MaxEnt: An Essential Maximum Entropy Based Tool for Interpreting Multiply-Charged Electrospray Data

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Introduction

The electrospray spectrum from a mixture of biopolymers (proteins or oligonucleotides) is complicated, in that each component in the mixture produces a series of multiply-charged ions on a mass-to-charge ratio ($m/z$) scale. On this scale, the positions of the multiply-charged positive ions from a single protein of molecular weight $M_r$, are given by: $m/z = (M_r+nH)/n$, where H is the mass of the proton and n is an integer in a series of consecutive integers. Thus, each ion in a series differs by one proton from its immediate neighbours.

Typically, a 20 kDa protein generates a series containing 10-20 peaks. Hence, in the form originally produced by the spectrometer, the spectrum from a mixture of proteins can be extremely complex.

Simplification of such data is almost mandatory before interpretation can be attempted. If each series from a given protein could be condensed into a single peak on a true molecular weight scale, the amount of data to consider would be reduced significantly and, furthermore, would then be in a form which is more readily associated with the originating proteins.

Various methods have been described to carry out this procedure, whereby each multiply-charged ion series is transformed to single peak on a molecular weight scale to give the so-called ‘zero-charge’ spectrum. An early method\(^1\) tended to produce artefacts and a baseline which increased with mass. A more recent approach\(^2\), the Micromass® Transform method, achieves an artefact-free zero-charge spectrum, but requires identification of the multiply-charged series from each protein before transformation. Although identification of the series is a simple procedure with mixtures containing few components, it becomes extremely tedious with complex mixtures, particularly those containing proteins of widely different molecular weights.

Furthermore, since the data in the original multiply-charged spectrum are directly transformed onto the true molecular mass scale, components which were unresolved in the original data remain unresolved in the zero-charge spectrum. Another method\(^3\) claims to obtain accurate estimates of molecular weight from spectra with poor signal-to-noise characteristics, but is not quantitative.
In 1992, a method was developed which is based on maximum entropy (MaxEnt) analysis. In collaboration with J. Skilling, the MemSys5 maximum entropy program was incorporated into the Micromass MassLynx software package. In this application note, the power of MaxEnt in simplifying the electrospray spectra from complex mixtures and in improving the signal-to-noise ratio is illustrated. Furthermore, the way in which MaxEnt enhances the resolution is demonstrated and thus allows otherwise unresolved components to be separated and their molecular weights and relative intensities measured.

The Benefits of MaxEnt

MaxEnt has the following distinct advantages over alternative methods of processing multiply-charged electrospray data:

1. **MaxEnt automatically finds the molecular masses of the components present in a mixture**

   With a completely unknown sample, a first analysis or survey is made over a deliberately chosen wide molecular mass range in order to localise the mass range or ranges for a second definitive analysis. MaxEnt is computationally intensive and the processing time increases with the number of data points (channels) in the zero-charge (output) spectrum. Consequently, when making a wide mass range survey, it is usual to employ a relatively coarse mass scale (3-5 Da/channel) to keep the processing time to within 5 minutes or so. However, when this is done, the molecular weights are not calculated with full accuracy. Therefore, once the approximate masses of the components have been found from the survey, the second definitive run is made over a narrower output mass range (or ranges) using a finer mass scale (0.5-1 Da/channel), in order to obtain fully accurate molecular mass values.

2. **MaxEnt enhances the resolution i.e. improves the ability to separate and accurately measure the molecular masses of otherwise unresolved components**

   The arguments for needing resolution enhancement in the analysis of haemoglobins by electrospray mass spectrometry have been described in an excellent review by Shackleton et al. In essence, there is a basic limit to peak width, and hence resolution, which is determined by the isotopic distribution of the elements in the protein molecule. For the haemoglobin chains (15-16 kDa), this basic width at half peak height is 8 Da. With state-of-the-art quadrupole and time-of-flight analysers, the instrumental contribution to peak width is small for the haemoglobin chains, and increases the overall width to a little less than 10 Da, thus giving rise to the practical limit for resolving two haemoglobin chains, without deconvolution, of about 12 Da. The Shackleton review shows how MaxEnt extends the practical limit for resolving and accurately measuring the masses of two haemoglobin chains from 12 to 6 Da.

