PYROLYSIS-MS IN THE IDENTIFICATION OF MICRO-ORGANISMS

Roger Berkeley and co-workers describe a clinical application of pyrolysis-MS

Rapid and accurate identification of micro-organisms is the goal of all diagnosticians. Most identification procedures involve obtaining an isolate in pure culture and characterising it in a reproducible manner. By using appropriate data handling techniques, it can then be discriminated from closely related, but non-identical organisms. After characterisation, the isolate is identified with a known microbial group, or not identified, if it is a hitherto unknown organism.

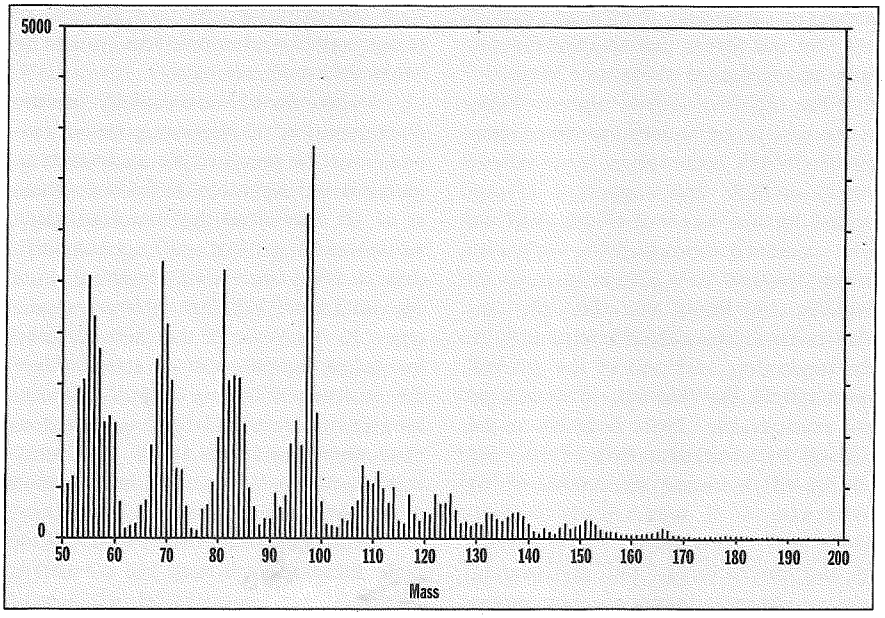
Traditionally, characterisation has been achieved by observation of morphological features and by the application of a battery of biochemical tests. Such an approach is time consuming and expensive but is generally applicable to the majority of organisms. Modern approaches include quick, specific serological tests, DNA probes and physicochemical methods. The first two of these techniques are powerful if an approximate identification is available, and provided the necessary sera and probes are at hand. In contrast, physicochemical, instrument-based approaches, depend neither on any prior knowledge of the organism's identity, nor on the availability of labile, expensive reagents. They also have the advantage that they are universally applicable.

Thus diagnosticians are increasingly adopting instrumented methods, thereby following in the path of analytical chemists who have long since turned from 'wet' chemistry to instrument-based techniques with great savings in time taken for analysis, economies in manpower and greatly increased analytical ability. Comparable benefits will certainly accrue as similar approaches are adopted in the microbiology laboratory.

Pyrolysis-mass spectrometry (Py-MS) is one physicochemical method for microbial identification which is currently developing rapidly. It has an advantage in that the size of sample required for analysis is very small.

Pyrolysis is the thermal degradation of a material in an inert atmosphere or a vacuum, and leads to the production of volatile fragments from non-volatile materials such as bacteria. Under controlled conditions the breakdown is reproducible and the fragments are characteristic of the original material. Following pyrolysis, the fragments are separated and analysed in a mass spectrometer.

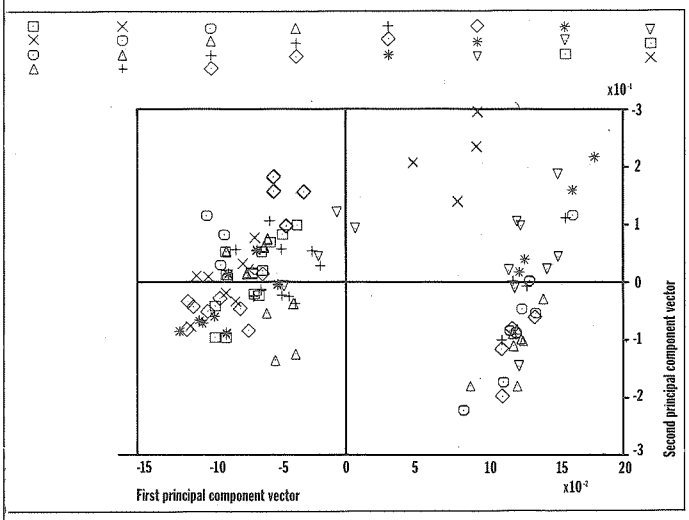
Figure 1. Example of a pyrolysis mass spectrum of normalised data in the range m/z 51–200 for the bacterium *Yersinia pseudotuberculosis*.



