SPECIAL FEATURE:
TUTORIAL

High-resolution Mass Spectrometry and Accurate Mass Measurements with Emphasis on the Characterization of Peptides and Proteins by Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry

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Fundamental concepts of high-resolution mass spectrometry as it relates to the issue of accurate mass measurements are presented. Important issues specific to magnetic sector instruments, Fourier transform ion cyclotron resonance and time-of-flight (TOF) methods are discussed. Recent high-resolution TOF measurements on peptides and proteins, ionized by matrix-assisted laser desorption/ionization (MALDI), are presented and discussed in terms of basic concepts important to accurate mass measurements. © 1997 by John Wiley & Sons, Ltd.

INTRODUCTION

Mass spectrometry is a complex discipline that has evolved through many stages, in terms of both the chemical problems that can be tackled and the instrumentation employed. Users of mass spectrometry fall into two general categories: those who know little about the hardware but employ mass spectrometry as a tool and must understand and interpret the data, and a second group composed of chemists who are familiar with the technique, employ it on a regular basis and perform research on some particular topic in mass spectrometry.[1] Clearly, the second group is in the minority, but the numbers and area of specialization of users intimately involved with mass spectrometers have increased enormously. The rapid growth in the mass spectrometer user base began with the introduction of quadrupole mass analyzers equipped with gas chromatograph introduction systems. More recently, rapid advances in electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) have brought with them many new instrument users and have changed the distribution of instruments in favor of quadrupole mass filters and time-of-flight (TOF) instruments.

The instruments routinely used for ESI and MALDI do not rival conventional magnetic sector instruments in terms of ultimate mass resolution, but their performance is improving. One particularly noteworthy development is the recent introduction of delayed extraction for TOF instruments. Another development which already competes in high-resolution measurement with the standard sector magnets is the ion cyclotron resonance (ICR) instrument, and advances are also occurring in quadrupole ion trap technology. It is these changes in and the needs of this user community that motivate the current tutorial on mass resolution and mass measurement accuracy. In this tutorial we reiterate important issues related to accurate mass measurements,[2,3] but we do not attempt to review the entire subject. High resolution is immensely helpful but neither a necessary nor a sufficient condition for high mass measurement accuracy. Therefore, we focus some of our discussion on how peak profiles and mass resolution affect mass measurement accuracy. We focus on TOF analyzers and MALDI ionization over other methods. We emphasize some of the differences between high resolution at low and high mass.

The traditional and continuing justification for high-resolution mass spectrometry is for the identification or confirmation of the molecular formulas of new compounds. In this experiment, high-resolution measurements are used to determine accurately the mass of the molecular ion and from this information a molecular formula is assigned (not necessarily uniquely). These

structural applications, were first used for organic compounds but have now been adapted to other areas of chemistry, e.g. to the characterization of organometallic and inorganic compounds and more recently to the study of biopolymers.[4–6] A general scheme for using mass spectrometry to identify an unknown compound is as follows: (i) determine the molecular mass of the compound from the mass spectrum; ideally the highest m/z ratio ion in the mass spectrum corresponds to an isotopic form of the intact, ionic molecule (see Table 1); (ii) identify functional groups present in the molecule on the basis of specific fragment ions and/or fragment ion series contained in the mass spectrum and characteristic fragment ions formed by loss of neutral molecules from the intact, ionic molecule that are characteristic of specific groups[11]; and (iii) assemble the identified functional groups to predict the structure of the molecule. Often some aspect of these three steps can be facilitated by applying more specialized methods, e.g. many compounds do not produce an intact molecular ion and an alternative ionization method may be used to produce a protonated, $[M + H]^+$, or deprotonated, $[M - H]^-$ or similar form of the molecule. Conversely, if the compound of interest is present in a mixture it may be desirable to use a chromatography/mass spectrometry or tandem mass spectrometry approach to solve the problem. Alternatively, it is possible to perform detailed compound identification or even complex mixture analysis using high-resolution mass spectrometry (HRMS).[2] An early example of the analytical utility of HRMS is for the detailed characterization of higher boiling fractions of petroleum in terms of functional group composition.[12] More recently, such methods have been used for the analysis of environmental samples, a specific example being TCDD[13–15].

HRMS INSTRUMENTATION

High-resolution mass spectrometers have evolved through many stages. In the 1960s, improved electronics and a better understanding of ion optics gave birth to a new generation of 'high resolution, double focusing' (see Table 1) mass spectrometers.[16] A sector magnetic analyzer is a momentum analyzer, thus ions of the same mass but with different velocities have different spatial focal points.[17] To overcome the limitations in mass resolution arising from ion sources that produce ions having a range of translational energies, an electrostatic energy analyzer can be added to magnetic sector instruments. Instruments having a combination of electrostatic and magnetic sectors focus ions according to both direction and velocity (double focusing) while dispersing according to mass-to-charge ratio. During the 1970s, considerable efforts were directed toward perfecting the

Table 1 Notes and Definitions

Molecular ion[a]	Mass spectrometrists refer to the intact, ionic molecule as the 'molecular ion,' however, this term should only be used to indicate the odd-electron ionic form, e.g. the radical cation, $M^{+\cdot}$ or radical anion $M^{-\cdot}$. Ions formed by ESI and MALDI are usually even-electron species such as $[M + H]^+$ or $[M - H]^-$ and should be referred to as the 'protonated molecule' or 'deprotonated molecule'
M_r	Average molecular mass calculated by using the average atomic mass of the individual elements (e.g. C = 12.011; H = 1.008; N = 14.007; O = 15.999)
M_i	Monisotopic molecular mass calculated by using the atomic mass of the most abundant isotope of each atom (C = 12.00000, H = 1.007825, O = 15.9949)
Double focusing mass spectrometer[b]	Strictly, the proper term is 'second-order double focusing' and it is related to coefficients of the direction and energy terms of the equation of motion for the ions
Resolving power (mass)[a]	The ability to distinguish between ions differing slightly in mass-to-charge ratio. It may be characterized by giving the peak width, measured in mass units, expressed as a function of mass, for at least two points on the peak, specifically for 50% and 5% of the maximum peak height
Resolution: 10% valley definition, $m/\Delta m$[a]	Let two peaks of equal height in a mass spectrum at masses m and $m - \Delta m$ be separated by a valley that at its lowest point is just 10% of the height of either peak. For similar peaks at a mass exceeding m, let the height of the valley at its lowest point be more (by any amount) than 10% of either peak height. Then the resolution (10% valley definition) is $m/\Delta m$. It is usually a function of m, and therefore $m/\Delta m$ should be given for a number of values of m.
Resolution: peak width definition, $m/\Delta m$[a]	For a single peak made up of singly charged ions at mass m in a mass spectrum, the resolution may be expressed as $m/\Delta m$, where Δm is the width of the peak at a height that is a specified fraction of the maximum peak height. It is recommended that one of three values, 50%, 5% or 0.5%, be used. For an isolated symmetrical peak, recorded with a system that is linear in the range between 5% and 10% levels of the peak, the 5% peak width definition is technically equivalent to the 10% valley definition. A common standard is the definition of resolution based upon Δm being the full width of the peak at half its maximum height, sometimes abbreviated to FWHM