3. **MaxEnt improves the signal-to-noise ratio**

   MaxEnt has the power to extract useful zero-charge spectra from noisy multiply-charged m/z data.
4. MaxEnt is Quantitative

MaxEnt produces quantitative relative intensity data(8), that is, it provides a zero-charge spectrum in which the areas under the peaks are a measure of the sum of the intensities of the peaks in the original multiply-charged data. This is in contrast to another maximum entropy based method(9), where it is implied that maximum entropy is not quantitative by stating: ‘The relative peak amplitudes in the entropy deconvolution method do not correspond to ion abundance, although in a qualitative sense more intense peaks generally produce stronger entropy peaks’.

A disadvantage of the technique is the processing time. MaxEnt is computer intensive, and, depending on the application, may take from a few minutes to an hour to process a spectrum. Nevertheless, it allows many otherwise intractable problems to be solved directly from mixtures without resorting to some form of chromatographic separation.

Since MaxEnt was introduced in 1992 as a means for deconvoluting multiply charged electrospray data, it has found wide application as a problem solving aid in many areas of biochemistry. These applications are listed in Micromass Technical Note 202.

Instrumental

Unless otherwise stated, data were acquired on a Micromass Quattro II using sample concentrations in the range 2-10 pM/µl. The sample flow rate into the electrospray source was typically 5 µl/min. MaxEnt processing employed the MemSys5 maximum entropy program (MaxEnt Solutions Ltd, Cambridge, UK) incorporated as part of the Micromass MassLynx software suite supplied with the instrument. MaxEnt processing times are based on the use of a 200 MHz Pentium PC (Digital Equipment Corporation) employing Windows NT.

In some examples, in order to illustrate the resolution enhancement produced by MaxEnt, the original multiply-charged data were also processed by the Micromass Transform method(2). This method presents the original data on a true molecular weight scale, whilst faithfully reproducing the resolution in the original data. It is fast and accurate and is generally used for processing data containing few components and in cases where the resolution enhancement produced by MaxEnt is not required.

Generally, mass scale calibration employed the multiply charged ion series from separate introductions of horse heart myoglobin (16951.5 Da). In the case of the haemoglobins, the series from the human α-chain (15126.4 Da) or from an appropriate animal β-chain were used.

The molecular weights calculated from the protein sequences are average values based on the following atomic weights of the elements: C=12.011, H=1.00794, N=14.00674, O=15.9994 and S=32.066(10). In the text, molecular weights calculated in this way are given in brackets after the name of the protein.
Results

Automatic Determination of the Molecular Mass

a) Methane monoxygenase A

This is a non-haem iron protein consisting essentially of 3 non-covalently bound protein chains, with approximate molecular masses of 20 (γ), 45 (β) and 60 (α) kDa. The objective of the electrospray analysis was to measure the molecular masses of the 3 chains by analysing the protein in a de-naturing solution so that the 3 chains were analysed as separate entities. The multiply-charged series in the m/z spectrum of these components overlap (Fig. 1(a)), yet the MaxEnt survey gave a clean, essentially artefact-free spectrum (Fig. 1(b)), from which the approximate molecular masses of the 3 major groups of components were determined.

Artefacts tend to feature to some extent in the zero-charge spectra produced by automatic methods. These can be particularly troublesome when the multiply-charged series from components with substantially different molecular weights overlap giving rise to phantom peaks in the wrong part of the zero-charge spectrum. Harmonics and sub-harmonics can also occur. MaxEnt, whilst not entirely artefact-free, generally produces spectra in which artefact peaks are at a low level compared with the genuine peaks.

The processing time for the survey using 5 Da/channel in the zero-charge spectrum was 1 minute. The insets in Fig. 1(b) are from a second definitive MaxEnt analysis using a finer molecular mass scale (1 Da/channel) to obtain fully accurate mass values. Note that when several components are present, a number of narrow output ranges may be specified in order to keep the processing time to a minimum, in this case 19.5-20.4, 44.7-45.5 and 59.3-61 kDa. The time to produce the definitive spectrum was 6 minutes.

