A comparison of mass spectrometric methods for the analysis of protein mixtures

I refer to the paper by Allen et al. [1] that seeks to compare electrospray (ES) and laser-assisted desorption time-of-flight (LDTOF) mass spectrometry for analysis of protein mixtures. In my opinion this paper does not compare the techniques impartially, but gives an impression, particularly to the non-expert in mass spectrometry, which misleadingly detracts from the utility of the ES technique.

The first major point on which I comment is that the authors state that gross suppression effects occur with ES m.s. they give an example where ES m.s. was used to analyse a mixture of haemoglobin and myoglobin and state "...when this mixture was first examined by ES, using equimolar concentrations of haemoglobin and myoglobin, ion signals from both haemoglobin chains were suppressed, compared with that of the myoglobin, with the haemoglobin-B-chain signal being so suppressed as to be unobservable. However, with the addition of a 25-fold molar excess of haemoglobin, signals adequate for the mass determination of all three species were present". The concentration of each component initially used was 6 \( \mu \)M.

This is not our experience with ES m.s. and, although there are sensitivity differences between proteins, we do not observe the gross effects described by Allen et al. Fig. 1 shows typical ES data, using our instrumentation, from an equimolar mixture of the same three proteins at similar concentrations (5 \( \mu \)M each) to those initially used by Allen. The transformed spectrum in the lower section of the Figure illustrates that the myoglobin (peak C) intensity is only 2.5–3 times higher than that of the \( \alpha \) and \( \beta \) globins (peaks A and B). Furthermore the \( \alpha \) and \( \beta \) globin peaks have comparable intensities. The differences in response observed between the two instruments are possibly due to differences in the design of the ES source and in the configuration of the ion transport system between the source and mass analyser.

The second major point is the omission from [1] of an adequate comparison between the resolution capabilities of the two techniques. In Fig. 1(a) of that paper is shown the matrix-assisted LDTOF spectrum of the equimolar myoglobin/haemoglobin mixture mentioned above. In this Figure, the \( \alpha \) and \( \beta \) globins are only resolved down to about 20\% valley, even though they are separated in mass by 741 Da (1 in 20 in mass). Furthermore the \( \beta \) globin/myoglobin peak resolution exhibits a similar valley even though the two peaks are separated by 1085 Da (1 in 15 in mass).

The resolution exhibited by the spectrum in Fig. 1(a) is significantly lower than is routinely produced by ES m.s. It is

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**Fig. 1.** Electrospray data from an equimolar mixture of haemoglobin \( \alpha \)-globin (A), \( \beta \)-globin (B) and myoglobin (C).

The upper spectrum shows the original data on an m/z scale and the lower spectrum the data after transformation onto a molecular mass scale.
partly compromised by the formation of the adducts of sample with the sinapinic acid matrix mentioned by Allen et al. The addition of a matrix is essential for analysis by the LDTOF technique to be successful. That there is a further limitation to resolution can be inferred from the Figure where the adduct peaks themselves are only partly resolved from the protonated molecular ion peaks at a mass difference of 206 Da (about 1 in 80 in mass).

Allen et al. make no comment on the resolution obtained by ES m.s. on this mixture and all the ES spectra in the paper are presented in a way which does not give the reader the opportunity to assess the resolution. In our ES m.s. data, the α and β globins are fully resolved down to the base line and, moreover, there is sufficient resolution available to partially separate, but nevertheless accurately measure, two globins differing in mass by as little as 14 Da at 16000 (1 in 1100), for example the two γ-globins in foetal haemoglobin with molecular masses of 15995.2 and 16009.3 [2].

The analysis of more than a thousand protein samples in our laboratory has demonstrated the utility of the higher resolution available with ES m.s. because a significant proportion of samples contain closely spaced components which would not be resolved by the LDTOF instrument. In such samples the molecular mass measured by LDTOF would, at best, be an average of the components present. The LDTOF spectra shown in Allen’s paper and those also given in a subsequent paper in the same issue [3] indicate performance where the resolution is at least a factor of 10 lower than with ES m.s.

These comments are not meant to imply that the LDTOF technique does not have a niche in protein mixture analysis. On the contrary, it reported higher sensitivity, mass range and resistance to the presence of buffers etc. in the sample make it complementary to ES m.s. However, I maintain that [1] presented the comparison between the two techniques in an unbalanced and misleading fashion and hence justifies this response.

