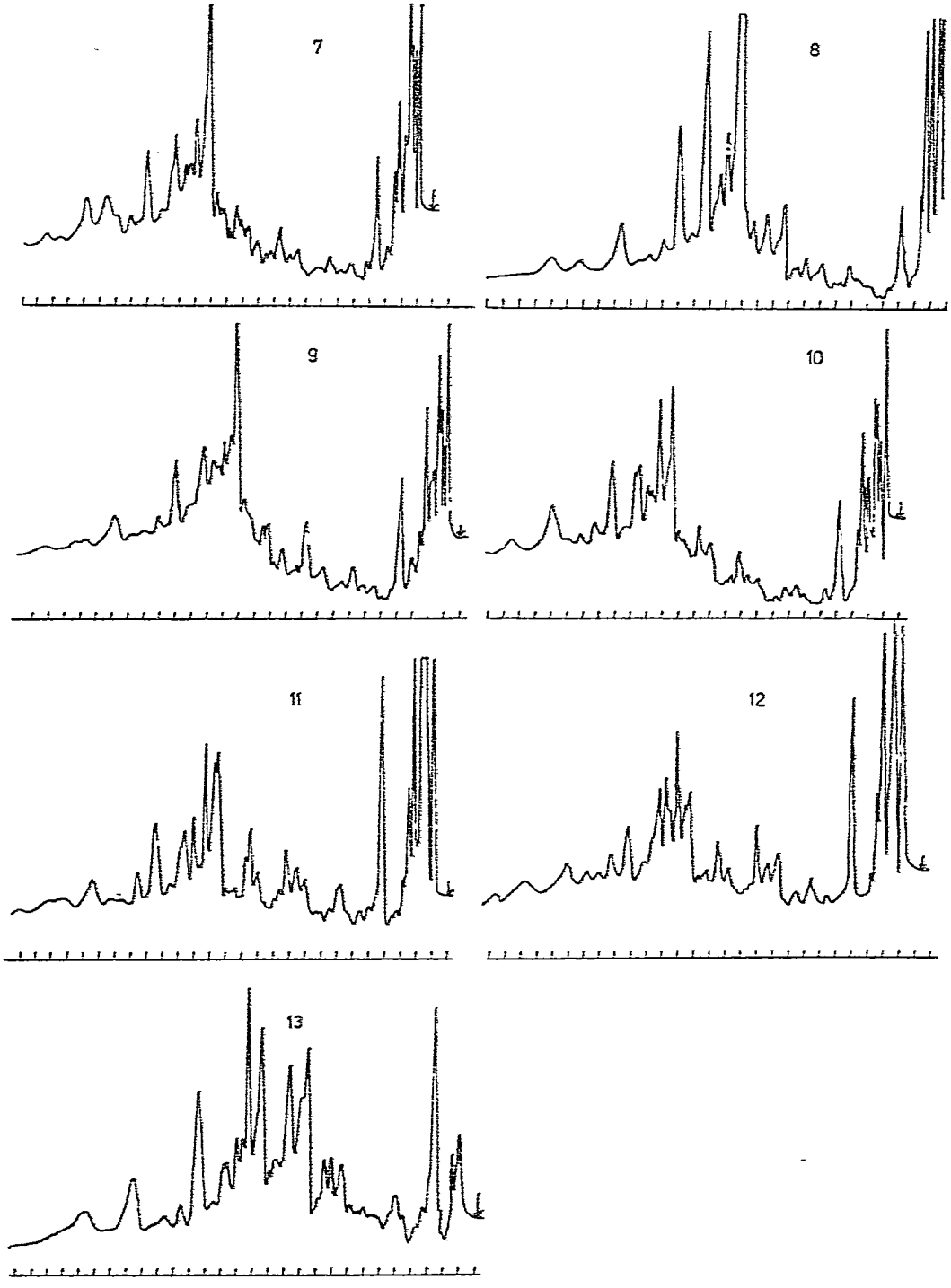


Fig. 1 (*continued*).