[a] Ref. 7.
[b] Refs 8–10.

ion optics of double focusing instruments in order to improve the overall ion transmission (sensitivity) and the maximumum mass resolution.[18] The ultimate object of much of this effort was to facilitate accurate mass measurements. Although it has long been recognized that accurate mass measurement can be performed with low-resolution instruments, e.g., single-focusing or quadrupole instruments, peak broadening and shifts due to the presence of interfering ions limit the use of such methods and instruments having higher mass resolution are always preferred.[19] The early high-resolution instruments could achieve a mass resolution of 10 000–20 000, but to achieve such resolution required optimum operating conditions, e.g. very clean ion source and the best possible vacuum, and the sensitivity of such instruments was correspondingly low. Instruments such as the Kratos MS-50[20] and VG ZAB[18] under optimum conditions can achieve a mass resolution of 75 000–150 000.

Note: these resolution values are based on the 10% valley definition commonly used for sector instruments, i.e. when the valley between two peaks of equal abundance is 10% of the peak heights the resolution will be given by the mass divided by the mass difference. All other types of mass spectrometers use the 50% valley definition (or, but see below, the full width of a single peak at half its maximum height), which gives numbers which are 2–10 times higher, depending on the peak shape.

Mass measurement errors for sector instruments operated under high-resolution conditions are typically less than 1–5 ppm. It is important to note that these values are independent of the mass-to-charge ratio of the ion, provided that the instrument is operated at optimum accelerating voltage. Resolution is not independent of mass for most other types of mass spectrometers.

Although recent developments in magnetic sector instruments have been over-shadowed by the increased interest in other mass analyzers that have opened new frontiers in biological mass spectrometry, these instruments still perform the bulk of mass spectrometric analysis in many, if not most, mass spectrometry laboratories.[21] Synthetic chemists, in all areas of chemistry, rely heavily on HRMS performed using magnetic sector instruments for the characterization of new compounds and for elemental composition determination.[22] In addition, conventional HRMS continues to play a vital role in solving may problems in the area of analysis of environmental pollutants.[14,15] Here the superior dynamic range of the sector instrument is particularly valuable. Another subtle but distinct advantage of sector instruments is that their high resolution is accompanied by high resolving power (see Table 1), that is 'the ability to distinguish between ions differing slightly in mass-to-charge ratios.'[7] This capability can be utilized to distinguish isobaric ions which differ by fractions of a mass unit and it might be thought that this is an automatic consequence of a high resolution capability. However, mass resolution is defined (see above) in terms of the linewidth of a single peak, as opposed to an ability to split doublets, which is mass revolving power. The ability to achieve high resolution for ions of a single mass-to-charge ratio (i.e. narrow peak widths in

terms of $m/\Delta m$) is not a strong test of high resolving power. One of the early limitations of Fourier transform (FT) ICR was that even when high mass resolution was obtained, isobaric ions were often unresolved, even when this should have been possible with the resolution apparently available. The limitations of mass resolving power are especially problematic if one ion is present in far greater abundance than the other. The effects of this problem have been diminished by recent developments in FTICR (see above), but the resolving power limitations of the quadrapole ion trap are similar and have not yet been overcome, in spite of very high resolution when single peaks are examined.

The principal factor which limits mass resolution is the distribution of kinetic energies of ions emitted by the ion source, as noted above, and to this can be added imperfections in ion optics and the focusing characteristics of the ion optical components of the instrument.[23] The principal limitations on mass measurement accuracy also include the peak profile and the signal-to-noise ratio of the data.[2] The directions taken during the 1970s and 1980s to optimize mass resolution and mass measurement accuracy in sector instruments were to perfect instrument design to minimize these limitations. In particular, the approach taken was to optimize performance by adding special lensing systems to correct for ion optical aberrations.[24] Great progress was made in this endeavor; however, the cost of such instruments is high, especially for instruments capable of measuring large mass-to-charge ratios, e.g. high molecular mass bipolymers.[25–27] A further consideration is that sector instruments are most difficult to adapt for MALDI and ESI.

Alternative instrument designs, e.g. FTICR mass spectrometry[28–30] reflectron-time-of-flight (R-TOF) instruments[31–33] and radiofrequency ion traps[34,35] that are capable of high resolution and accurate mass measurements have been introduced, but there is still some doubt as to whether these instruments have developed to the level that they can produce data that are comparable to those obtained with high-performance magnetic sector instruments. Marshall and co-workers[29] recently reported a detailed evaluation of high-resolution FTICR for the analysis of aromatic neutral fractions of petroleum distillates. They discussed the effects of various instrument parameters on the precision of the mass measurements and how such effects dictate the acquisition of the data.[30] For example, ions of m/z 100 must be sampled at a higher rate than higher mass ions to satisfy the Nyquist criterion,[36] hence the time required to acquire a high-resolution mass spectrum depends on the m/z of the ion. Another important consideration is computer data set length, typically 1–4 Mwords per file. Because the optimum operating parameters of the FTICR instrument strongly depend on the mass range that is sampled, it is advantageous to eliminate ions that lie outside the mass range of interest. This is typically done using tailored excitation waveforms referred to as SWIFT.[37–43] Although the analysis by Marshall and co-workers[29] was limited to a relatively narrow mass range, e.g. m/z 260–310, advantages of ICR over conventional high-resolution magnetic sector instruments are clearly evident. For example, from a mass range of 42 Da, ions with 358 distinct
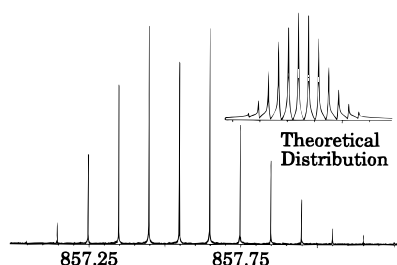
Figure 1. Partial ESI/FTICR mass spectrum of ubiquitin (M_r = 8565).

