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

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SUMMARY

Using pyrolysis gas chromatography it was possible to identify each of nine *Aspergillus* species. The effects of hyphal age and the non-differentiation with spores are discussed.

INTRODUCTION

The characterisation of material of biological origin by pyrolysis gas–liquid chromatography (GLC) is now a reasonably well established method of chemotaxonomy. Current interests are in determining which tissue or microorganism groups can be differentiated and in the pyrolysis process, in particular, the nature of the products formed using various forms of mass spectroscopy (MS).

The first systematic chemical identification of components in bacterial pyrolysates was Simmonds' use of a quadrapole MS–GLC system[1]. He compiled an extensive list of identified pyrolysate components and tentatively assigned their origin to protein, carbohydrate, lipid, nucleic acid, and porphyrin sub-structure components. The potential of field ionisation MS, which yields prominent molecular parent ion peaks, coupled to gas chromatography (GC) has been examined by Schulten *et al.*[2]. Some 177 fragments and 80 compounds of their possible origin were observed in the field ionisation MS data for the pyrolysate of *Pseudomonas putida*. A Curie point pyrolysis direct quadrupole MS system was shown to give rapid identification of *Neisseria sicca* and *Leptospira*[3]. The direct combination of Curie point pyrolysis with low-voltage electron impact ionisation MS was shown to be of potential using insulin and trypsin as samples[4]. This method was examined because it involves less fragmentation of material in the ion source than with the quadrupole system. Characteristic *m/e* peaks allow ready identification of the protein nature of samples. The direct coupling of pyrolysis to field desorption MS allowed the identification of all five bases in herring DNA as well as that of some nucleosides, nucleotides, and dinucleotides using precision mass measurements and computer analysis of spectral patterns[5].

The computer matching of pyrolysis chromatograms of pathogenic organisms has been shown to be viable using six bacteria, each belonging to a different Kauffman–White serological group of *Salmonella* spp.[6]. However, in many cases, simple statistical or even visual examination of pyrograms suffices for identification.

A preliminary report[7] indicates the classification of anaerobic bacteria, *Bacteroides fragilis*, *Fusobacterium gonidiaformans*, *Propionibacterium acnes* and *Pepto-Streptococcus anaerobius*, identified by examination of extractable volatile fatty acids and alcohols. Identification of oral *Streptococci* by pyrolysis GC is particularly helpful with the *Streptococcus mitior* and *Strep. sanguis* groups[8]. Pyrolysis GLC was preferred to transesterification for the identification of *Streptococci* of several Lancefield groups[9].

The present communication concerns *Aspergilli* following a previous study of *Penicillium* species[10]. The *Aspergilli* belong to the Class, Fungi Imperfecti; Order, Moniliales; Family, Moniliaceae.

Aspergillus species are widely distributed and are responsible for spoilage of food products, particularly in the post-harvest period when stored seeds are involved. *Aspergillus repens* is active in spoiling grain in the moisture range 13.2–15%[11], above 18% moisture *A. candidus* has been reported to be a predominant spoilage type[12]. The spontaneous heating of grain, *i.e.*, the production of "hot spots" is characteristic of rapid fungal growth. Gilman and Barron[13] showed in laboratory tests that *A. flavus*, *A. niger* or *A. fumigatus* raised the temperature of wheat, barley or oats, at 18% moisture, 26° above an initial temperature of 17°.

Toxin production can also be of concern in food products spoiled with certain members of the genus *Aspergillis*[14], the best known being the production of aflatoxins by *A. flavus* and other *Aspergilli*. In addition, the pyrone derivative patulin is produced by *A. clavatus* and kojic acid by *A. oryzae*[15], which also produces maltoryzine, which caused toxicosis in cattle in Japan[16].

The inhalation of spores produced by *A. fumigatus* growing on decaying vegetation is the cause of the pulmonary disease aspergillosis[17].

EXPERIMENTAL

Preparation of samples

The *Aspergillus* species studied were: (1) *A. niger*, C.M.I. 31,821; (2) *A. clavatus*, C.M.I. 54,399; (3) *A. candidus*, C.M.I. 19,446; (4) *A. flavus*, C.M.I. 89,717 (ii); (5) *A. ustus*, C.M.I. 16,045; (6) *A. fumigatus*, C.M.I. 16,062; (7) *A. repens*, C.M.I. 16,113; (8) *A. wentii*, C.M.I. 39,510; (9) *A. oryzae*, C.M.I. 12,6842.