TABLE I

RELATIVE PEAK HEIGHTS OF PYROGRAMS OF *P. parvum*

| *Peak No.* | *Relative peak height* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *Range* |
| 1 | 158.8 | 122.5 | 134.3 | 141.2 | 160.6 | 129.8 | 38.1 |
| 2 | 13.7 | 10.3 | 14.6 | 8.7 | 9.4 | 11.5 | 5.9 |
| 3 | 15.7 | 13.3 | 10 8 | 13.2 | 10.6 | 14.2 | 5.1 |
| 4 | 5.8 | 7.5 | 3.8 | 5.9 | 8.5 | 7.6 | 4.7 |
| 5 | 21 5 | 23.3 | 29.2 | 19.6 | 27.5 | 25.2 | 9.6 |
| 6 | 10.8 | 11.6 | 13.2 | 16.5 | 9.5 | 15.2 | 7.0 |
| 7 | 19.6 | 25.0 | 27.3 | 18.2 | 24 5 | 19.8 | 9.1 |
| 8 | 6.9 | 8.3 | 10.2 | 12.4 | 9.7 | 6.5 | 5.9 |
| 9 | 47.0 | 50.0 | 55.5 | 47.2 | 51.2 | 48.1 | 8.5 |
| 10 | 8.8 | 15.0 | 15.8 | 7.6 | 9.3 | 12.1 | 8.2 |
| 11 | 5.5 | 5.0 | 5.6 | 7.8 | 9.9 | 4.9 | 5 0 |
| 12 | 23.5 | 31.6 | 32.1 | 20 2 | 24.1 | 30.1 | 11.9 |
| 13 | 29.4 | 35.0 | 37.2 | 29 9 | 38.6 | 31.3 | 9.2 |
| 14* | 35.3 | 37.5 | 37.2 | 34.9 | 37.0 | 36.7 | 2.6 |
| 15 | 23.5 | 30.0 | 32.3 | 23.6 | 24 2 | 26.5 | 8.8 |
| 16 | 29.4 | 21.6 | 24.2 | 26.2 | 19.8 | 20.5 | 9.6 |
| 17 | 311.7 | 278 3 | 291.3 | 279.1 | 285.2 | 301 2 | 33.4 |
| 18* | 78.4 | 76.6 | 77.5 | 76.8 | 78.5 | 76 9 | 1.9 |
| 19 | 13.7 | 6.6 | 12.5 | 8.1 | 11.7 | 9.7 | 7.1 |
| 20 | 17.6 | 21.6 | 22.3 | 15 6 | 18.1 | 19.3 | 6.7 |
| 21* | 92.1 | 88.3 | 91.1 | 89.8 | 88.7 | 89.7 | 3.8 |
| 22* | 100.0 | 100 0 | 100.0 | 100.0 | 100.0 | 100.0 | 0.0 |
| 23 | 18.6 | 25.0 | 20.0 | 15.2 | 26.1 | 21.1 | 10.9 |
| 24 | 11.7 | 8.3 | 6.2 | 12.2 | 11.0 | 8.4 | 6.0 |
| 25 | 32.3 | 18.3 | 30.0 | 19.3 | 25.2 | 31.3 | 14.0 |
| 26 | 43.1 | 51.6 | 43.2 | 57.1 | 47.2 | 45.1 | 14.0 |
| 27 | 3.9 | 5.8 | 4 0 | 5.9 | 6.2 | 4.3 | 2.3 |
| 28 | 10.2 | 14.3 | 11.2 | 12.3 | 15.1 | 14.0 | 4.9 |
| 29* | 15 3 | 15 8 | 15.7 | 16.1 | 16.0 | 15.4 | 0.8 |

* Most reproducible peaks.

TABLE II

RELATIVE PEAK HEIGHTS AND AREAS FOR THE MOST REPRODUCIBLE PEAKS IN PYROGRAMS OF *P. parvum*

| | *Height* | | | | | *Area* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *14* | *18* | *21* | *22* | *29* | *14* | *18* | *21* | *22* | *29* |
| | 35.3 | 78.4 | 92.1 | 100 | 15.3 | 34.6 | 75.2 | 91.1 | 100 | 16 8 |
| | 37.5 | 76.6 | 88.3 | 100 | 15.8 | 35.2 | 75.7 | 89.4 | 100 | 17.3 |
| | 37.2 | 77.5 | 91.1 | 100 | 15.7 | 37.0 | 75.0 | 92.2 | 100 | 17.4 |
| | 34.9 | 76.8 | 89 8 | 100 | 16.1 | 35.5 | 76 2 | 90.9 | 100 | 16 8 |
| | 37.0 | 78 5 | 88.7 | 100 | 16.0 | 36.1 | 75.3 | 90.7 | 100 | 16.9 |
| | 36 7 | 76.9 | 89.7 | 100 | 15.4 | 35.7 | 74.9 | 94.7 | 100 | 16.8 |
| Average | 36.4 | 77.3 | 89.9 | | 15.7 | 35.6 | 75.5 | 91.6 | | 16.9 |
| % Variation | 3.6 | 1.2 | 2.1 | | 2.5 | 3.4 | 0.9 | 2.9 | | 1.8 |

agents in food production and in industrial fermentations. The taxonomy of the genus is very complex[20] and because of the paucity of obvious taxonomic characters and the arbitary nature of many of the groupings, the genus remains taxonomically difficult even to the expert[21]. It was considered that the use of pyrolysis GLC could aid in rapid and reproducible identification of this genus and this idea is examined herein.

