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Rapid and quantitative analysis of recombinant protein expression using pyrolysis mass spectrometry and artificial neural networks: application to mammalian cytochrome b_5 in *Escherichia coli*

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Abstract

Recombinant *Escherichia coli* clones encoding between 0 and 6 copies of the mammalian cytochrome b_5 gene were subjected to pyrolysis mass spectrometry (PyMS). To deconvolute the pyrolysis mass spectra so as to obtain quantitative information on the amount of cytochrome b_5 produced fully-interconnected feedforward artificial neural networks (ANNs) were studied. It was found that the combination of PyMS and ANNs could be used to predict the amount of cytochrome b_5 expressed in *E. coli*. PyMS is a novel, convenient and rapid method for the screening and analysis of microbial and other cultures producing recombinant proteins.

Key words: Pyrolysis mass spectrometry; Artificial neural network; Recombinant protein; Mammalian cytochrome b_5; Chemometrics; Biotechnology

1. Introduction

There is a continuing need within biochemistry and biotechnology for the development of rapid, efficient and reliable methods for the analysis of microbial cultures (over)producing biomolecules of interest. This requirement is of importance in terms of novel isolates (e.g., Ōmura, 1986; Crueger and Crueger, 1989), during the development of derivatives of existing productive strains in strain improvement programs (e.g., Crueger and Crueger, 1989; Azuma, 1992), and, in particular, during a batch growth or a production run itself (Clarke et al., 1985; Kell et al., 1990; Locher et al., 1992; Schügerl, 1992; Schügerl et al., 1993).

The ideal method for such analyses would have minimum sample preparation, would analyse samples directly (i.e., be reagentless), would give information about recognizable chemical characters, and would prove to be rapid, automated, quantitative and (at least relatively) economical. Pyrolysis mass spectrometry (PyMS; Ir-

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win, 1982; Meuzelaar et al., 1982) is an automated, instrument-based technique which possesses the above properties, in that it is reagentless, rapid (the typical sample time is less than 2 min) and may easily be automated. A particular goal lies in the rapid and quantitative analysis of recombinant protein production in (bacterial) hosts over-expressing the gene of interest, since existing chromatographic and electrophoretic methods (e.g., Randal et al., 1990; Marko-Varga and Barceló, 1992; Perrett and Ross, 1992; Aebersold, 1993; Paliwal et al., 1993) remain far from ideal. We, therefore, considered that PyMS might be a very suitable method for exploitation in the rapid analysis of microbial cultures producing recombinant proteins.

Kaderbhai and colleagues (see Kaderbhai et al., 1990; Gallagher et al., 1992; Karim et al., 1993) have constructed a variety of strains of *Escherichia coli* strains containing different copy numbers of a synthetic structural gene encoding a mammalian cytochrome b_5, the extent of the production of the haemoprotein in simple batch culture being an approximately linear function of the gene copy number (at least when this is < 5; Gallagher et al., 1992). The cytochrome gene(s) were placed under the control of λP_L promoter in a high level expression plasmid vector. High expression of the recombinant genes, or indeed of cognate genes coding for fusion proteins containing cytochrome b_5, leads to a noticeable pink colour in the strains E (Kaderbhai et al., 1990), and the addition of saturating amounts of haem to bacterial lysates provides a simple and accurate spectrophotometric assay for the amount of recombinant protein produced (Gallagher et al., 1992).

In the present work, we compared the extent of production of recombinant cytochrome b_5 in pλ-*n*cyt as deduced spectrophotometrically with that assessed by PyMS in combination with artificial neural networks (ANNs). We found good agreement between the two methods, suggesting (given that PyMS is applicable to all biological systems, including those lacking suitable chromophores) that PyMS provides a convenient method for the rapid analysis of recombinant protein production.

2. Materials and methods

2.1. Cultures and growth conditions

E. coli TB-1 clones harbouring plasmid pλ-*0*cyt, which did not express cytochrome b_5, or pλ-*n*cyt, expressing a variable number (n) of the gene copies of cytochrome b_5, under the control of the thermo-inducible λ promoter (Gallagher et al., 1992), were employed. Bacteria were propagated in Luria broth (LB) composed of 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. The pH of the medium was adjusted to 7.0 prior to autoclaving.

2.2. Expression and analysis of cytochrome b_5

For expression of cytochrome b_5, *E. coli* pλ-*n*cyt were cultivated in 20 ml LB medium at 30°C to $OD_{600} = 1$, after which they were thermoinduced at 38.5°C for 5 h. The harvested cells ($5000 \times g$ for 5 min) were washed with 20 ml water and suspended in 200 ml of TE (10 mM Tris-HCl (pH 8.0) 1 mM EDTA).