chemical formulas were resolved using FT-ICR, whereas, double focusing sector mass spectrometry resolved only 216 of those ions. Marshall and co-workers suggested that further development in several areas is needed, e.g. algorithms for locating positions of overlapping mass spectral peaks and apodization and/or data reduction methods to improve ion abundance precision and accuracy, and the dynamic range should be extended. The most significant limitation of FTICR is the dependence of mass measurement accuracy on trapping voltage, the density of trapped ions and the cyclotron radius of the ion packet following r.f. excitation. For the analysis of large molecules, a further consideration is the fact that mass resolution in FTICR decreases as the m/z of the analyte ion increases. This fundamental limitation of FTICR is one of the factors that motivated the development of ESI/FTICR. That is, ESI produces multiply charged ions of the analyte, thus reducing the m/z ratio. McLafferty and co-workers[44] have shown that the ulra-high resolution of FTICR is advantageous for the correct assignment of the charge state of multiply charged protein ions. The high mass resolution of FTICR allows the separation of the isotopic envelope of proteins having high charge states where the isotopes are separated by ($1/z$) Da, where z is the charge state. For example, Fig. 1 contains a portion of an ESI/FTICR mass spectrum of ubiquitin (M_r = 8565). The mass separation between the isotopes is $1/10$ Da; therefore, the ions contained in Fig. 1 all correspond to the ubiquitin $[M + 10H]^{10+}$ ion. R-TOF instruments are capable of mass resolution of 10000–15000, and mass measurement accuracy of 5 ppm for biomolecules <5 kDa, but the mass resolution (and consequent mass measurement accuracy) is often limited by jitter in the electronics or drift of high voltage power supplies, as discussed here. Quadrapole (Paul) ion traps have also achieved impressive mass resolutions[45–51] and recent theoretical treatments of the operation and parameters of the trap provide new insights into the analytical potential of this device.[35]

PEAK SHAPES AND MASS ASSIGNMENT

Limitations in the accurate determination of masses from mass spectral data are imposed by the mass resolution and also the peak shapes;[2] this assumes that an accurate calibraton curve can be applied to the data. Ideally, the signals in the mass spectrum are composed of Gaussian peak profiles which can be easily cen-

troided and converted to accurate mass values. The accuracy in determining the peak centroid is influenced by peak shape and signal-to-noise (S/N) ratio. A list of possible elemental compositions having masses within an established error range (1–10 ppm) is then compared with the measured values. It is important to limit the possible elemental compositions considered, otherwise the data set becomes unmanageable; however, this is generally not an arduous task because there will generally be additional information available on the compound, e.g., other analytical data (IR, NMR, etc.) or some knowledge as to the origin of the sample. Depending on how the mass data will be utilized, it may be advantageous to set the error limits to be very small in order to limit the total number of possibilities for elemental composition, but in other cases it may be desirable to set the error limits relatively large. As will be shown in a later section, the same considerations apply in the analysis of large biomolecules. A specific example of a problem where large error limits are desirable is illustrated later when discussing protein database searching.

Mass measurements of proteins are typically made using average mass values, and accurate mass measurements of proteins can be made when the isotopic envelope remains unresolved,[52] provided that the peak shape is Gaussian. Figure 2 displays a MALDI mass spectrum of horse heart myoglobin (M_r = 16951.5). The mass resolution of the $[M + H]^+$ ion is >2000, corresponding to an FWHM of ~8.4 Da. Note that the peak profile is Gaussian and that the peak width agrees well with the calculated theoretical distribution (Fig. 2(b)), which predicts the FWHM of the unresolved ^{13}C isotope distribution to be 8 Da. The accuracy of mass measurements made using average mass values is ultimately limited to ~10 ppm owing to variations in the natural abundance of ^{13}C which causes the average mass of carbon to vary between 12.0107 and 12.0111[53].