## EXPERIMENTAL

### *Preparation of samples*

The *Penicillium* species studied were: (1) *P. chrysogenum*, CMI 26211, (2) *P. luteum*, A40, (3) *P. griseofulvum*, A41, (4) *P. lanosoviridae*, CMI 39818, (5) *P. charlesii*, CMI 40232, (6) *P. lavendulum* CMI 40570, (7) *P. parvum*, CMI 40587, (8) *P. thomii*, CMI 40027, (9) *P. rubrum*, CMI 40036, (10) *P. patulum*, CMI 28808, and (11) *P. pulvillorum*, CMI 29215. For comparative purposes (12) *Aspergillus niger*, CMI 31821, and (13) *Neurospora crassa*, CMI 75723, were also used.

To remove any possibility of contamination with complex materials, the

TABLE III

RETENTION DATA OF THE COMPONENTS DERIVED FROM *P. parvum*

| *Peak No.* | *Retention time (min)* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *Average* |
| 1 | 7.5 | 7.6 | 7.5 | 7.8 | 7.5 | 7.6 | 7.6 |
| 2 | 8.4 | 8.5 | 8.4 | 8.5 | 8.5 | 8.5 | 8.5 |
| 3 | 10.4 | 10.5 | 10.4 | 10.5 | 10.3 | 10.4 | 10.4 |
| 4 | 11.5 | 11.6 | 11.4 | 11.4 | 11.5 | 11.5 | 11.5 |
| 5 | 13.0 | 13.2 | 13.1 | 13.3 | 13.2 | 13.1 | 13.2 |
| 6 | 14.3 | 14.4 | 14.2 | 14.5 | 14.1 | 14.3 | 14.3 |
| 7 | 17.0 | 17.2 | 17.0 | 17.4 | 17.0 | 17.2 | 17.1 |
| 8 | 17.8 | 17.6 | 17.7 | 17.9 | 17.8 | 17.6 | 17.7 |
| 9 | 19.2 | 19.4 | 19.0 | 19.4 | 19.5 | 19.3 | 19.3 |
| 10 | 20.3 | 20.4 | 20.4 | 20.5 | 20.2 | 20.2 | 20.3 |
| 11 | 21.2 | 21.2 | 21.4 | 21.5 | 21.2 | 21.3 | 21.3 |
| 12 | 22.2 | 22.3 | 22.4 | 22.5 | 22.3 | 22.4 | 22.4 |
| 13 | 23.2 | 23.4 | 23.1 | 23.5 | 23.1 | 23.3 | 23.4 |
| 14 | 24.6 | 24.6 | 24.5 | 24.9 | 24.8 | 24.8 | 24.7 |
| 15 | 26.5 | 26.5 | 26.6 | 26.8 | 26.7 | 26.7 | 26.6 |
| 16 | 27.3 | 27.5 | 27.2 | 27.5 | 27.2 | 27.3 | 27.3 |
| 17 | 28.2 | 28.4 | 28.2 | 28.5 | 28.2 | 28.3 | 28.3 |
| 18 | 29.9 | 29.8 | 30.0 | 30.0 | 29.7 | 29.7 | 29.7 |
| 19 | 30.5 | 30.3 | 30.3 | 30.2 | 30.1 | 30.3 | 30.3 |
| 20 | 31.2 | 31.0 | 31.0 | 31.4 | 31.3 | 31.2 | 31.2 |
| 21 | 32.5 | 32.5 | 32.5 | 32.5 | 32.6 | 32.5 | 32.5 |
| 22 | 36.0 | 36.2 | 36.2 | 36.4 | 36.1 | 36.2 | 36.2 |
| 23 | 38.2 | 38.4 | 38.4 | 38.2 | 38.2 | 38.2 | 38.3 |
| 24 | 39.9 | 39.5 | 39.5 | 40.0 | 39.7 | 40.0 | 39.8 |
| 25 | 41.4 | 41.3 | 41.3 | 41.3 | 41.3 | 41.4 | 41.3 |
| 26 | 43.7 | 43.6 | 43.6 | 43.8 | 43.6 | 43.6 | 43.6 |
| 27 | 47.2 | 47.0 | 47.0 | 47.2 | 47.3 | 47.5 | 47.2 |
| 28 | 48.6 | 48.8 | 48.8 | 48.7 | 48.9 | 48.9 | 48.7 |
| 29 | 52.5 | 52.4 | 52.4 | 52.1 | 52.4 | 52.5 | 52.4 |

organisms were grown on a synthetic medium containing: $K_2HPO_4$, 1 g/l; KCl, 0.5 g/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l; $FeSO_4$, 0.01 g/l; vitamin-free casamin acids (Difco), 1 g/l, and sucrose, 20 g/l. When solidified media were required Oxoid agar No. 3, 15 g/l, was added. The pH of all media was adjusted to 5.5 prior to use.