Soluble fractions (10–50 μl) of the lysozyme-mediated cell lysates, prepared as previously described (Gallagher et al., 1992), were used for the spectral estimation of cytochrome b_5 content. In a final 1 ml volume containing 5 μM haem, against a comparable blank, the amount of cytochrome b_5 was derived from the Soret absorption peak at 413 nm. Protein content was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.3. Pyrolysis mass spectrometry

5-μl aliquots of the bacterial suspensions were evenly applied onto iron-nickel foils. Prior to pyrolysis the samples were oven dried at 50°C for 30 min. Samples were run in triplicate.

The pyrolysis mass spectrometer used in this study was the Horizon Instruments PYMS-200X, as initially described by Aries et al. (1986), and see Goodacre and Kell (1993) and Goodacre et al. (1991, 1993). The sample tube carrying the foil was heated, prior to pyrolysis, at 100°C for 5 s. The pyrolysate was generated in a vacuum by the

heating of a ferro-magnetic foil carrying the sample. Heating was achieved by passing a radio-frequency current for 3 s through a pyrolysis coil which surrounds the sample-coated alloy foil. The foil and sample heated rapidly, 0.5 s, to the temperature corresponding to the Curie-point of the iron-nickel foil. At this temperature, 530°C, the alloy ceased to exhibit ferro-magnetic properties and heating finished; on cooling below the Curie-point, inductive heating resumed, so that the foil-pyrolyser system acted as a thermo-static switch maintaining the sample at the Curie-point, until current ceased to flow through the pyrolysis coil. This pyrolysis temperature was chosen because it has been shown (Windig et al., 1980; Goodacre, 1992) to give a balance between fragmentation from polysaccharides (carbohydrates) and protein fractions.

The pyrolysate then entered a gold-plated expansion chamber heated to 150°C, whence it diffused down a molecular beam tube to the ionisation chamber of the mass spectrometer. To minimize secondary fragmentation of the pyrolysate the ionisation method used was low voltage electron impact ionisation (25 eV). Non-ionised molecules were deposited on a cold trap, cooled by liquid nitrogen. The ionised fragments were focussed by the electrostatic lens of a set of source electrodes, accelerated and directed into a quadrupole mass filter. The ions were separated by the quadrupole, on the basis of their mass-to-charge ratio, and detected and amplified with an electron multiplier. The mass spectrometer scans the ionised pyrolysate 160 times at 0.2 s intervals following pyrolysis. Data were collected over the m/z range 51 to 200, in one tenth of a mass-unit interval. These were then integrated to give unit mass. Given that the charge of the fragment was unity the mass-to-charge ratio can be accepted as a measure of the mass of pyrolysate fragments. The IBM-compatible PC used to control the PYMS-200X, was also programmed (using software provided by the manufacturers) to record spectral information on ion count for the individual masses scanned and the total ion count for each sample analysed.

Prior to any analysis the mass spectrometer was calibrated using the chemical standard perfluorokerosene (Aldrich), such that m/z 181 was one-tenth of m/z 69.

2.4. Numerical analysis

The data from PyMS may be displayed as quantitative pyrolysis mass spectra (e.g., as in Fig. 1). The abscissa represents the m/z ratio whilst the ordinate contains information on the ion count for any particular m/z value ranging from 51–200. Data were normalised as a percentage of total ion count to remove the influence of sample size per se.

The data (normalised as above but not weighted by their standard deviations) were analysed by principal components analysis (PCA) using the program Unscrambler II Version 4.0 (CAMO A/S, Olav Tryggvasonsgt. 24, N-7011 Trondheim, Norway) (and see Martens and Næs, 1989) which runs under Microsoft MS-DOS on an IBM-compatible PC. PCA is a well-known technique for reducing the dimensionality of multivariate data, whilst preserving most of the variance (Chatfield and Collins, 1980; Flury and Riedwyl, 1988; Martens and Næs, 1989) and is an excellent technique for observing the natural relationships between samples.

All ANN analyses were carried out using a user-friendly, neural network simulation program, NeuralDesk (version 1.2) (Neural Computer Sciences, Lulworth Business Centre, Nutwood Way, Totton, Southampton, UK), which runs under Microsoft Windows 3.1 on an IBM-compatible PC. For in-depth descriptions of the modus operandi the reader is referred to Goodacre and Kell (1993) and Goodacre et al. (1993, 1994b).