To avoid any possibility of contamination with complex materials, the organisms were grown on a modified Barnes medium containing: K_2HPO_4, 1 g/l; NH_4NO_3, 1 g/l; KNO_3, 1 g/l; glucose, 1 g/l; vitamin-free casamino acid (Difco), 1 g/l made up in distilled water. When solid media were required, Oxoid agar, 15 g/l, was added. The pH of all media was adjusted to 5.5 prior to use.

The organisms were grown and harvested after three, five and seven days' growth. The three- and five-day samples were used to examine different stages of mycelial growth and were grown in liquid culture, with 150 rpm orbital shaking, at 25°. Samples were harvested by filtration on Whatman No. 1 papers. This was more effective than centrifugal separation in this case. The seven-day spore samples

were grown on solidified media. After harvesting spores were examined microscopically for mycelial strands; if present, samples were subjected to agitation in an homogeniser. The mycelial strands could then be almost completely removed by filtration through non-absorbent cotton wool. The filtrate was then subjected to 5-min vibration at 15 kHz. Spores were harvested by centrifugation at 4000 *g* at 4° for 10 min and washing twice with distilled water. Mycelia and spores were dried by lyophilisation.

Apparatus and conditions

Pyrolysis investigations were carried out on 50–80 μg samples of fungi using a Pye 104 Series (Model 64) chromatograph with a P.V. 4080 pyrolysis module. Samples were held in the bent end, $\frac{1}{2}$ in. flattened, of a ferromagnetic wire in a 2-mm quartz tube. A new wire and tube were used for each sample. The optimum conditions were established in preliminary experiments to be similar, but not identical, to those previously used for *Penicillia*[10], namely: A pyrolysis time of 10 sec at 770° was employed in all but preliminary experiments. Chromatography was carried out using 10 ft. × 6 mm O.D. dual glass columns packed with 10% Carbowax 20M on 60–80 mesh A.B.S. (Phase Separations, Queensferry, Great Britain). The temperature was programmed to increase at a linear rate of 6°/min from 60–220°, initial hold 0, final hold ∞. The detector temperature was 250°. The air pressure was 8.6 p.s.i., the hydrogen pressure 16.6 p.s.i. The nitrogen flow-rate was 40 ml/min. The recorder was set at 1 mV f.s.d.; attenuation, various. The chart speed was 10 mm/min.

RESULTS

Three samples of each organism were separately grown and each was harvested after three, five, and seven days. The pyrograms for young mycelia and for spores of each organism showed greater variation than for mature mycelia. The latter also show the most features and are thus preferred for identification purposes. No single common peak was available for relative internal standard purposes; peak patterns can be distinguished by visual inspection, as may be seen from the data in Fig. 1.

DISCUSSION

The taxonomy of the genus is, up to a point, not difficult owing to the excellent scheme of classification given by Thom and Raper[18]. The most recent revision of the genus by Raper and Fennell[19] follows the same scheme but provides for the inclusion of extra species. The conidiophore apex is the morphological feature used to differentiate between *Aspergillus* and the closely related genus *Penicillium*. The conidia of the *Aspergilli* are dry, dusty, typically spherical and ornamented with small spines and these spores are dispersed by air currents. They vary in colour from almost colourless to green or blackish in some species. The wide range of colony colour exhibited facilitates identification, these colours being relatively stable, and when correlated with morphological and biochemical characters permits classification. Some of the species are fairly definite in their characters and use of these has produced the concept of "group species". However, when a great number of strains are ex-