A useful feature of the software is that the peaks in both the m/z spectra and the MaxEnt spectra may be automatically annotated with operator defined letters and symbols once the molecular weights of the components present have been determined. The peaks in Figs 1-3 were annotated in this way.

Fig. 1(a). Original electrospray data from the protein chains (α, β & γ) of methane monoxygenase A and (b) the MaxEnt survey spectrum showing the major components. Note that artefacts are minimal, despite overlap of the multiply-charged series in the original data. The insets in Fig. 1(b) are from a definitive MaxEnt analysis made to obtain fully accurate molecular weights.
b) Leech Haemoglobin

This sample, from leech haemoglobin, is a complex mixture of about 30 proteins and glycoproteins. In the original m/z data (Fig. 2(a)), there is considerable overlap between the multiply-charged series from these components, yet, as in the previous example, the MaxEnt survey spectrum (Fig. 2(b)) clearly reveals three groups of components (2 major and 1 minor) with minimal artefacts. The processing time for the survey spectrum was 30 seconds using 4 Da/channel in the output spectrum. The original data were reprocessed to present the regions indicated by the survey (16.2-18.5, 24.2-26.2 and 32.8-35.0 kDa) on a finer mass scale (1 Da/channel), and the zero-charge spectra of the two major groups are given in (Figs. 2(c) and 2(d)). These show several series of proteins and glycoproteins clearly separated by hexose units, as indicated by the mass differences in each series being close to the calculated mass difference for hexose of 162.1. The processing time was 4 minutes.

Fig. 2(a). Raw electrospray spectrum from a leech haemoglobin sample containing approximately 30 components and (b) the MaxEnt survey spectrum revealing the major components in spite of the complexity of the raw data.

Figs. 2(c) and (d) show definitive MaxEnt spectra produced as a result of processing the raw data over the molecular weight ranges indicated by the survey.
Signal-to-Noise Ratio Improvement

**Fc fragment from an immunoglobin (IgG)**

This example illustrates how MaxEnt can extract a clean spectrum and the molecular weight from electrospray data which exhibit a very poor signal-noise-ratio. The sample was subsequently found to contain large quantities of buffer, hence the poor quality of the m/z spectrum. Fig. 3(a) shows the original data and Fig. 3(b) the same data after processing by MaxEnt. Note that the search window for molecular components was very wide (15-115 kDa). Nevertheless, 3 major Fc glycoforms differing by approximately 291 Da (sialic acid) were clearly revealed and artefacts (harmonics and sub-harmonics of the true molecular weight) were insignificant. MaxEnt salvaged meaningful results from an otherwise intractable m/z spectrum. The processing time to obtain this MaxEnt survey spectrum was 1 minute using an output mass scale resolution of 3 Da/channel.

**Fig. 3(a).** Raw electrospray data from the Fc fragment of an immunoglobulin (IgG) showing very poor signal-to-noise ratio and (b) after processing by MaxEnt to reveal 3 major glycoproteins differing by sialic acid (291.3). Note the wide mass range used for the component search.
Resolution Enhancement

a) Haemoglobin analysis

The improvement in resolution produced by MaxEnt when analysing haemoglobins has been illustrated for the Quebec-Chori and Hafnia β-chain variants\(^{5-6}\). These differ in mass from the normal β-chains by 12 and 9 Da resp. The Shackleton review\(^6\) also illustrates how MaxEnt separates the two α-chains from a mixture of sheep and cow haemoglobins, which differ by only 6 Da from one another. Fig. 4 shows two examples of the analysis of haemoglobin heterozygotes where, in order to establish the mass of the variant, it was necessary to resolve the variant chain from the normal chain by MaxEnt. In the first example, the mass of the variant (α20(His→Gln), Hb Le Lamentin) differs from the normal α-chain mass (15126.4 Da) by -9.0 Da and is not resolved from the normal α-chain in the original m/z data (Fig. 4(a) and inset). However, the MaxEnt deconvoluted spectrum (Fig. 4(b)) shows the two α-chains well resolved, allowing the mass difference of 9.0 Da to be determined with an error of only 0.1 Da.