The algorithm used was standard back-propagation (BP) (Rumelhart et al., 1986). This algorithm employs processing nodes (neurons or units), linked by abstract interconnections (connections or synapses). Connections each have an associated real value, termed the weight, that scale signals passing through them. Nodes sum the signals feeding to them and output this sum to each driven connection scaled by a 'squashing' function.

For training the ANNs, each of the inputs were the normalised triplicate pyrolysis mass spectra derived from *E. coli* pλ-*0*cyt, pλ-*2*cyt, and pλ-*5*cyt, and were paired with the desired outputs which were the actual (true) amount of cytochrome b_5 (as determined spectrophotometrically) expressed as a percentage of the total protein, viz. 1.625, 9 and 18.1; together these are called training pairs, and collectively make up the training set. The input is applied to the network, which is allowed to run until an output is produced at each output node. The differences between the actual and the desired output, taken over the entire training set are fed back through the network in the reverse direction to signal flow (hence back-propagation) modifying the weights as they go. This process is repeated until a suitable level of error is achieved.

The structure of the ANN used in this study to analyse pyrolysis mass spectra consisted of three layers; 150 input nodes, one output node, and one 'hidden' layer containing eight nodes (i.e., a 150–8–1 architecture). Before training commenced the values applied to the input nodes were normalised between 0 and 1, and the connection weights were set to small random values (Wasserman, 1989). The output layer was scaled to exploit less than the full range of the normalised scale between 0 and 1 (Goodacre et al., 1993), from −10 to 30 (thus approx. doubling the original scaling range of approximately 1.6 to 18). Each epoch represented 1217 connection weight updatings and a recalculation of the root mean squared (RMS) error between the true and desired outputs over the entire training set. Finally after training, all pyrolysis mass spectra of the *E. coli* were used as the 'unknown' inputs (test data); the network then output its estimate in terms of the amount of cytochrome b_5 in *E. coli*.

3. Results and discussion

The results of the spectrophotometric measurements of cytochrome b_5, expressed as a percentage of the total protein, from the seven *E. coli* strains are given in Table 1. The error in these values was typically 3–5%. A small amount of cytochrome b_5 was apparently found in the *E. coli* strain not containing the *cyt* gene (pλ-*0*cyt). This was due to endogenous bacterial *b*-type haemoprotein.

Pyrolysis mass spectral fingerprints of *E. coli* pλ-*0*cyt, *E. coli* pλ-*6*cyt, and mammalian cytochrome b_5 are shown in Fig. 1. The pyrolysis mass spectra of the *E. coli* strains are complex (Figs. 1A and B), such that their visual distinction is very difficult. Pure cytochrome b_5 shows a somewhat simpler pyrolysis mass spectrum, with intense peaks at m/z 102, 108 and 152 (Fig. 1C). These peaks are relatively minor in the mass spectra of the *E. coli* analysed (Fig. 1A and B), and may be considered to be characteristic of cytochrome b_5. However, the intensity of these peaks was not well correlated with increasing gene copy number, nor were these peaks prominent in the difference spectrum of the two *E. coli* strains (not shown). Thus, changes in single ions could not be used to estimate the amount of cytochrome b_5 that was produced in the *E. coli* strains.

The next stage was therefore to look at the relationship between the pyrolysis mass spectra of *E. coli* producing various amounts of cytochrome b_5 using principal components analysis (PCA). PCA is the best method for reducing the dimensionality of multivariate data whilst preserving most of the variance; in our pyrolysis mass spectral data this reduction will be from the 150 m/z values to the first two or three principal components (PCs). Plots of the first two PCs of the variance in the PyMS for cytochrome b_5 in *E. coli*

Table 1
The amount of cytochrome b_5 produced in the different strains of *E. coli* studied, as measured spectrophotometrically

Strain	Cytochrome b_5 as % of total protein
E. coli pλ-*0*cyt	1.625
E. coli pλ-*1*cyt	4.8
E. coli pλ-*2*cyt	9
E. coli pλ-*3*cyt	14.8
E. coli pλ-*4*cyt	17
E. coli pλ-*5*cyt	18.1
E. coli pλ-*6*cyt	12.6

