8:1 The differentiation of meat species by Direct Probe Mass Spectrometry

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INTRODUCTION

Recent cases of adulteration of beef supplies by horse, sheep and kangaroo meats have highlighted the necessity for rapid and reliable methods of determining the species origin of meats. Methods currently in use for unprocessed meats include the Ouchterlony immunodiffusion method [1], isoelectric focusing methods [2] and enzyme-linked immunosorbent assay (ELISA) techniques [3]. A novel alternative approach for the classification of meat species is direct probe mass spectrometry (DPMS), a technique which has been under investigation in this laboratory for some considerable time for the differentiation of micro-organisms [4,5]. It has the potential of providing a rapid method of analysis, requiring minimal sample preparation and is applicable to a wide variety of biological materials.

The technique employs a standard direct inlet probe, an accessory available on most mass spectrometers. Samples are thermally degraded over a fixed temperature range adjacent to the ion source of the instrument and the resultant spectra are averaged across the temperature profile. The technique is similar to pyrolysis mass spectrometry (Py-MS) [6-8] except that lower temperatures are used, a maximum of 350°C as opposed to 500-800°C. In common with Py-MS the differences between the spectra are generally small and require careful quantitative analysis of the ion intensities to differentiate samples. As each spectrum may contain up to three or four hundred individual ions, computerized techniques are essential to reduce the data and determine which ions provide the greatest degree of characterization.

Three experiments have been carried out; the first examines the discrimination within three animal species with samples taken from different muscle areas within each animal. The second looks at inter- and intra-species separation using four individual animals from six species. The third experiment examines in greater detail three of the species which were found more difficult to separate.

EXPERIMENTAL

Selection of samples

In the first experiment samples were taken from six different muscle areas of one beef animal, one chicken and one rabbit. The beef samples were obtained from the fore and hind quarter of one side of the animal carcass. The chicken samples consisted of three areas of breast muscle and three leg muscles, and the rabbit samples were taken from different muscles across the animal. In the second experiment a single sample was taken from each of four animals from each of six species, cattle, chicken, horse, pig, rabbit and sheep. Muscle from the loin was used for the beef, pig, rabbit and sheep meat samples while breast muscle was used for the chicken and neck muscle for the horse samples. For the third experiment three species, cattle,horse and sheep were re-examined using a single sample from each of six individual animals of each species. These samples were all taken from neck muscle.

Preparation of meat extracts

Aqueous extracts of uncooked meats were prepared from 20g portions of each muscle, trimmed of fat and excessive connective tissue, finely chopped and homogenised (M.S.E. homogeniser) in 80 ml of distilled water. Quantities were adjusted for some of the small muscles of rabbit and chicken which weighed less than 20g. The clear filtrates, obtained through Whatman No.3 paper were frozen (-20°C) and then freeze-dried in glass containers. The resulting residue was ground down to a fine powder and stored in glass vials until use.

Mass Spectrometry

Approximately 50µg of sample was taken with a platinum wire and placed in a quartz glass tube which was inserted into the stainless steel probe. Analyses were performed in a similar manner to that described previously [5], on a Finnigan 4000 quadrupole mass spectrometer coupled to an Incos 2100 data system. The mass spectrometer was operated at an electron energy of 25 eV and a source temperature of 270°C. After insertion into the instrument the probe was temperature programmed at 60°C min^{-1} from ambient to 300°C (350°C for experiment 3). Although the sample is incompletely pyrolysed at these temperatures, the maximum of 350°C is limited by the instrument configuration. Spectra were recorded over the mass range m/z 33-400 using a 2 sec. scan cycle.

As the direct probe was heated the total ionisation produced from the sample in the mass spectrometer was recorded as a function of time in an ion current profile. A single spectrum was produced for each sample by averaging across the profile from the beginning to the end of the pulse of ions and subtracting an averaged ten scan background taken before the pulse to remove any underlying contamination. Although the actual number of scans varied from sample to sample the averaging procedure provided reproducible and representative spectra because it was applied to the same section of each ion current profile.