In the second example, the variant (β5(Pro→Ser), Hb Tyne) is 10.0 Da lower in mass than the normal β-chain (15867.2 Da). Fig. 4(c) and inset show the two β-chains unresolved in the m/z spectrum. Again, in the MaxEnt deconvoluted spectrum (Fig. 4(d)) the two β-chains are well resolved allowing their masses to be accurately determined.

Both of these data sets were acquired on a Quattro LC by simply diluting whole blood 500 fold in 50% aqueous acetonitrile containing 0.2% formic acid. The time to process each data set by MaxEnt was 2 minutes.
The precision and accuracy of the molecular weight measurements to be expected from three doublets which are separated by 9, 8 and 6 Da are given in Table 1. None of these measurements would have been possible without applying some form of deconvolution, because the doublets were unresolved in the original m/z spectrum. To obtain these data, 7 repeat analyses were made on the haemoglobin from a heterozygote for the Hafnia variant and on equimolar mixtures of pig and sheep and of sheep and cow haemoglobins.

It can be seen from Table 1 that the reproducibility in the determination of the molecular weights of the components was about 0.8 Da standard deviation. This is about 3 times higher than is normally obtained for haemoglobin chains which are already resolved in the original m/z data (Table 2), the occurrence of overlap in the original data necessarily introducing some extra uncertainty in the resolved spectrum. Nevertheless, MaxEnt allows the molecular weights of unresolved proteins to be determined, albeit with slightly lower accuracy, a result not possible using alternative methods.

<table>
<thead>
<tr>
<th>Doublet</th>
<th>Measured ( M_R \pm \text{S.D.} )</th>
<th>Calculated ( M_R )</th>
<th>Error (Da)</th>
<th>Mass Diff ( \pm \text{S.D.} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hafnia ( \beta )</td>
<td>15858.4 ± 0.8</td>
<td>15858.2</td>
<td>0.2</td>
<td>9.2 ± 0.4</td>
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<tr>
<td>Normal ( \beta )</td>
<td>15867.6 ± 0.7</td>
<td>15867.2</td>
<td>0.4</td>
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<tr>
<td>Pig ( \alpha )</td>
<td>15038.7 ± 0.7</td>
<td>15039.1</td>
<td>-0.4</td>
<td>7.8 ± 0.6</td>
</tr>
<tr>
<td>Sheep ( \alpha )</td>
<td>15046.5 ± 0.7</td>
<td>15047.1</td>
<td>-0.6</td>
<td></td>
</tr>
<tr>
<td>Sheep ( \alpha )</td>
<td>14047.2 ± 0.8</td>
<td>15047.1</td>
<td>0.1</td>
<td>6.6 ± 0.8</td>
</tr>
<tr>
<td>Cow ( \alpha )</td>
<td>15053.8 ± 0.7</td>
<td>15053.2</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Results from 7 repeat analyses on each of 3 haemoglobin doublets separated by 9, 8 and 6 Da.

<table>
<thead>
<tr>
<th>Haemoglobin Chain</th>
<th>Measured ( M_R \pm \text{S.D.} )</th>
<th>Calculated ( M_R )</th>
<th>Error (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig ( \alpha )</td>
<td>15039.3 ± 0.2</td>
<td>15039.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Human ( \alpha )</td>
<td>15126.4 ± 0.2</td>
<td>15126.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Human ( \beta )</td>
<td>15867.5 ± 0.3</td>
<td>15867.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Pig ( \beta )</td>
<td>16035.3 ± 0.6</td>
<td>16035.4</td>
<td>-0.1</td>
</tr>
</tbody>
</table>

Table 2. Results from 8 repeat analyses of an equimolar mixture of pig and human haemoglobins after processing by MaxEnt. Before processing, the m/z scale of each spectrum was calibrated using the human \( \alpha \)-chain multiply charged ion series as an internal standard.
b) Porcine pepsin

Whilst attempting to rationalise 3 different published sequences of porcine pepsin A (Table 3), an initial approach was to measure the molecular weight of this 35 kDa protein. It was analysed using negative ions because there are only 4 basic amino acids in the pepsin molecule which is insufficient to bring the multiply charged positive ion series into the m/z range (4000) of the Micromass Quattro II. Results from commercial samples of the pepsin indicated the presence of more than one component.