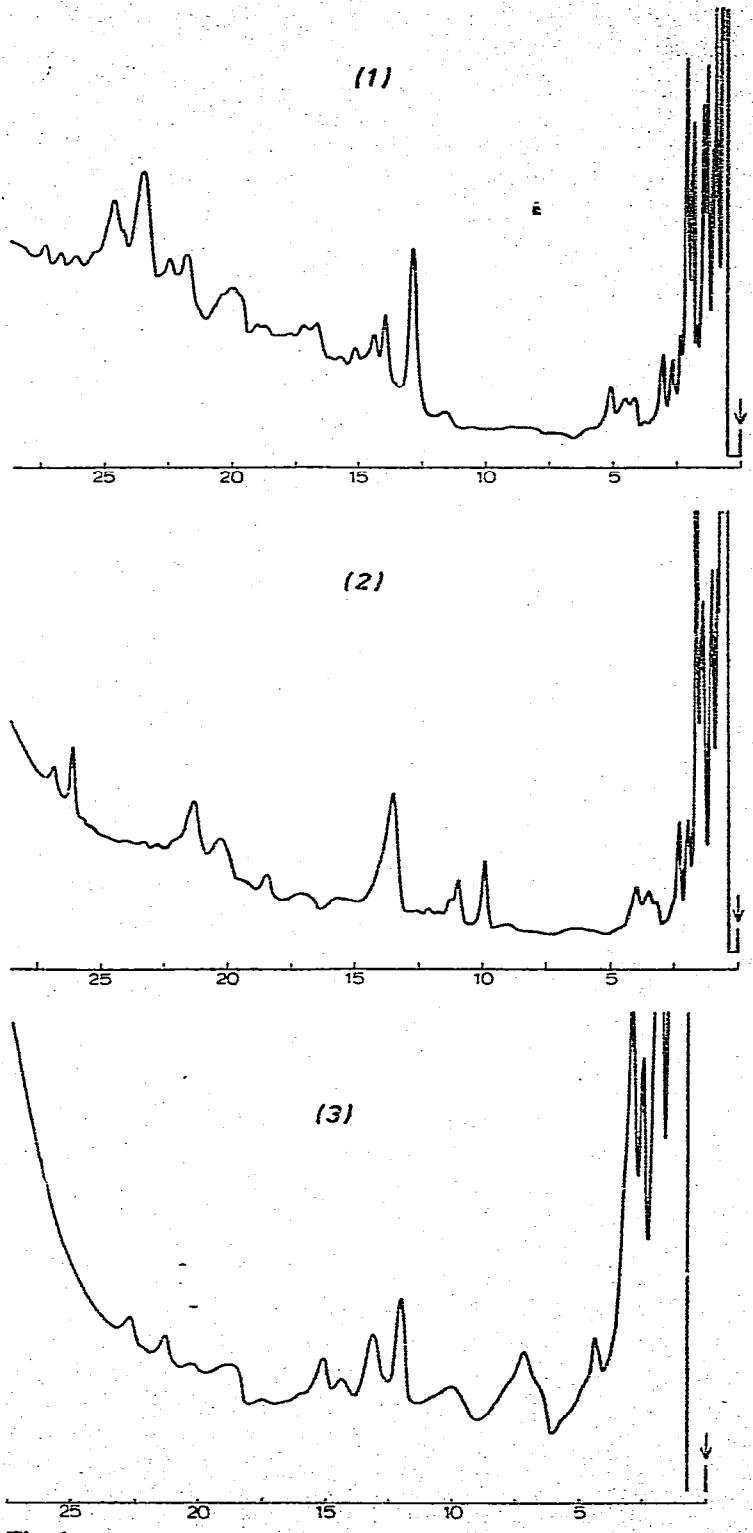

Fig. 1.