In all cases at least three replicates of each sample were run. A fourth replicate was run for six of the samples in experiment 1, two muscles from each animal, to act as "unknown" test samples. In experiment 2 twelve test samples were obtained by running a fourth replicate for two animals of each species. In experiment 3 six test samples were provided by again running a fourth replicate for two animals from each species. The choice of which replicate should be treated as a test sample and the order of analysis was determined by generating random numbers. In this context each individual replicate was regarded as a separate sample, i.e. the replicates were spread in a random manner over the period of each study. The first study therefore had a total of 60 samples, the second a total of 84 and the third a total of 54. All three studies were treated in a similar manner with respect to mass spectral and data analysis.

Data analysis

Averaged spectra were produced by the standard Incos software for visual comparison of the samples. The raw data was also converted to a Fortran readable format and each spectrum normalised to its total ion count to remove variations in sample size. The spectra were then analysed using a package of multivariate data analysis routines specially written in the laboratory for the Incos system.

Comparisons between spectra of the data base, i.e. excluding test samples, were made on replicate means of three spectra for each of the 18 samples of experiment 1, 24 samples of experiment 2 and 18 samples of experiment 3. The extra individual replicates were only added at a later stage to test the validity of the discrimination produced by the analysis. These comparisons were made using a subset of ions produced after initial data reduction. The reduction, which was based on the characteristicity concept of Eshuis et al. [9] was necessary to remove redundant data and reveal those ions which provided genuine discrimination.

Characteristicity is a measure of the ability of an ion to discriminate samples and, for data with non-defined groups is determined from the ratio of the inter-sample to inter-replicate variance for each ion. This can be used to derive a subset of ions which will differentiate samples without any pre-definition of the groups involved. A variation of this can be used to derive a more specific set of ions if the original classification is known, then the ratio of the inter to intra group variance for each ion may be determined. This latter approach was used in these studies. The optimum combination of ions was then determined using a stepwise discrimination procedure which examines the success of the classification at each stage of a sequential addition of ions in order of their characteristicity; generally, an optimum result is achieved with less than 20.

To examine the relationships between the samples two methods were used, the first was a simple plot, or scatter diagram, of the normalised intensities of the two most characteristic ions. In many cases this is sufficient to show clear differentiation between samples, particularly where only a few groups are present. In the second method, which combines the discriminating ability of a greater number of ions, similarity values between the spectra were determined using the subset derived above. The similarities were calculated using proportional similarity coefficients [10, 11] and the matrix of similarities visualised using multidimensional scaling [12].

RESULTS

As is generally the case with DPMS of biological materials the spectra showed a high degree of qualitative similarity and differed mainly in the relative ion intensities. Ions were detected up to m/z 400 but were generally weak above m/z 200, with m/z 45, 58, 84, 113 and 136 prominent in all the samples run. Visual examination of the spectra did not reveal any obvious signs of discrimination into the species groups in any of the studies so the more sensitive data analysis procedures described above were implemented.

Experiment 1

The 54 sample data set was first analysed to provide detailed information on the degree of characterization of the ions. Subsequent analysis using the stepwise discrimination procedure showed that complete separation could be easily demonstrated by a plot of the two most characteristic ions, m/z 70 against m/z 61, without the necessity for a multidimensional solution. Figure 1 is a plot of the normalised intensities of these two ions for the 18 sample replicate means, plus the 6 test samples (shown in circles). It can be seen that the three groups are clearly separated, although the beef samples are rather more widely spread than the rabbit and chicken. This is probably due to a combination of two factors: the variation in composition of the samples taken from across the animal and the greater difficulty which was encountered in obtaining homogeneous beef samples after freeze drying. The slight separation of the chicken samples into two clusters is interesting in that it reflects the origin of these samples, the top cluster came from leg muscle, the lower cluster from