When subjected to ion exchange chromatography, the pepsin was separated into 2 major components (pepsins A1 and A2). Figs 5(a) and 5(c) show the transformed spectra from these two components. These spectra faithfully represent the resolution in the original m/z data and indicate the presence of unresolved components. Therefore, the same original datasets were processed by MaxEnt to enhance the resolution (Figs 5(b) and 5(d)) giving two spectra showing almost identical detail, but displaced in molecular weight by approximately 48 Da.

Table 4 summarises the results from 7 repeat analyses on pepsins A1 and A2. The molecular weights of the major peaks agree well with two of the literature sequences (within 2 Da, 60 ppm), and are consistent with the presence of two variants in commercial porcine pepsin A, in which amino acid 242 is aspartic acid (D) in one variant and tyrosine (Y) in the other. The mean measured mass difference between the two variants was 48.4 Da, which compares with 48.1 Da for the expected mass difference between D and Y. The mass spectral data are inconsistent with the presence of the isoleucine at position 230 in the sequence given in Ref. (a) of Table 3.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Mr (1 to 326)</th>
<th>Mr (-2 to 326)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>34696.9</td>
<td>34881.2</td>
</tr>
<tr>
<td>b</td>
<td>34583.8</td>
<td>34768.1</td>
</tr>
<tr>
<td>c</td>
<td>34631.9</td>
<td>34816.2</td>
</tr>
</tbody>
</table>

Table 3. Variations in some published sequences of porcine pepsin and the molecular weights calculated from these sequences. Note that I-230 is absent in references b and c and is not included in the numbering of the sequences.

<table>
<thead>
<tr>
<th></th>
<th>1 to 326</th>
<th>-2 to 326</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Porcine pepsin A1.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measured Mr ± S.D.</td>
<td>34585.5 ± 0.8</td>
<td>34769.3 ± 3.6</td>
</tr>
<tr>
<td>Sequence Mr (ref. b)</td>
<td>34583.8</td>
<td>34768.1</td>
</tr>
<tr>
<td>Error (Da)</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Porcine pepsin A2.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measured Mr ± S.D.</td>
<td>34633.9 ± 0.5</td>
<td>34817.7 ± 1.4</td>
</tr>
<tr>
<td>Sequence Mr (ref. c)</td>
<td>34631.9</td>
<td>34816.2</td>
</tr>
<tr>
<td>Error (Da)</td>
<td>2.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 4. Results from 7 repeat analyses of porcine pepsins A1 and A2.

The MaxEnt processed data also revealed minor components in pepsins A1 and A2, measured to be 183.8 Da higher than the major components. These correspond to the pepsins with 2 additional amino acids (A-L, 184.2 Da) from the activation peptide i.e. they correspond to the protein with residues -2 to 326.
Figs 5(a) and (c). Transformed spectra from porcine pepsins A1 and A2 indicating unresolved components. These are a faithful representation of the resolution shown by the original electrospray spectrum.

Figs 5(b) and (d). After processing the same original data by MaxEnt showing significantly higher resolution.
The Quantitative Aspects of MaxEnt

To assess the quantitative aspects of MaxEnt in protein mixture analysis, 2 series of measurements were made. In one series of measurements, a mixture of two proteins, which were resolved from one another in the original m/z spectrum, were used. In the other series of measurements, a mixture of 3 proteins, which were unresolved in the original spectrum, were used.

a) Resolved 2 protein mixture

A series of mixtures of horse heart (16951.5 Da) and whale (17199.9 Da) myoglobins was analysed, in which the horse heart myoglobin was kept at a constant concentration of 1 pM/µl and the whale myoglobin concentration varied from 0.1 to 40 pM/µl. Data were acquired over the m/z range 650-1500. After processing the data by MaxEnt, a plot (Fig. 6) of the relative intensities of whale to horse myoglobins vs the relative concentrations of the 2 proteins gave a straight line with a linear regression correlation coefficient of 0.9997. The data showed good linearity over 2 orders of magnitude concentration ratio. The intensities were based on the areas under the peaks in the MaxEnt output spectrum.