The presence of unresolved multiplets requires improved mass resolution to separate the overlapping peaks. For example, Fig. 3 contains data for bovine insulin (M_r = 5733.5) ions obtained at different mass resolution. This region of the MALDI mass spectrum contains the $[M + Na]^+$ and $[M + H]^+$ ions, in addition to fragment ions that arise by the loss of H_2O and NH_3 from the intact ions. At low mass resolution, the signals due to the individual ions are not resolved and the mass obtained from the peak centroid is 5717.7 Da, which corresponds to an error of 0.3%. At the highest mass resolution the measured mass for the $[M + H]^+$ ion (5735.1 Da) corresponds to an error of 0.01%. In the case of bovine insulin, the distribution of the ^{13}C isotopes yields a peak shape for the $[M + H]^+$ ion that is Gaussian.[54] Thus, accurate mass measurements can be made from signals where the isotopic profile is not resolved. However, a high level of mass measurement accuracy is unattainable if the peak shape is abnormal, owing to instrumental reasons or unresolved multiplets due to overlapping masses, or to poor S/N ratio. In any of these cases, the error in centroiding the peak will result in significant errors for the measured mass. Limitations imposed by the S/N ratio due to random noise can be overcome by signal averaging, but this requires careful alignment of the scan variables, e.g. magnetic
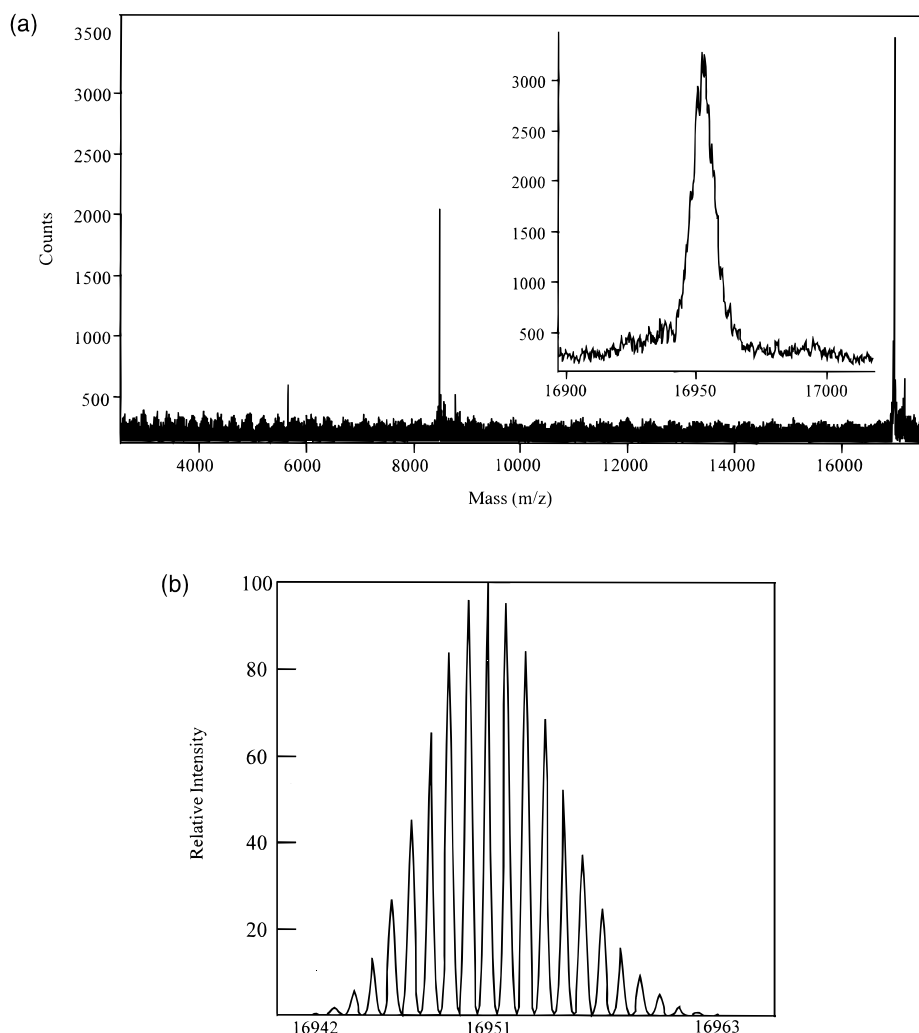
Figure 2. (a) MALDI-DE/TOF mass spectrum of horse heart myoglobin. Peaks corresponding to the $[M + H]^+$, $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ ions are observed. The inset contains an expansion of the $[M + H]^+$ ion region, illustrating a mass resolution ($m/\Delta m$) of 2000. (b) Simulated isotopic distribution for horse heart myoglobin.
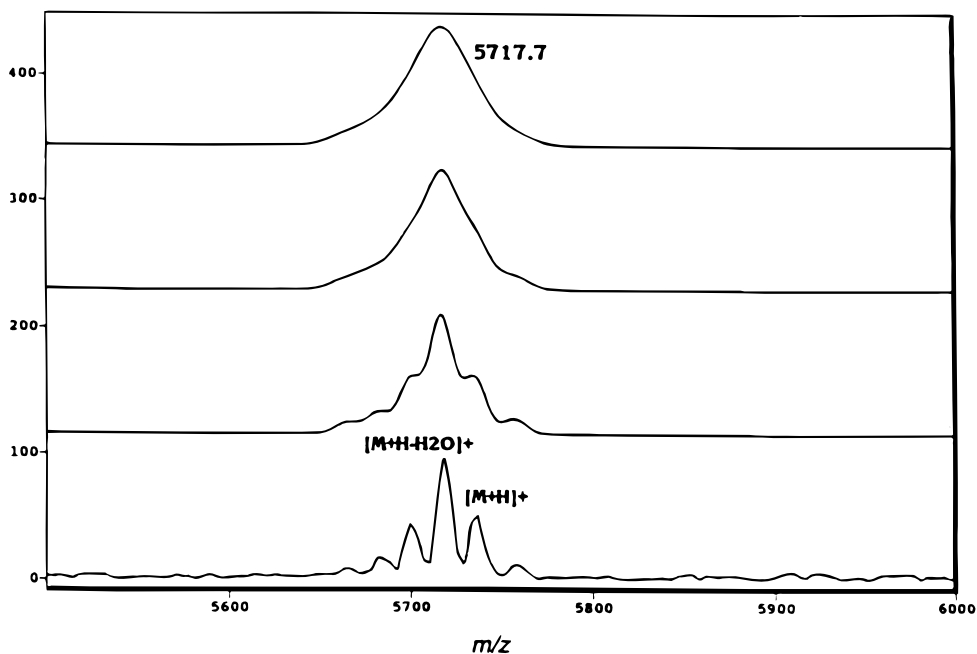
Figure 3. MALDI/FTICR mass spectra of bovine insulin. The top three traces correspond to artificially broadened peaks obtained by summation of Gaussian peaks with widths of (top) 25, (second) 20 and (third) 15 Da. The positions and heights of the individual Gaussian peaks were determined by a fit to the original mass spectrum (bottom).

field strength in the case of sector instruments, ion frequency in the case of ICR and time in the case of TOF instruments, as the individual mass spectra are summed.

Even when artificial peak broadening does not exist, i.e. the width of the peak is the same as the isotopic envelope, and individual isotopes are not resolved, there are problems with accurate (< 50 ppm) mass calibration for peptides with $M_r < 5$ kDa because the calculated distribution of ^{13}C isotopes is not Gaussian.[54] Just as asymmetric peaks due to unresolved ions cause difficulties in mass assignment, asymmetry of the peak profile due to the natural abundance of ^{13}C isotopes gives rise to mass errors for smaller peptides. An asymmetric carbon isotope distribution which contains theoretical peak profiles for peptides of 1000, 2000, 3000 and 4000 Da is illustrated in Fig. 4.

HIGH-RESOLUTION AND MASS MEASUREMENT ACCURACY WITH TIME-OF-FLIGHT INSTRUMENTS

The introduction of MALDI/TOF has greatly contributed to the development of a routine mass spectrometer for the analysis of biomolecules. The analytical advantages of TOF include low cost, high sensitivity, large mass range (in excess of 100 kDa), and the ability to record a complete spectrum in a single acquisition (so called Felgett advantage). The major disadvantage of TOF has traditionally been low mass resolution. The mass resolution of MALDI/TOF is especially poor because the ions are formed with a broad kinetic energy distribution[55] and a mass-independent initial velocity.[56,57] The mass resolution of TOF and MALDI/TOF can be improved by incorporating an ion reflector,[58] but metastable ion decay in the field-free region can skew the peak shapes.[59] Because isotope peaks are rarely resolved in MALDI mass spectra, mass calibration is performed using some method of centroiding a broad unresolved ion signal. Mass calibration using

peak centroiding methods gives reasonably good mass measurement accuracy, e.g. Beavis and Chait[60] reported mass accuracies of 0.01% (100 ppm) for MALDI/TOF in 1990. As illustrated above, errors in peak centroiding arise if the peak is skewed due to unresolved ions. The issue of peak shape is especially problematic in the analysis of proteins which have sample heterogeneity due to phosphorylation, glycosylation or other types of covalent modifications. MALDI can also produce multiple ions of the analyte, e.g. $[M + H]^+$, $[M + Na]^+$ and $[M + matrix]^+$ adduct ions. In the example of myoglobin shown above, these ions are resolved, but these ions would not be resolved in a typical linear MALDI/TOF mass spectrum.

Historically, the first significant improvement in TOF mass resolution was achieved by incorporating time-lag focusing (TLF).[61] Recently, several researchers have discussed the principles of TLF, or delayed extraction (DE) as it relates to MALDI-formed ions, and have shown its use to improve the resolution significantly.[31–33,62–64] In fact, the data contained in Fig. 2(a) were obtained by using DE. In this experiment, the mass resolution achieved with continuous extraction is ~ 200 compared with 2000 using DE. DE involves forming the ions in a field-free region and applying a high voltage pulse to accelerate the ions from the source a few hundred nanoseconds after ion formation.