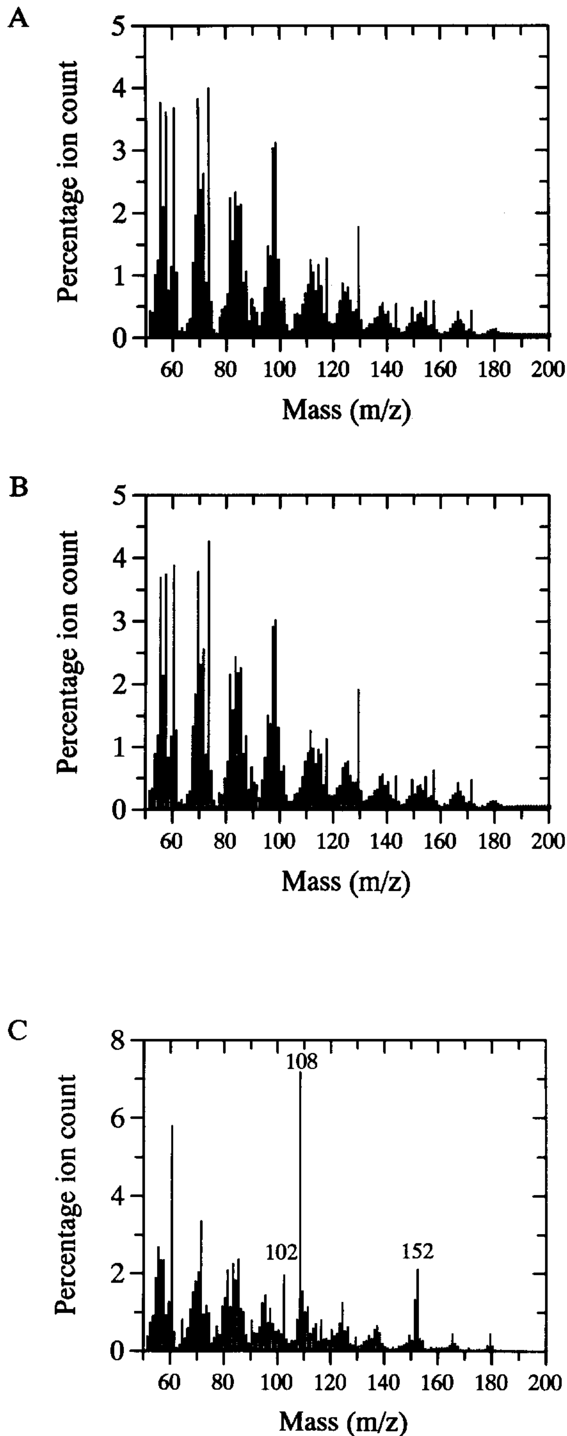

Fig. 1. Normalised pyrolysis mass spectra of *E. coli* pλ-*0*cyt (A), *E. coli* pλ-*6*cyt (B), and of a purified preparation of mammalian cytochrome b_5 (C).

(Fig. 2), which account for 55.0% and 14.0% of the total variation, show that most of the variation was preserved in the first PC. It was also evident that the first PC served roughly to account for (or describe) the difference in the amount of cytochrome b_5 being expressed in *E. coli*. That the second PC did, however, contain pertinent information in some of the samples (especially in bacteria expressing 14.8 and 18.1% cytochrome b_5 by weight of protein), indicates (as does the lack of similarity between the difference spectrum of Fig. 1A and B and the normalised spectrum of pure cytochrome b_5) that the expression of cytochrome b_5 in *E. coli* changes the mass spectra in a *nonlinear* fashion. This type of phenomenon is due to the occurrence of intermolecular reactions within the pyrolysate (Van de Meent et al., 1982; Schulten and Lattimer, 1984).

ANNs were then trained, using the standard back-propagation algorithm, with normalised ion intensities from the triplicate pyrolysis mass spectra from *E. coli* pλ-*0*cyt, pλ-*2*cyt, and pλ-*5*cyt as the inputs with outputs of 1.625, 9 and 18.1% cytochrome b_5, respectively; these values are the amounts of cytochrome b_5 previously estimated (Table 1). The effectiveness of training was expressed in terms of the RMS error between the actual and desired network outputs; to assess the