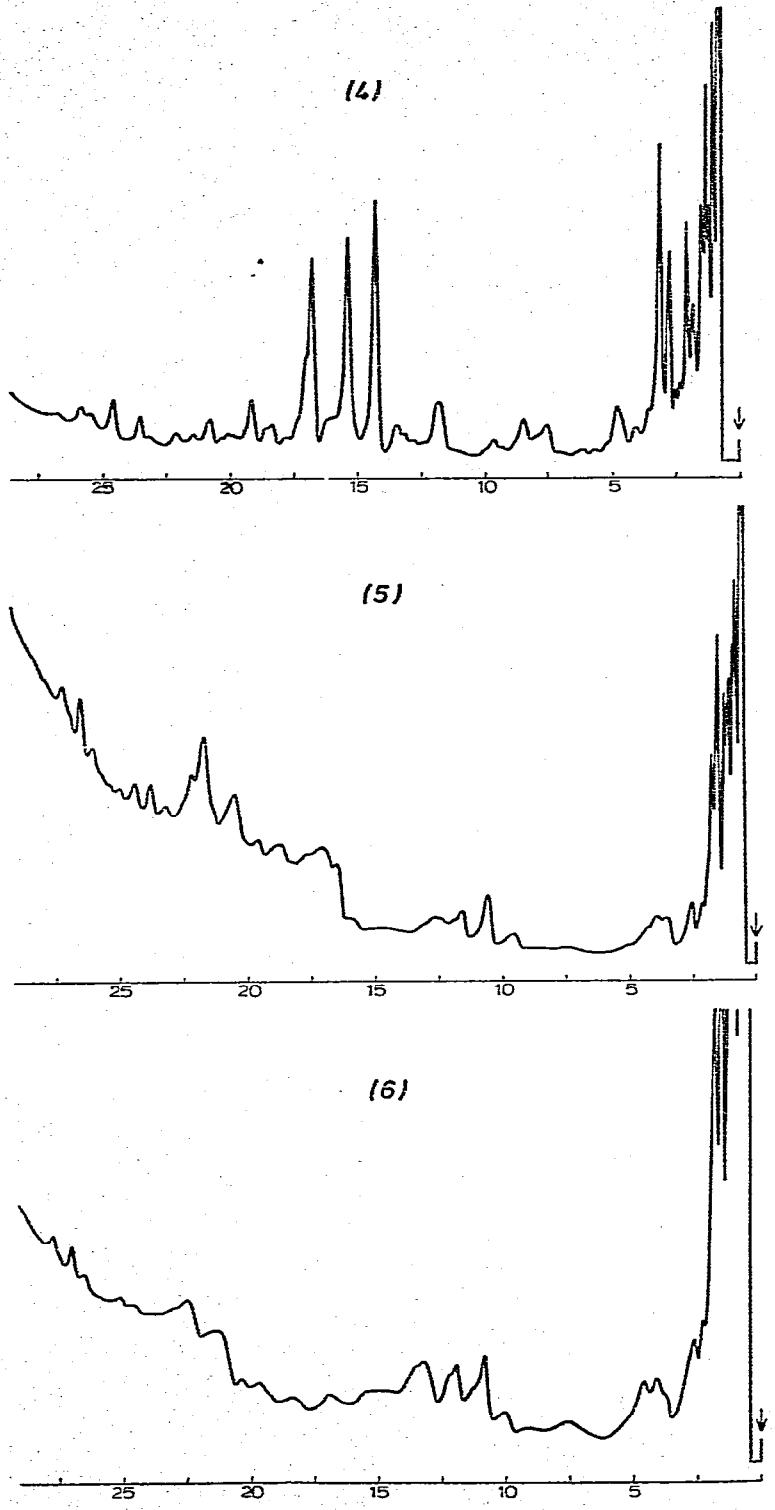

Fig. 1.

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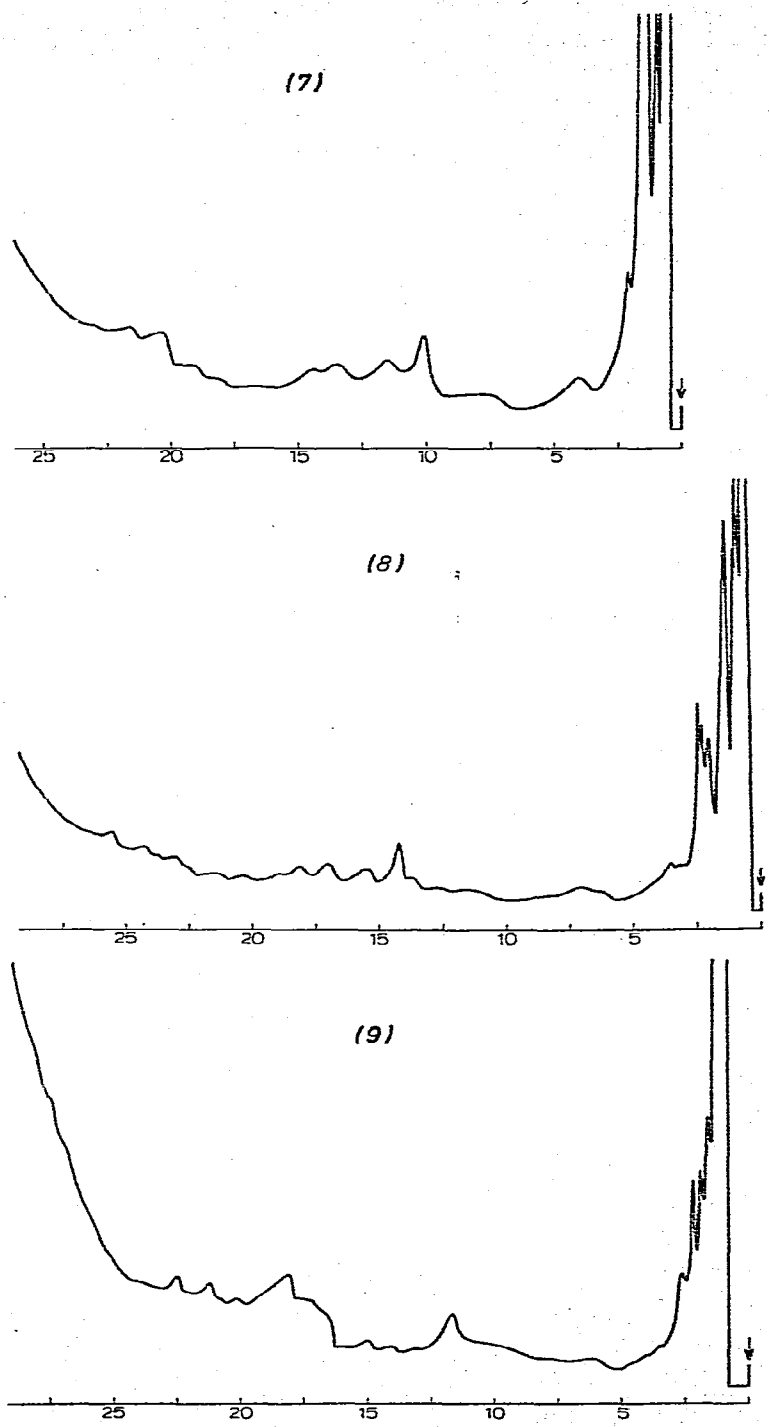