The mass resolution that can be obtained using DE and the mass range over which unit resolution is achieved (m/z up to ~ 5000) is very well suited to the analysis of peptides and for peptide mapping. As illustrated by the data contained in Fig. 4, the isotopic abundance of peptides having M_r 1000–4000 are markedly asymmetric, hence it would be difficult to centroid the unresolved peak profile accurately.[54] Note also that the abundance of the all ^{12}C isotope is sufficiently high to give a good S/N ratio up to $M_i \approx 5000$. Because higher M_r values have a low abundance of the all ^{12}C isotope, the S/N ratio of the all ^{12}C is low, making it difficult to use this peak for accurate mass assignment. The high-resolution MALDI-DE/R-TOF mass spec-
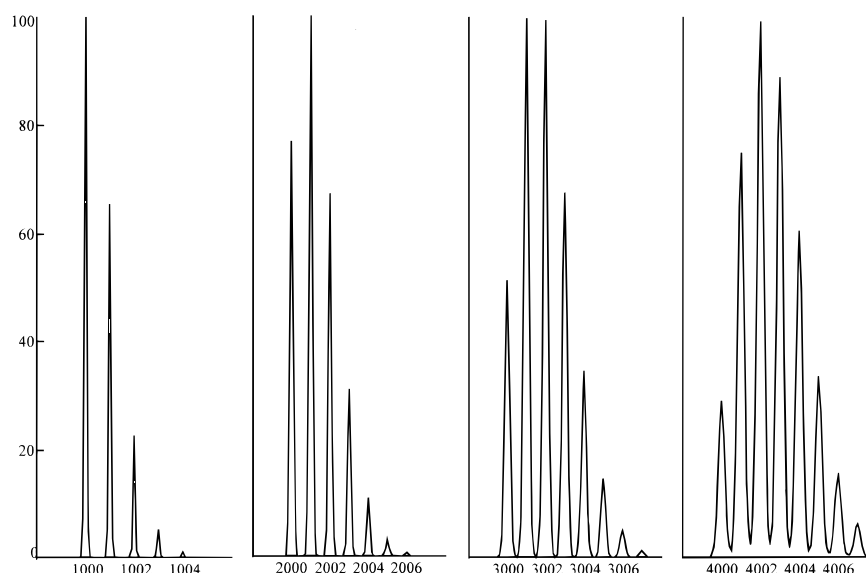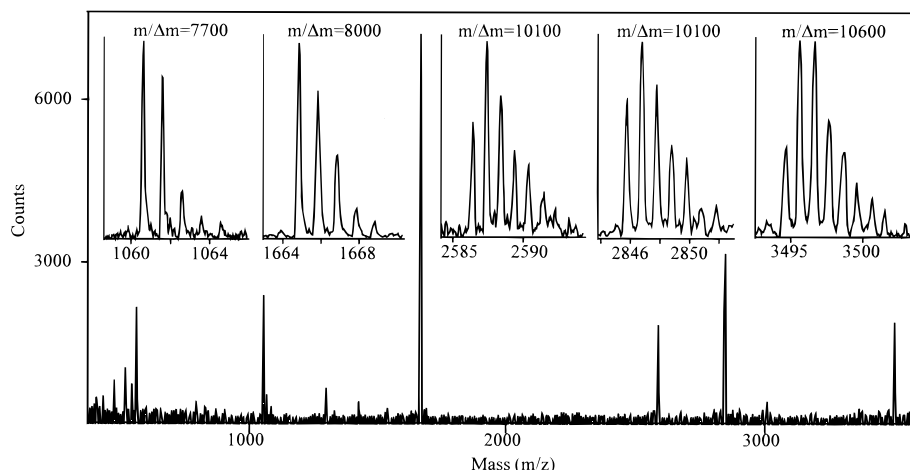

Figure 4. Theoretical isotope distributions of peptides of 1000, 2000, 3000 and 4000 Da.

Figure 5. MALDI-DE/TOF mass spectrum of a peptide mixture demonstrating that high resolution can be obtained over a range of m/z values.

trum of a peptide mixture is displayed in Fig. 5. The mixture contains peptides ranging in mass from ~ 1 to 3.5 kDa, and demonstrates that a range of m/z values can be simultaneously measured at good mass resolution. Mass resolution $>10\,000$ is routinely observed in this type of experiment, but under optimum conditions mass resolution as high as $15\,000$ can be achieved.[32] An important factor in achieving high-resolution TOF mass spectra is minimizing or eliminating factors that result in flight time drift or jitter.[65] Because TOF requires two events to be triggered simultaneously (i.e. ion formation and data acquisition), care must be taken to eliminate variations (jitter) associated with laser firing and the start of the data acquisition. This is usually accomplished with the use of fast photodiodes to synchronize laser firing and the start of data acquisition. Because TOF spectra are averages of many (50–100) individual spectra, the jitter will 'average in' and this is a major cause of signal broadening. Jitter will have a significant effect on mass measurement accuracy, especially if the jitter is large enough to cause the carbon isotope peaks to coalesce. A factor that has even more impact on the mass measurement accuracy than jitter is flight time drift. The most significant source of flight time drift is long-term drift of the high-voltage power supplies used to accelerate the ions. The effect of power supply drift can be greatly reduced by allowing them to warm up for at least 1 h prior to data acquisition. As the observed peak widths in TOF spectra become narrower, an ultimate limitation may be imposed by the response, typically 1–10 ns, from the microchannel plates used for ion detection.[65]

Sample surface inhomogeneity can also affect the accuracy of the flight time measurement. Sample preparation techniques that minimize these effects by providing a homogeneous area of matrix/analyte crystals have been described.[33,66,67] Variations in the sample thickness due to surface irregularities change the distance between the plate and the first extraction grid and result in variations of ion flight times. A variation of only 10 μm in the sample surface height produces a flight time shift of 1 ns, which corresponds to a deviation of 13 ppm for a 150 μs flight time, e.g. for ions of m/z 2000. When using external calibration, both flight

time drift due to power supply instability and sample hetereogeneity will affect the accuracy of mass calibration since the calibration spectrum is taken from a separate sample spot and at a different time from the spectrum containing the analyte.