Analysis of biological materials by Py-MS was first achieved by Zemany in 1952, but it was not until 1973 that a fully integrated instrument was produced by Meuzelaar and Kistemaker. Since then, only a handful of other instruments have been produced. Recently, however, the Horizon PYMS-200X (Horizon Instruments) was launched. Its existence has much to do with current rapid developments in the use of pyrolysis for the examination of biological materials. Much of the work reviewed below has been carried out with this instrument.

Characterisation

Characterisation of a bacterium using the PYMS-200X gives a spectrum such as that shown in figure 1. This has a mass range of m/z 51–200. Below m/z 50, fragments corresponding to methane (15,16), ammonia (16,17), water (17,18), methanol (31,32) and hydrogen sulphide (32–34) are found. As these fragments are



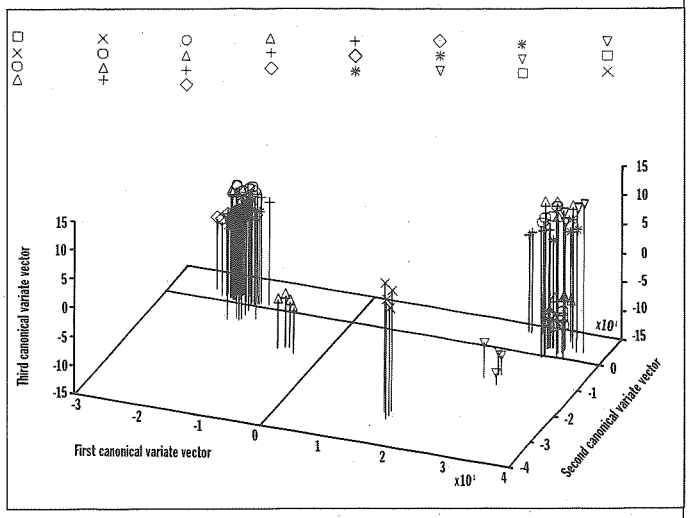


Figure 2 (left). A typical Principal Components Analysis (PCA) plot demonstrating that strains of *Acinetobacter anitratum* separate into at least two broad groups.

Figure 3 (right). Canonical Variates Analysis (CVA) plot derived from the same data as used in figure 2 illustrating the improvement in discrimination gained using the technique. At least six groups can now be clearly discerned.

likely to originate from pyrolysis of any biological material, this data is discarded. For fragments of m/z greater than 200, data capture may increase the cost of the instrument, and evidence exists that there is little benefit, in terms of extra discrimination, in extending the mass range.

Reproducibility

In an early study of Py-MS reproducibility, Windig *et al.* [1] showed good short-term comparability between spectra using biological standards with a mean dissimilarity of only 6%. Further work by Shute *et al.* [2] showed a mean dissimilarity of 4% for pyrolysis spectra from replicate samples over eight weeks. Over longer periods the reproducibility of Py-MS deteriorates. Windig *et al.* [1] attempted to stabilise operating parameters, but still found that spectral dissimilarity had increased to 8% after 34 days.

The observed lack of reproducibility is thought to be caused by mass spectrometer drift rather than by pyrolysis variations [1,3]. This suggestion is supported by the work of Shute *et al.* [4] who examined reproducibility over a 14 month period using four species of *Bacillus*, and found that although samples were discriminated comparably at the beginning and end of the period, spectra from the two sets of data were not comparable. Drift is likely to be caused by contamination of the ion source of the mass spectrometer by pyrolysate [4, 5], which leads to alterations in ion transmission, electron energy and ion focusing, with concomitant spectral changes. Ageing of other components, such as the electron multiplier, may also affect reproducibility.

In an attempt to effectively eliminate the adverse effects of instrument drift, two complementary approaches are now being taken. A corrective algorithm to relate mass intensities of samples run on different occasions to a common standard has been written. This reduces the problem of drift (Kelley and Berkeley, unpublished). Also, the instrumental reasons for lack of reproducibility are being systematically investigated in conjunction with the manufacturer and efforts are being made to eliminate the causes by hardware modifications.

To use Py-MS successfully for the discrimination of micro-organisms, cultures must be prepared for analysis in an identical and reproducible fashion. Shute *et al.* [2] illustrated that batch-to-batch variations in growth media are insufficient to affect spectra and therefore strain discrimination, but that different media types do. However, if the methods used for sample preparation are standardised, the process will have a minimal effect on long-term reproducibility compared to instrument variations [1, 3]. Following reproducible characterisation, the data produced is analysed as described below.