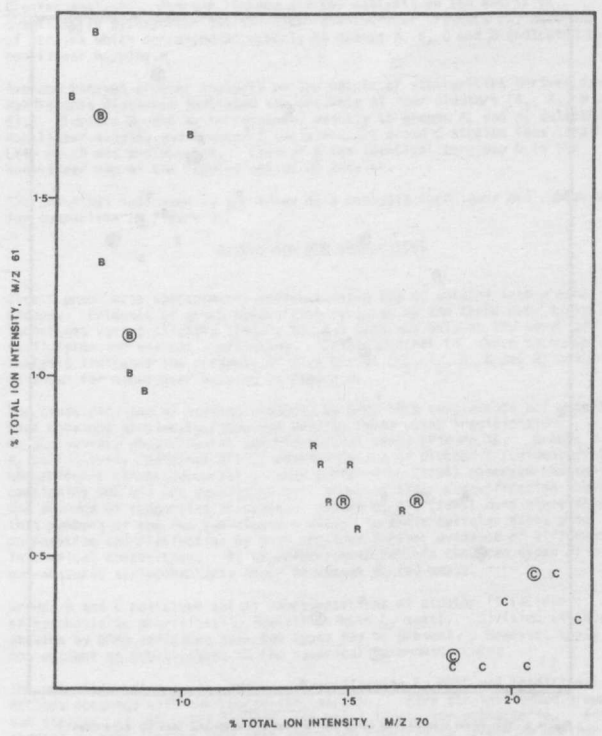

Figure 1. Plot of the relative intensities of ions m/z 70 against m/z 61 for experiment 1. B = beef, C = chicken, R = rabbit

breast muscle. The test samples fit extremely well into their respective groups including correct assignment into the two chicken subgroups.

Experiment 2

The analysis procedure followed for this 6-species study was similar to that for the first experiment. The spectra of the 72 sample data set were analysed to determine the most characteristic ions and the stepwise discrimination procedure employed to ascertain the optimum number of these to produce the best discrimination amongst the samples. Figure 2 is a non-linear map of the 24 sample replicate means plus the 12 test samples (shown in circles), produced using the 10 most characteristic ions, the optimum number indicated by the stepwise discrimination procedure. The ions were, in order of characteristicity, m/z 59, 96, 95, 58, 70, 55, 74, 102, 116 and 152. The diagram indicates that the chicken, rabbit, pig and sheep meat samples are quite well defined but there is some overlap between the horse and beef. The test samples generally fit well into their respective groups with the possible exception of one of the beef samples which appears intermediate between the beef and pig meat clusters.

The stress value is a measure of the distortion of the two-dimensional picture compared to the original multidimensional configuration. Values of 10% or lower have been found to give reliable representations of the data and values of 2 or 3% can be regarded as an indication of an almost perfect relationship between the data.

Experiment 3

As the horse, beef and sheep meat clusters were relatively close together in the previous experiment a further study was carried out on only these three species using a new set of samples. The analysis procedure was similar to the previous experiments and optimum discrimination was indicated using the seven most characteristic ions, m/z 119, 61, 146, 107, 75, 81 and 60. Figure 3 is a non-linear map of this data showing the 18 sample replicate means plus the 6 test samples (circled). The samples have clustered into the three groups with the horse completely separate from the other two species. However, there is now some overlap between the beef and sheep meat groups. This overlap results from the relatively large difference between the horse samples and those of the other two species disproportionately influencing the selection of characteristic ions, the sub-set used mainly reflects the separation of the horse group. A sequential analysis was therefore performed with the horse samples removed and characteristicities recalculated for the beef and sheep meat data only. Complete separation was then achieved between these two groups using four ions, m/z 60, 73, 150 and 74.

The test samples fit into their respective clusters, particularly for the well-defined horse group in Figure 3 and also for the beef and sheep meat groups in the separate analysis of these two species.

DISCUSSION

The technique of direct probe mass spectrometry coupled with multivariate statistical analysis has successfully discriminated meat species in the three studies undertaken. In addition, the concept of the characteristicity of an ion has been shown to be a very valuable tool in eliminating redundant data and revealing clustering tendencies amongst samples.