The effects of jitter and long-term drift on mass resolution and mass measurement accuracy are shown to be slight in the following data. Consecutive MALDI-DE/R-TOF mass spectra of oxidized bovine insulin b-chain ($M_i = 3493.643$) were acquired at a mass resolution of $10\,000$ and the spectra overlaid. Insets of the overlaid spectra containing the $[M + H]^+$ ions are shown in Fig. 6, where the letters A–F represent the first six carbon isotope peaks. Table 2 contains the measured flight times of the ions shown in Fig. 6. The average deviation in flight time for each carbon isotope peak is ~ 0.6 ns or ~ 3 ppm. The most intense ion signals (B, C and D) have the lowest average deviation in flight time, illustrating the effect of the S/N ratio of the peak on errors in centroiding the peaks.

Flight time reproducibility of MALDI-DE/R-TOF spectra taken from different sample spots is compared in Fig. 7. Six sample spots were laid down near the center of the sample plate, with each spot containing three analytes: angiotensin II, substance P and mellitin. A spectrum, consisting of an average of 50 laser shots, was taken at each sample spot. The flight time deviation, in ppm, from the average flight time for each analyte is plotted in Fig. 7. The average deviation in time for analytes acquired from different sample spots is ~ 5–6 ppm, significantly larger than the deviation observed for consecutive spectra from the same sample spot (Tables 2 and 3). The error associated with flight time drift should be the same for the two sample sets, and therefore the likely cause of the increased deviation from the data obtained from different sample spots is either inhomogeneity in the matrix/analyte crystals or, more likely, surface aberrations of the sample support. The sample support used on the Voyager Elite XL is a large ($\sim 5 \times 5$ cm) plate that moves in the x and y directions.[31] If the alignment of the plate is not perfectly parallel with the extraction grid, even slight movements of the plate cause the distance between the sample plate and extraction grid to change.
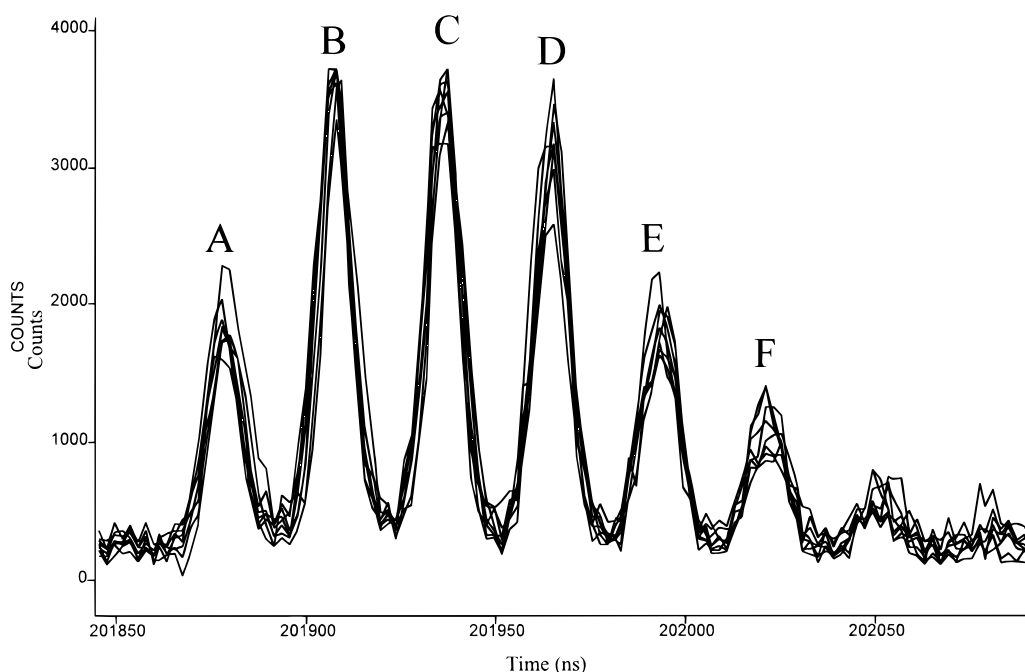
Figure 6. An overlay of nine MALDI-DE/R-TOF mass spectra of bovine insulin b-chain showing the $[M + H]^+$ ions acquired at a total acceleration of 20 kV, a pulse voltage of 6 kV and a delay time of 300 ns. A–F denote the first six isotope peaks.

The ability to measure accurately the flight time of ions is a requirement for TOF accurate mass measurements; however, accurate measurements of flight time do not necessarily result in accurate mass measurements. The mass spectra must be calibrated so that the flight time is related to the appropriate m/z value. The standard TOF calibration equation is $t = k_1(m/z)^{1/2} + k_2$, where k_1 is a constant related to the relationship between time and $(m/z)^{1/2}$ and k_2 is a time offset that arises from the time difference between ion formation/ion extraction and the data acquisition start trigger. The calibration equation is derived from the equation for ion kinetic energy and assumes that each ion has the same kinetic energy (i.e. translational energy from the acceleration voltage). This assumption mat not be valid for MALDI formed ions since mass-independent initial velocities derived from the desorption event have been measured in several laboratories.[56,57] Thus the trans-

lational energy of MALDI-formed ions is a sum of the energy acquired by acceleration and the initial velocity. Consequently, the calibration equation should contain a third term that includes the initial velocity, and this third time is mass dependent. In an effort to evaluate the accuracy of the calibration curve generated using the standard TOF calibration equation, we acquired MALDI-DE/R-TOF mass spectra of a peptide mixture containing α-melanocyte stimulating hormone (α-MSH) ($M_i = 1633.793$ Da), mellitin ($M_i = 2844.754$ Da) and oxidized bovine insulin b-chain ($M_i = 3493.643$ Da), using the matrix α-cyano-4-hydroxycinnamic acid. Each mass spectrum was calibrated using the standard TOF calibration equation for two ions, the matrix dimer $[2M + H]^+$ ion and the $\alpha - MSH$ $[M + H]^+$ ion. The flight times and m/z values measured for mellitin and bovine insulin b-chain are given in Table 3. Even though the measured masses for the two analytes are