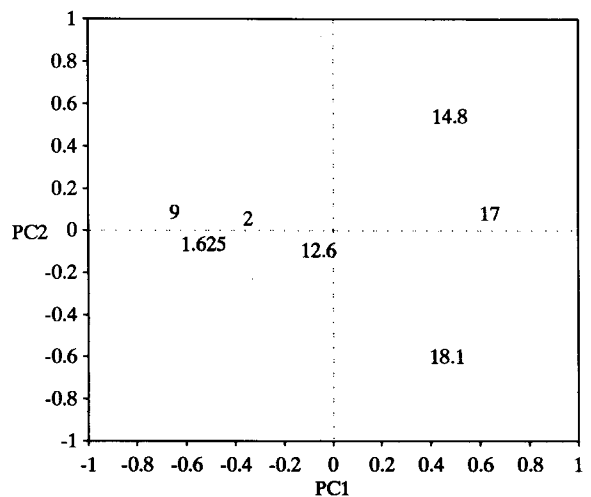

Fig. 2. Principal components plots based on PyMS data showing the relationship between all the samples.

extent of training the RMS error of the test set was plotted against the RMS error of the training set (Fig. 3). The RMS error in the test set reached a plateau at 6.7% when the RMS error of the training set was 0.2% and optimal training had occurred; additional training failed to increase or decrease the %RMS error in the test set. This point was after 6×10^3–6.5×10^3 epochs and took a little under 3 min. The %RMS errors in the test data are somewhat higher than those usually observed using defined mixtures (Goodacre et al., 1993, 1994a); this is probably because of the low numbers of exemplars in the training set (Goodacre et al., 1993). The ANN was then interrogated with the training and test sets, and a plot of the network's estimate vs. the amount of cytochrome b_5 expressed in *E. coli* (Fig. 4A) gave an approximately linear fit (i.e., $y = x$). It was therefore evident that the network's estimate of the quantity of mammalian cytochrome b_5 in *E. coli* was very similar to the true quantity (as judged by spectrophotometric methods) both for spectra that were used as the training set and, most importantly, for the 'unknown' pyrolysis mass spectra.

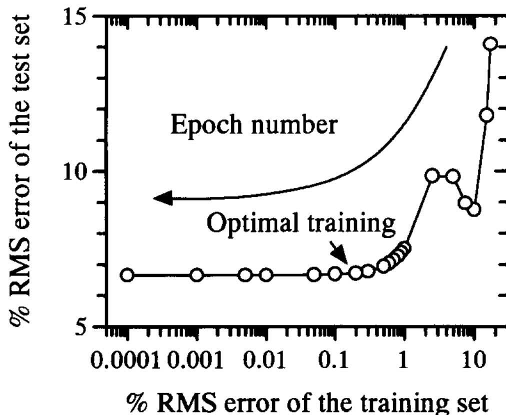

Fig. 3. Effect of the extent of learning of the data in the training set on the ability of the ANN to generalise. The generalisation of the ANNs and the extent of training was elucidated by plotting the percentage RMS error of the test set vs. the percentage RMS error of the training set during learning; this shows that optimal training occurred at 0.2% RMS error (indicated by the arrow). The number of epochs (and hence extent of training) increases from right to left.

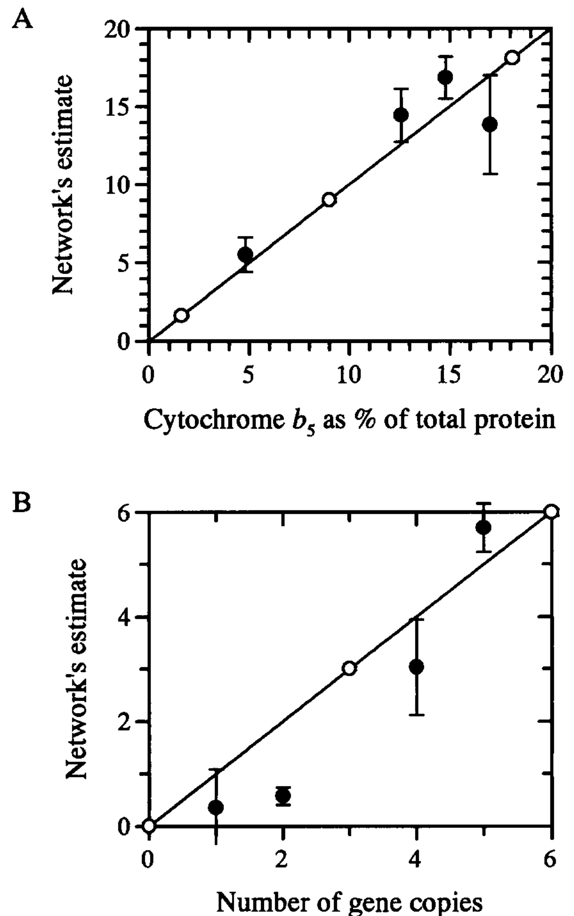

Fig. 4. Estimation of the biochemical properties of *E. coli* pλ-*n*cyt using pyrolysis mass spectrometry and artificial neural networks. Networks were trained using the standard back propagation algorithm to the optimal points (see text). Data points are the averages of the triplicate pyrolysis mass spectra. Open circles represent spectra that were used to train the network and closed circles indicate 'unknown' spectra which were not in the training set. Error bars show standard deviation. The expected proportional fits are shown. (A) Estimates of trained 150-8-1 neural networks of the true amount of cytochrome b_5. (B) Estimates of trained 150-8-1 neural networks of the *cytchrome* b_5 gene dose. ○, Results from data used to train the ANNs; ●, results from unseen data; —, expected proportional fit.