Multivariate data (often from 150 or more samples, each with 150 masses) is processed using a computer, which may be off line, or the IBM AT compatible microcomputer which controls the PYMS-200X may be used. Spectral information is first normalised, using either the instrument software or the PYSTAT program, to eliminate effects due to differing sample sizes. Subsequent analysis is aimed at identifying significant differences between spectra. This can be achieved using the statistical package GENSTAT [6], supplied with the instrument.

Principal Components Analysis (PCA) is normally carried out next. With small data sets all the available information can be analysed on the microcomputer. With larger amounts of information, a data reduction step, also carried out with PYSTAT, is necessary in order to allow microcomputer analysis. Reduction involves removing masses that vary little between samples by choosing only those masses with a high 'characteristicity' [7]. Discrimination is, however, often improved by analysing data for all 150 masses per sample using, for example, an IBM 3090 mainframe computer.

PCA reduces the number of dimensions required to display most of the variance within the data; typically 95% of the variance is accounted for in the first three dimensions. PCA does not take account of groupings within the data set, but is useful in displaying relationships between data [2], as shown in figure 2.

The next stage in the process, Canonical Variates Analysis (CVA), separates the samples into groups using these principal components and an input of *a priori* groupings, if knowledge about likely groupings is available [8]. Using the

maximum available number of principal components, a better between-group variance compared to within group-variance, that is a better split, is obtained. A typical CVA plot is shown in figure 3.

Cluster analysis, dendrogram production and minimum spanning tree generation are also possible using GENSTAT. The use of this information in achieving identification is discussed later. Analysis of the information contained in such spectra enables the discrimination of different organisms.

Discrimination

Amongst the microbes discriminated by Py-MS are the bacterial genera *Bacillus* [2]; *Corynebacterium* [9]; *Fusobacterium* [10]; *Klebsiella* [3]; *Legionella* [11]; *Listeria* [9]; *Mycobacterium* [12] and *Neisseria* [13]; certain yeasts [5, 14] and other fungi [15].

An example of the discriminatory power of Py-MS is the separation of the four closely related organisms: *B. subtilis, B. pumilus, B. licheniformis* and *B. amyloliquefaciens*. This is a demanding test because there are few classical tests that distinguish these species [2]. Another illustration of the ability of Py-MS to achieve discrimination is the division of 25 strains of *B. cereus* into two groups; one of twelve strains with no history of involvement in food poisoning and one of the organisms isolated in connection with emetic type food poisoning outbreaks [16]. A third example showing the extremely powerful discrimination of Py-MS is the demonstration that it is possible to distinguish between four strains of *Escherichia coli* which differ only in the presence or absence of a single antibiotic resistance plasmid [17].

These studies demonstrate the ability of Py-MS to discriminate between very closely related micro-organisms, and to divide them into groups. It is one of the strengths of the technique that distinctions between very closely related types are possible.

Although Curie-point Py-MS has been shown to be capable of fine bacterial discrimination, identification can only be carried out at present by 'operational fingerprinting' where suitable reference strains are analysed at the same time as unknown strains. Figure 4 demonstrates the identification of two unknown clinical isolates in this way. This procedure, though rapid and usually successful, has the disadvantage that it may be difficult to decide which reference strains are likely to be closely related to the fresh isolates. It is, however, not affected by instrument drift.

An alternative approach, avoiding the need to choose reference strains, is identification by the matching of pyrolysis spectra against an existing data-base of spectra of such strains. Long-term reproducibility is essential for this method to be viable. Current work to define instrument variability in the PYMS-200X and to reduce the effects of drift by the use of the corrective algorithm, is bringing the routine application of this method to identification nearer.

Conclusions

Pyrolysis-mass spectrometry is already a rapid and effective method for the discrimination and identification of micro-organisms. The availability of a low-cost dedicated Py-MS system has led to considerable progress towards the ultimate goal of diagnosticians, the identification of freshly isolated micro-organisms within minutes of their arrival in the lab.

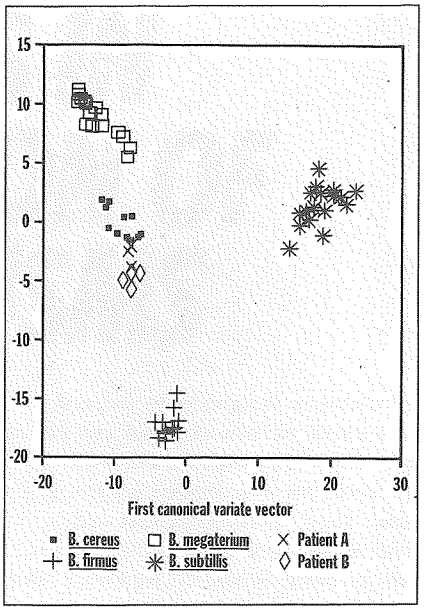


Figure 4. The identification, by operational fingerprinting, of two isolates from patients as *B. cereus*.

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