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Received 17 December 1991

Specificity constants in the context of protein engineering of two-substrate enzymes

It has become popular in recent years to combine the two constants of the Michaelis–Menten equation in a ratio, the so-called specificity constant, $k_{cat}/K_m$ [1,2]. This is frequently used for comparing the activities of a single enzyme towards two alternative substrates. It has a clear physical meaning, representing the proportionality of rate to substrate concentration at low concentrations (i.e. well below $K_m$). Thus, with the same amount of enzyme and equal low concentrations of two substrates (separately), the rates should be in the ratio of their specificity constants.

This constant has also been adopted as a convenient way of comparing the activities of protein-engineered mutants with the activity of the corresponding wild-type enzyme. With effectively single-substrate enzymes such as subtilisin or chymotrypsin this presents no problems, but with two- or three-substrate enzymes such as tyrosyl-tRNA synthetase, glutathione reductase or lactate dehydrogenase it needs to be used with care. In the Dalziel representation [3] of the initial-rate equation (eqn. 1)

$$
\frac{v}{K_m} = \frac{1}{K_m} + \frac{1}{K_{cat}} + \frac{1}{K_{cat}^A} + \frac{1}{K_{cat}^B} + \frac{1}{K_{cat}^{AB}}
$$

$K_m$ for substrate A is $\frac{1}{K_{cat}^A}$, $k_{cat}$ is $\frac{1}{K_m}$ and the specificity constant for substrate A is simply $\frac{1}{K_{cat}^A}$. Similarly for B the constant is $\frac{1}{K_{cat}^B}$. It is immediately apparent that both the $K_m$ for A and the $V_{max}$ must be measured with, or rather extrapolated to, saturating concentrations of B. If the concentration of A is varied only at a single, supposedly saturating, concentration of B it cannot be assumed that the same concentration of B is necessarily saturating for a mutant form of the enzyme. It is nonetheless increasingly common to present only the constants without the evidence for a valid procedure of extrapolation. This poor documentation devalues much of the published information on the results of protein engineering.

Nevertheless the specificity constant remains a meaningful concept in the two-substrate context. It has been used, for example, to describe the strikingly successful conversion of lactate dehydrogenase to malate dehydrogenase by a single mutation. In the mutant Q102R, derived from Bacillus stearothermophilus lactate dehydrogenase [5], the specificity constant for lactate decreased from $4.2 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ to $5 \times 10^3 \text{M}^{-1} \text{s}^{-1}$ and that for oxaloacetate increased from $4 \times 10^3 \text{M}^{-1} \text{s}^{-1}$ to $4.2 \times 10^4 \text{M}^{-1} \text{s}^{-1}$.

The same group of workers have now addressed the specificity of their enzyme for its second substrate, NAD+, and attempted to engineer improved selectivity for NADP+ [6]. In presenting their results they employ a new parameter, which they term the ‘overall catalytic efficiency’, given by

$$
\frac{k_{cat}}{K_m} = \frac{k_{cat}^A}{K_m^{pyruvate}} \times \frac{k_{cat}^B}{K_m^{coenzyme}}
$$

To quote, “in evaluating the relative efficiency of an enzyme with alternative coenzymes, the substrate $K_m$ must be taken into account, i.e. the ability of the enzyme–coenzyme to bind the substrate must be considered …… A realistic measure of overall catalytic efficiency must take account of the maximal turnover rate and $K_m$ values for both substrate and coenzyme.”

It is puzzling, first of all, that these authors have not similarly addressed the possible modulation of apparent selectivity for lactate versus malate by the coenzyme concentration. However it is not at all clear that the proposed new parameter is either needed or valid. It is indeed true that the apparent selectivity for either substrate is likely to be affected by the concentration of the other, but if there is concern for the way in which coenzyme selectivity might be affected by substrate concentration, it has to be met by reference to the rate behaviour at low concentrations of both substrates. As eqn. (1) shows, whereas $\frac{1}{K_m}$ is the proportionality constant with respect to [A] at high fixed [B], $\frac{1}{K_{cat}}$ is the corresponding constant at low fixed [B]. The constant which Feeney et al. have introduced is $\frac{1}{K_{cat}^A}$ and this is only equal to the constant $\frac{1}{K_m^A}$ under the exceptional circumstances that the $K_m$ for A is independent of [B] and vice versa, i.e. the one set of circumstances where varying the fixed substrate does not affect the apparent specificity constant! This constant thus has no easily identifiable physical significance, and to describe it as a measure of overall catalytic efficiency is misleading.

The original concept of a specificity constant, carefully applied,