The organisms were grown for fourteen days in liquid culture at 25° to obtain mature mycelium, for three days to give young mycelium and for thirty days for production of spores. The spores of *P. chrysogenum* were readily obtained from the surface of cultures grown on solidified media. It proved easier to obtain spores from the other species from liquid culture, by removing the mycelium by filtration, resuspending in water and disrupting the mycelium in a blender. The mycelial strands could then be almost completely removed by filtration through non-adsorbent cotton wool. The filtrate was then subjected to 5 min vibration at 15 kHz. The suspension was then centrifuged to obtain the spores. Microscopic examination revealed the absence of mycelium.

All samples of mycelium and spores were harvested by centrifugation at 4000 *g*, at 4° for 10 min, washed twice with distilled water and dried by lyophilisation.

*Apparatus and conditions*

Pyrolysis investigations were carried out on 50–80 $\mu$g samples of the fungi using a Pye 104 series (Model 64) chromatograph with a P.V.4080 pyrolysis module. As the samples were of a powdery nature the coil method of Thompson[22], in a 2-mm quartz tube, was used, a new ferromagnetic wire and quartz tube being used for each sample. The optimum conditions for analysis, determined in preliminary studies were: A pyrolysis time of 10 sec at 770° was employed throughout. Chromatography was carried out using 10 ft. × 6 mm O.D. dual glass columns packed with 10% Carbowax 20M on 80–100 mesh Phase Separations A.B.S. (Queensferry, Great Britain). The temperature was programmed to increase at a linear rate of 4°/min from 60–140°,

TABLE IV

RELATIVE HEIGHTS OF REPRODUCIBLE PEAKS AND THEIR RETENTION TIMES (min)

| *No.* | *Relative peak height* | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *7–7.9* | *8–8.9* | *9–9.9* | *10–10.9* | *11–11.9* | *12–12.9* | *13–13.9* | *14–14.9* | *15–15.9* | *16–16.9* | *17–17.9* | *18–18.9* | *19–19.9* | *20–20.9* | *21–21.9* | *22–22.9* | *23–23.9* | *24–24.9* | *25–25.9* | *26–26.9* | *27–27.9* | *28–28.9* | *29–29.9* |
| 1 | | | | | | | | | | | | | 40 | | | | | | | | 37 | | |
| 2 | | | | | | | | | | | | 8 | 54 | 9 | | | | | | | | | |
| 3 | | 17 | | | | | | | | | | | | | | | | | | | | | |
| 4 | | | | | | 13 | | | 37 | | | | 13 | | | | | | | | | | |
| 5 | | | | | | | | | | | | | | | | | | | | | | | |
| 6 | | | | | | | | | | | 21 | | | | | | | | | 31 | 21 | | |
| 7 | | | | | | | | | | | | | | | | | | 36 | | | | | 76 |
| 8 | | | | | | | | | | | 9.5 | | | | | | | 34 | | | | | |
| 9 | | | | | 16 | 11 | | | | | | | | | | | | 32 | | | | | |
| 10 | | | | | | | | 6 | | | | | | | | | | | 42 | | 15 | | |
| 11 | | | | | | | | 12 | | | | | | 13 | | | | | | | | | |
| 12 | | | | | | | | 14 | | | 83 | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | | 29 | | 125 | | 74 | | 11 | | |

initial hold 10 min, final hold infinity. The detector temperature was 250°. The air pressure was 50 lb./sq.in.: the hydrogen pressure, 25 lb./sq.in. and the carrier gas (nitrogen) pressure, 50 lb./sq.in. The nitrogen flow-rate was 40 ml/min. The recorder was set at 1 mV f.s.d.: attenuation, various. The chart speed was 5 mm/min.

## RESULTS

Preliminary pyrograms obtained with mature mycelia showed variation in relative peak heights for each species studied. In an attempt to eliminate this variation the three basic components of the fungal colonies —the mature mycelia and young mycelia and the spores— were separated and individually studied. The pyrograms for young mycelia and for spores showed greater variation than for mature mycelia, which were then solely studied but in more detail.