Fig. 1. Pyrograms for *Aspergilli*. (4) *A. niger*, C.M.I. 31,821; (1) *A. clavatus*, C.M.I. 54,399; (2) *A. candidus*, C.M.I. 59,446; (5) *A. flavus*, C.M.I. 89,717(ii); (8) *A. ustus*, C.M.I. 16,045; (6) *A. fumigatus*, C.M.I. 16,062; (7) *A. repens*, C.M.I. 16,113; (3) *A. wentii*, C.M.I. 39,510; (9) *A. oryzae*, C.M.I. 126,842. Time scale, 2½ min/division.

amined, the apparently sharp demarcation lines disappear and instead of a few definite species there is a perfectly graduated series.

Delineation of a species in such a group is also complicated by the fact that a single strain may cover a considerable range in the series of variations in response to changes in cultural conditions, or even in successive cultures grown under the same conditions.

A further problem is that some of the smaller and more delicate species bear a strong resemblance to certain of the monoverticillate *Penicillia*[20].

Because of these difficulties, for the non-specialist there is clearly a need for a technique for the rapid identification of these important groups of organisms, on a routine basis.

Simple visual examination of pyrograms is considered to be sufficient to distinguish between the nine *Aspergillis* species studied here. For identifications in this manner it should be stressed that pyrograms should be compared to pyrograms of mycelia of similar age grown under identical metabolic conditions[21,22] and examined under the same pyrolysis and chromatographic conditions[3].

The spores produced by these members of the genus *Aspergillus*, although they give reproducible pyrograms, cannot be used for their classification because the numbers and variety of peaks are too low to permit adequate differentiation. This may be due to the fact that spores, being a survival stage, do not contain the variety of metabolites present in the hyphal stage and there could be a considerable degree of similarity in type and amount of molecules present in the spores of the various species.

The use of five-day samples, which give more useful pyrograms than three-day samples, suggests that the older hyphae are probably producing a variety of secondary metabolites and so perhaps give a wider range of compounds and enzymes which give the distinctive peaks.

The technique has potential for the rapid identification of fungi and other microorganisms[2,3,6,8,9], but is limited in application at the moment because of its empirical nature. One difficulty is that a standardised set of conditions may be difficult to achieve when comparing genera, although the conditions can be standardised when working at the species level. Other physical techniques, like an analysis of the fatty acids present in organisms[7,23], may appear more useful because the nature of the components investigated is known and fewer parameters are studied in any one analysis. Pyrolysis GC, because of the difficulty in obtaining quantitative samples, requires a study of retention times and the assigning of peaks to a fingerprint region and 5–25 min retention herein, in a manner similar to the consideration of much mass spectral data on a qualitative basis. Both fatty acid analysis and pyrolysis require cells of the same age obtained under identical cultural conditions to avoid variations in the cell depending on physiological age. Further progress may be possible with a mass spectral link to identify compounds but for routine identification use pyrolysis GC is considered sufficient.

Current work is concerned with the examination of strains of single species from a wide variety of sources and effects of storage on lyophilised mycelia with the object of aiding interlaboratory identification studies.

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