Table 2 Flight time reproducibility of bovine insulin b-chain isotopes

| Spectrum No.	Flight time (ns)					
	A	B	C	D	E	F
1	201 879.23	201 907.89	201 936.90	201 965.49	201 994.99	202 022.60
2	201 877.64	201 907.72	201 936.30	201 965.47	201 994.38	202 021.28
3	201 877.51	201 906.90	201 936.35	201 964.94	201 992.61	202 022.73
4	201 878.21	201 907.06	201 935.40	201 965.18	201 993.35	202 022.42
5	201 876.93	201 906.91	201 935.55	201 964.82	201 993.42	202 021.15
6	201 878.66	201 906.90	201 936.31	201 964.16	201 993.35	202 020.92
7	201 878.92	201 908.32	201 937.41	201 965.79	201 993.85	202 023.29
8	201 878.61	201 906.23	201 935.20	201 963.13	201 992.37	202 021.49
9	201 879.13	201 907.39	201 935.95	201 964.86	201 993.88	202 021.56
Average	201 878.32	201 907.26	201 936.15	201 964.87	201 993.58	202 021.94
Average deviation (ns)	0.66	0.51	0.56	0.56	0.62	0.73
Average deviation (ppm)	3.3	2.5	2.8	2.8	3.1	3.6
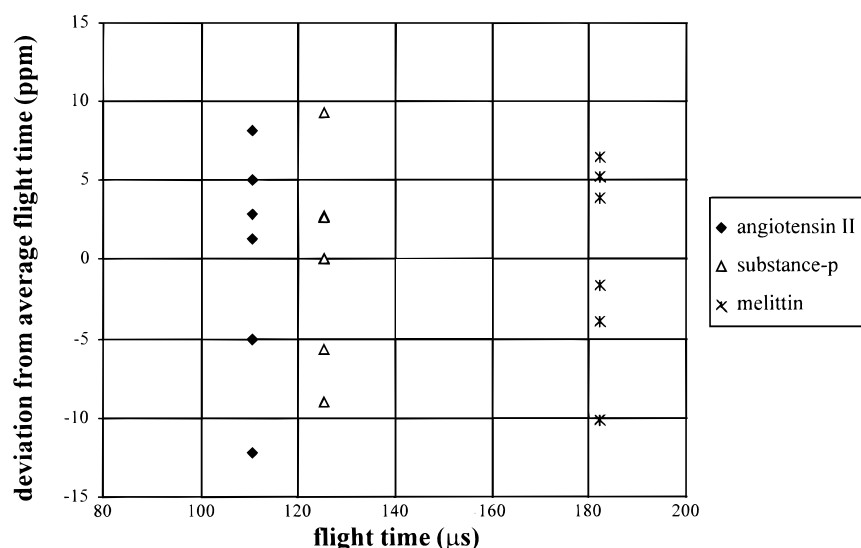
Figure 7. Plot of the variation in the flight time (expressed as ppm) of analyte ions taken from six different sample spots.

obtained by extrapolating the calibration curve 1–2 kDa, the average mass error for mellitin and bovine insulin b-chain is only 2–3 ppm. Such a high accuracy over a large mass range is remarkable especially considering that other methods for mass measurement, e.g. magnetic sector and FTICR, are limited to narrow mass ranges and do not allow accurate mass values to be obtained by using extrapolated mass calibration. Calibration curves employing a third term involving ion initial velocities have shown improvements in mass calibration of large biomolecules,[68,69] and bovine insulin cluster ions.[70,71]

The mass measurement accuracy of polymers other than biopolymers was investigated by acquiring MALDI-DE/R-TOF mass spectra of polypropylene glycol 2000 (PPG) (Fig. 8). The most prominent ions observed in this spectrum are $[M + Na]^+$ ions. Figure 8 also contains expanded regions of the spectrum near

the $[M + Na]^+$ ions at m/z 1492 and 2537, the two ions used for mass calibration. The calculated and measured m/z values and flight times of the $[M + Na]^+$ ions of PPG are listed in Table 4. The calibration curve is very accurate, resulting in an average mass error of only 0.6 ppm across the 1000 Da mass range. To illustrate how accurately the flight time must be measured in order to achieve 0.6 ppm accuracy, the measured flight times were compared with calculated flight times. The calculated flight times were generated using the calibration curve and the calculated m/z of the polymer ions. The last column in Table 4 lists the flight time (ns) difference between the measured and calculated values, and shows that the average flight time difference is only 0.05 ns. The limits of accurately centroiding a peak are determined by the peak profile, especially when unresolved peaks are present, as was mentioned above. However, in this case we are dealing with a well defined peak profile

Table 3 Mass measurement data for melittin and bovine insulin b-chain acquired by MALDI-DE/R-TOF using internal calibration

Spectrum No.	Mellitin		Insulin b-chain	
	Flight time (ns)	m/z	Flight time (ns)	m/z
1	182 179.23	2845.749	201 867.71	3494.600
2	182 179.80	2845.789	201 868.79	3494.667
3	182 180.41	2845.813	201 868.39	3494.659
4	182 178.14	2845.755	201 868.27	3494.672
5	182 179.03	2845.761	201 868.10	3494.636
6	182 178.56	2845.743	201 867.19	3494.599
7	182 179.58	2845.783	201 868.35	3494.652
8	182 179.48	2845.764	201 868.21	3494.625
9	182 178.49	2845.772	201 866.67	3494.622
10	182 179.42	2845.793	201 868.86	3494.688
Average	182 179.21	2845.772	201 868.05	3494.642
Average deviation	0.53	0.018	0.52	0.026
Average deviation (ppm)	2.9	6.2	2.6	7.3
Calculated m/z		2845.762		3494.651
Mass error		0.010		−0.009
Mass accuracy (ppm)		3.6		−2.6
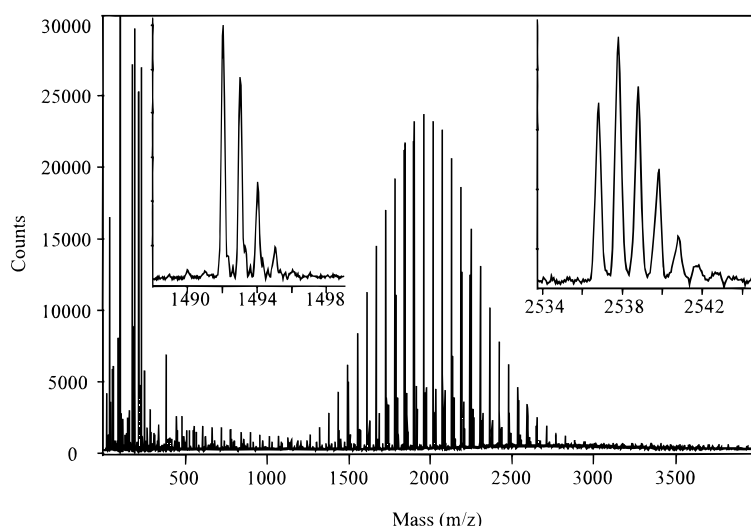
Figure 8. MALDI-DE/R-TOF mass spectrum of polypropylene glycol (PPG) 2000, with $[M + Na]^+$ ions as the most prominent species. The spectrum was acquired at a total acceleration of 25 kV, a pulse voltage of 6.25 kV and a delay time of 300 ns. The insets contain expansions of the $[M + Na]^+$ ions of PPG with $n = 25$ and $n = 43$.

and the limiting factors in estimating the peak centroid are the S/N ratio and the ion signal digitization rate. The digitization rate used for the TOF data shown is 500 mHz (2 ns per point), and the PPG ions were acquired at a resolution of ~ 7500. The lower m/z peaks have a FWHM of about four data points (8 ns) and the higher m/z species about five data points (10 ns). From the accuracy of the PPG data it is evident that an accurate centroid can be determined using a 500 MHz digitizer to measure peak profiles with FWHM in the range of 4–5 data points. Note that an increase in the mass resolution above 7500 would necessitate a higher digitization rate in order to achieve comparable mass measurement accuracy.