In this case, since the peaks from the two myoglobins were resolved in the original data, the Micromass Transform method was also used to process the data, and gave comparable results to the MaxEnt results (Fig. 6).

Fig. 6. Whale to horse myoglobin intensity ratios determined by MaxEnt plotted against whale to horse myoglobin concentration ratios showing good linearity over a factor of 100 in concentration ratio.

\[
\text{MaxEnt. } \quad I = -0.11 + 0.75 C \quad (r = 0.9997) \\
\text{Transform. } \quad I = -0.09 + 0.76 C \quad (r = 0.9997)
\]

where I is Intensity Ratio, C is Concentration Ratio.
Fig. 7. Comparison of transformed with MaxEnt processed data in the region of the $\alpha$-chains from mixtures of horse and human haemoglobins, in which the concentration ratios of horse to human haemoglobin were (a) 1:5, (b) 1:1 and (c) 2:1. The resolution enhancement given by MaxEnt allows the relative intensities of these chains to be measured, in contrast to the Micromass Transform and original data where the resolution is insufficient.
b) Unresolved 3 protein mixture

In this case, a series of analyses was made on mixtures of horse and human haemoglobins in which the concentration ratio of horse to human haemoglobin was varied in steps from 1:10 to 10:1.

In these mixtures, there are 3 α-chains with molecular weights 15098.3 Da (horse α1), 15114.3 Da (horse α2) and 15126.4 Da (human α), separated by mass differences of 16 and 12 Da. These 3 proteins were insufficiently resolved in the m/z spectrum for them to be quantified without the resolution enhancement afforded by MaxEnt. The original m/z data were processed by MaxEnt to obtain the relative intensities of the 3 α-chains, and were also processed by the Micromass Transform method to illustrate the resolution before enhancement by MaxEnt. Note that the transform method mimics the resolution in the original m/z spectrum.

Fig. 7 compares the transformed with the MaxEnt derived spectra for the cases where the concentration ratios of horse to human haemoglobins were (a) 1:5, (b) 1:1 and (c) 2:1. The heights of the bars in the lower of the two MaxEnt spectra (MaxEnt Intensities) represent the areas under the peaks in the MaxEnt profile spectra and were used as a measure of their intensities. It can be seen that the relative intensities of the 3 α-chains cannot be measured from the transformed data, because the peaks are not sufficiently resolved. However, the peaks are well resolved in the MaxEnt processed data.

Fig. 8 shows the MaxEnt intensity ratios of horse α1 and α2-chains to human α-chain plotted against the concentration ratios of horse to human haemoglobin. The plots are linear over a range of about 50:1 in concentration. The maximum usable ratio of horse to human haemoglobin is approximately 5:1 because at higher ratios, interferences between the horse α1 sodium adduct and the human α become significant.

![Fig. 8. Horse α1 and α2 to human α intensity ratios determined by MaxEnt plotted against the concentration ratio of horse to human haemoglobins showing good linearity over about 50:1 in concentration ratio.](image-url)
Conclusions

The examples shown above demonstrate that MaxEnt automatically produces artefact-free zero-charge (molecular weight) spectra from the multiply-charged electrospray data from complex mixtures. MaxEnt achieves this feat without any prior knowledge of the molecular weight.

MaxEnt has also demonstrated an ability to improve the signal-to-noise ratio by salvaging meaningful results from an otherwise intractable m/z spectrum exhibiting very poor signal-to-noise ratio.

Furthermore, MaxEnt enhances the resolution beyond that achievable by quadrupole mass spectrometry alone i.e. beyond the theoretical limit imposed by the isotopic distribution of the elements in the protein molecule.

Finally, MaxEnt is quantitative in that it faithfully reproduces the intensities in the originating multiply-charged spectrum. MaxEnt is the only reported method of processing multiply-charged electrospray data which demonstrates all of these benefits.
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Prof. S. N. Vinogradov, Dept. of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan, USA, for the leech haemoglobin.

Dr. B. J. Wild, Dept. of Haematological Medicine, King’s College Hospital, Denmark Hill, London, SE5 9RS, UK, for the Tyne haemoglobin.

References


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