During over-production of cytochrome b_5 Gallagher et al. (1992) found that haem became deficient at higher gene doses (two to six). Gallagher and colleagues (1992) also found that a supplement of 5 μM bovine haem increased pro-

ductivity of the holo-cytochrome. To investigate the robustness of ANNs trained with pyrolysis mass spectra from haem deficient cells, accurately to assess the cytochrome b_5 content of haem-containing *E. coli*, a second test set was prepared which contained the same seven samples spiked with 5 μM haemin (Sigma). When the above ANN was interrogated with this new test set it was found that the network's preditions were the same as those observed from cells without haemin added (data not shown). It was therefore evident that the addition of 5 μM haemin to the *E. coli* strains did not cause significant spectral changes nor did it affect neural network generalisation.

It is worth considering whether the differences that the ANNs are learning in these pyrolysis mass spectra are due directly to differences in the amounts of cytochrome b_5 or alternatively simply to variations in the pλ-*n*cyt gene dose, which would be expected to alter the amount of mRNA in a proportionate manner (Gallagher et al., 1992). Ions at m/z 126 and m/z 135 are known to be derived from thymine and adenine (Voorhees et al., 1992) and thus may be considered representative of RNA and DNA. However, plots of the intensities of these ions against the copy number of the *cyt* gene (data not shown) showed that the intensities of m/z 126 and m/z 135 were quite independent of the gene copy number. Further, to investigate the possible effect of RNA and/or DNA on the mass spectra of these *E. coli*, ANNs were set up with the same architecture as those used above to estimate the gene dose. The inputs were the triplicate mass spectra from *E. coli* pλ-*0*cyt, pλ-*3*cyt, and pλ-*6*cyt with the outputs being the number of copies of the *cyt* gene, the output node was scaled from −3 to 9. After training for approx. 4×10^3–5×10^3 epochs, the point at which optimal training occurred, and when the %RMS error in the training set and test sets were 0.5% and 9.5%, respectively, the network was interrogated with the mass spectra from all seven *E. coli*. Fig. 4B shows a plot of the network's estimate vs. the *cyt* gene dose in *E. coli*, it is evident that the network was much less successful in estimating the gene dose than the cytochrome content. It is plausible that this ANN failed to generalise well because the production of cytochrome b_5 is only linear with gene dose up to four copies of the *cyt* gene (Table 1; Gallagher et al., 1992), i.e., there is not a direct correlation between gene copy number and amount of cytochrome b_5 expressed. In addition, quantitative considerations alone suggest that changes in protein, rather than nucleic acids, should dominate the pyrolysis mass spectra observed. We therefore conclude that the pyrolysis mass spectral patterns that the ANNs are learning are indeed due to differences in the cytochrome b_5 of the organisms.

As well as quantification, there is a continuing need within biochemistry, medicine and biotechnology for the rapid identification of proteins (Geisow, 1991; Chait and Kent, 1992; Wada, 1992; Aebersold, 1993; Moore, 1993). In addition, Shaw (1993) has recently pointed out that the amino acid composition, as well as the sequence, of known proteins is unique, such that measuring the former (together with the molecular weight) and interrogating a database can allow a rapid putative identification. The present study demonstrates that PyMS is a rapid technique which can be used to quantify proteins. The pyrolysis mass spectrum of a protein contains a wealth of information regarding its chemical characteristics, and it is reasonable, therefore, that with the correct exemplars in ANN training sets that it would be possible to effect a rapid estimation of the amino acid composition of the protein. This information could then be manipulated by suitable algorithms (Shaw, 1993) to gain the identity of the protein analysed.

In conclusion, we have shown that the combinations of PyMS and ANNs can quantitate the amount of mammalian cytochrome b_5 expressed in *E. coli*, and that the PyMS approach provides a rapid and convenient method for the analysis of recombinant protein production generally.

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