The difficulty in controlling the sample size in replicate experiments made it necessary to use relative rather than absolute values of peak heights and areas. The peak heights and areas were calculated relative to a well resolved peak which appeared in the pyrograms at a retention time of 36 min for all the species studied. No significant difference was found between relative peak height or area measurements (estimated by planimeter) so that peak heights were used after the initial studies were completed. The peak heights in each case were measured above a base line contructed by linear interpolation between valleys.

Typical pyrograms for each species studied are shown in Fig. 1.

Each mature mycelium was examined in six replicates. Results showing relative peak heights for *P. parvum* are shown in Table I, relative peak areas and peak heights for the peaks with the most reproducible heights/areas are given in Table II and the retention data in Table III.

The relative peak heights for the most reproducible peaks for the series of species examined is given in Table IV.

| 30–30.9 | 31–31.9 | 32–32.9 | 33–33.9 | 34–34.9 | 35–35.9 | 36–36.9 | 37–37.9 | 38–38.9 | 39–39.9 | 40 40.9 | 41–41.9 | 42–42.9 | 43 43.9 | 44–44.9 | 45–45.9 | 46–46.9 | 47–47.9 | 48–48.9 | 50–50.9 | 51–51.9 | 52–52.9 | 53–53.9 | 54–54.9 | 55–Inf. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 100 | | | | | | | | | | | | | | | | | | |
| | | | | | | 100 | | | | | | | | | | | | | | | | | | |
| | | | | | | 100 | | 23 | | | | | 63 | | | | | 21 | | | | | | |
| | | | | | | 100 | | | | | | | 61 | | | | | | | | | | | |
| 80 | 55 | | | | | 100 | | 29 | | 90 | | | | | | | | | | | | | | |
| | | | | | | 100 | | | | | | | | | | | | 15 | | | | | | |
| | | 90 | | | | 100 | | | | | | | | | | | | | | | 16 | | | |
| | | | | | | 100 | | | | | | | | | | | | | | | | | | |
| 48 | 28 | | | | | 100 | | | | | | | | | | | | | | | 7 | | | |
| | 13 | | | | | 100 | | 23 | | 12 | | | | | | | | | | | | | | |
| | | | | | | 100 | | | | | | | | | | | | | | | | | | |
| 225 | | | | | | 100 | | | | 10 | | | | | | | | | | | | | | |
| | | | | | | 100 | | | | | | | | | | | | | | | | | | |

## DISCUSSION

The taxonomy of the important genus, *Penicillium*, is extremely complex[20]. Most species exhibit shades of green which change as the colonies age and, in addition, vary with cultural conditions. Their fruiting (spore bearing) structures are small and disintegrate with age, the asexual spores are among the smallest in true fungi and only rarely show distinctive features. Sexual states, if occurring at all, may require months to reach fruition. The most definitive work is by Raper and Thom[19], but because of the paucity of obvious taxonomic characters and the arbitrary nature of many of the manual's groupings the genus remains taxonomically difficult[21].

Such problems suggest the need for alternative approaches to classification and species identification. Hence a chemotaxonomic approach which has been useful in identification of other materials of biological origin was considered.

The data given in Table IV show that it is possible to clearly distinguish between eleven *Penicillia* and the two related species *Aspergillus niger* CMI 31821 and *Neurospora crassa* CMI 75723 by pyrolysis GLC using mature lyophillised mycelia. Each pyrogram contained reproducible peaks, amongst the more variable ones, with characteristic relative heights and retention times.

The existence of the reproducible peaks in relatively non-reproducible pyrograms was an interesting feature of the results. This effect is in contrast to those obtained with bacteria where a high degree of reproducibility of the pyrograms was reported[7–9]. This feature might not be unexpected on the basis of the varied metabolic activities going on in different areas of the mycelium, *e.g.* the production of secondary metabolites causes the chemical composition to vary depending on the "physiological age" of the mycelial strand and the age of the culture[23]. Although many workers have emphasised the importance of the "physiological age"[24–26], very little cytochemical differentiation has been reported on the mycelia of fungi.

For identification purposes mature mycelia were found to be necessary, as pyrograms containing young and mature mycelia showed great variability in relative peak heights.

For spores a greater uniformity in chemical composition would be expected. However, pyrograms for individual species showed too great a variability to be used for identification purposes. This effect might be due to physiological age; the only feasible method of spore separation does not discriminate on age.

Work is currently being carried out in the identification of *Aspergillus* species and to examine the chemical origin of the reproducible peaks within a species.

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