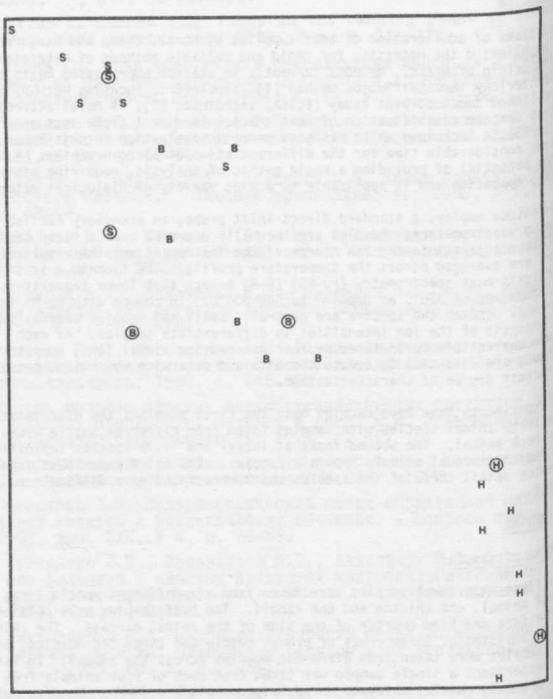

Figure 3. Non-linear map derived using the 7 most characteristic ions for experiment 3.
B = beef, H = horse, S = sheep. Stress = 1·92%

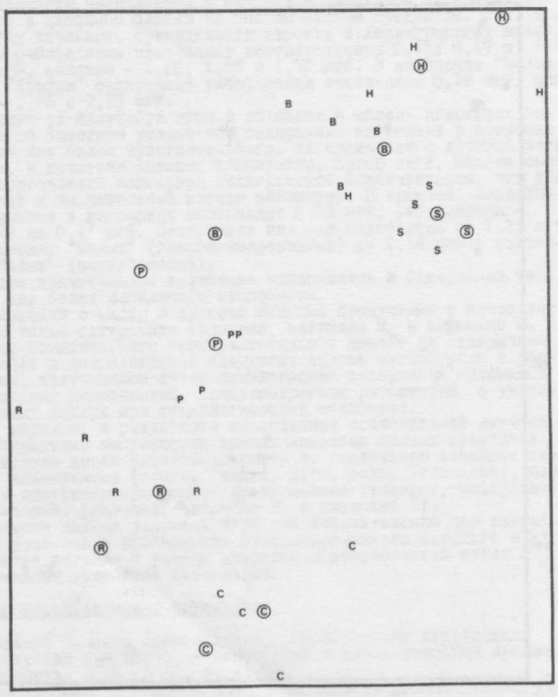

Figure 2. Non-linear map derived using the 10 most characteristic ions for experiment 2.
B = beef, C = chicken, H = horse, P = pig, R = rabbit, S = sheep. Stress = 7·41%

The first study demonstrated that there is greater difference between species than there is between different muscles of the same animal, hence it is not necessary to know precisely which part of the animal the samples came from to ensure correct classification. This is obviously important if samples of unknown origin are analysed. The relative position of the species groups, particularly in experiments 1 and 2, reflect the expected separation between meats, horse and beef will be expected to be closer than, for example, horse and chicken. Bearing in mind that we have used aqueous extracts the overall pattern seems to correlate with the colour of the meat, going from the red meats of horse and beef through to the white meat of chicken. The characterising factor may therefore be associated with the degree of pigment the meat contains. Experiment 3 confirmed that the separation achieved between three closely related species, horse, beef and sheep, was genuine, although a sequential analysis was necessary to optimise the parameters.

The analyses described in this paper have been applied to pure samples of each animal species. However, a major problem facing the analyst in species identification is the determination of the identity and relative amounts of different meats in mixtures. In this context the present method could be applicable particularly if the overall reproducibility of the technique could be improved with a consequent tightening of the parameters defining the species groups. As indicated earlier, the main differences between the spectra of different meat species is of a quantitative rather than a qualitative nature, hence it is impossible to define species by the presence or absence of any particular ion. It is more a question of interpolation between the typical values for ions characteristic of the species under examination. Non-linear maps could be used to get some idea of how an unknown mixture fits into the overall pattern. However, because of the distortion of the distances which may be produced by the mapping procedure, as indicated by the stress value, the maps should only be used to obtain qualitative impressions of the data. If absolute measurements are required the similarity, or distance, matrix should be used.

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