The data for bovine insulin b chain acquired at two different mass resolutions can be compared to examine the effects of a limited number of data points on peak centroiding. The data in Table 2 were acquired at 10 000 resolution and those in Table 3 at 6500 resolution. At these resolution values, the FWHM of the insulin b chain peaks are five data points (~ 10 ns) and 7–8 data points (~ 15 ns), respectively. The average deviation for each of these measurements is ~ 3 ppm, further suggesting that the accuracy of the calculated peak centroid is not reduced by the limited number of data points used to centroid the peak. It should be noted that the data for the insulin b chain were acquired on a TOF mass spectrometer with an effective flight path of ~ 6.6 m.[31,32] The long flight tube employs a clear advantage in terms of mass measurement accuracy over shorter instrument designs owing to the long flight times of the ions. The effects of deviations in sample homogeneity and jitter impact the reproducibility of the spectra half as much as for an instru-

Table 4 Mass measurement accuracy of polypropyleneglycol $[M + Na]^+$ ions

Flight time (ns)	Calculated m/z	Measured m/z	$\Delta m/z$	Error (ppm)	Δ Flight time (ns)
118 005.48	1492.0469	1492.0469	0.0000	0.0	0.00
120 276.26	1550.0888	1550.0917	0.0029	1.9	0.11
122 504.72	1608.1307	1608.1315	0.0008	0.5	0.03
124 693.45	1666.1725	1666.1749	0.0024	1.4	0.09
126 844.27	1724.2144	1724.2154	0.0010	0.6	0.04
128 959.17	1782.2563	1782.2555	−0.0007	0.4	−0.03
131 040.01	1840.2981	1840.2986	0.0005	0.3	0.02
133 088.23	1898.3400	1898.3401	0.0001	0.0	0.00
135 105.44	1956.3818	1956.3836	0.0017	0.9	0.06
137 092.85	2014.4237	2014.4244	0.0007	0.3	0.02
139 051.89	2072.4656	2072.4670	0.0014	0.7	0.05
140 983.60	2130.5074	2130.5071	−0.0004	0.2	−0.01
142 889.27	2188.5493	2188.5502	0.0009	0.4	0.03
144 769.75	2246.5912	2246.5907	−0.0005	0.2	−0.02
146 626.22	2304.6330	2304.6352	0.0022	1.0	0.07
148 459.34	2362.6749	2362.6761	0.0012	0.5	0.04
150 270.16	2420.7168	2420.7196	0.0029	1.2	0.09
152 059.28	2478.7586	2478.7593	0.0007	0.3	0.02
153 827.62	2536.8005	2536.8005	0.0000	0.0	0.00
			Average error (ppm)	0.6	

ment of half the length. A further disadvantage of a shorter flight tube is that it necessitates the use of a faster digitizer. One might argue that the same effect could be observed on a smaller instrument by lowering the ion acceleration voltage, thus increasing the flight time; however, we observe both collection efficiency and resolution to decrease as the acceleration voltage decreases.

PRACTICAL UTILITY OF ACCURATE MASS MEASUREMENTS

It is practical to use accurate mass measurements to determine the elemental composition of ions with low $M_i (<500$ Da).[3] In this mass region unique elemental compositions are separated by at least ~ 1 ppm and, when combined with complementary chemical information, the required mass measurement accuracy is of the order of 5 ppm. On the other hand, accurate mass measurement for the characterization of higher molecular mass species is a more recently developed capability and has not yet been fully utilized. It is not feasible to use accurate mass measurements in determining, *a priori*, the elemental composition of molecules with M_i 1–5 kDa, because this would require mass measurement accuracy of better than 1 ppb, well outside the accuracy limits provided by any mass spectrometer. Thus, what is the practical utility of accurate mass measurements for molecules with M_i of 1–5 kDa? One practical use of accurate mass measurements for peptide analysis is in distinguishing between amino acid residues that differ only slightly in mass. For example, the masses of lysine and glutamine differ by only 0.03639 Da.[72] Recognition of a lysine for glutamine substitution in a M_i 1000 peptide requires a mass measurement accuracy of 36 ppm, whereas the same substitution in a M_i 5000 peptide requires a mass measurement accuracy of 7 ppm.

Highly accurate mass measurements also have practical utility in the analysis of peptides in the rapidly expanding area of protein database searching.[34] Proteins can be identified using the masses of peptides that result from enzymatic digestion because the generated map of peptide fragments serves as a 'fingerprint' for a particular protein.[73,74] The number of peptides required to identify a protein correctly is directly related to the accuracy with which the peptide masses are measured. If each of the predicted digest fragments are observed in the mass spectrum, accurate mass measurements are not required for correct protein identification. When many digest fragments are used in the database search, the mass window for matching peptides can be large (~ 1 Da). Several proteins in the database will have peptides with similar (within 1 Da) mass values to the observed digest fragments, resulting in a large number of possible proteins, but the correct protein is identified by having the largest number of matched peptides. Large error limits cannot be used in database searching when the number of observed peptide fragment ions is small. There are several scenarios that can lead to the observation of only a few peptide fragments

in the mass spectrum after a protein has undergone enzymatic digestion. Certain proteins resist enzymatic digestion, especially proteins that have a large number of cysteine residues that form intramolecular disulfide bonds. To facilitate digestion, the disulfide bonds can be broken with the addition of a reducing agent such as DTT and then the cysteines alkylated to prevent recombination, but this method of reduction/alkylation is not practical when the aim of the study is to locate the cross-linked residues. Even when a complete map of peptide fragments is generated from the enzymatic digestion, the number of observed peptides in the mass spectrum may be small owing to analyte ion suppression. The peptide fragments that contain acidic residues may not be observed in positive ion MALDI mass spectra. The buffers and salts used in protein isolation/purification can also lead to suppression of analyte ion signals in both MALDI and ESI mass spectra. The choice of the MALDI matrix used, and also the pH of the matrix/analyte solution, strongly influences peptide ion yields.[75] Suppression of the analyte ion signal is especially daunting when the amount of peptides being produced is near the detection limit. Under the conditions outlined above, accurate mass measurements of the peptide fragments are crucial to correct protein identification. In cases where only a few peptide fragments are observed, the mass error limits for matching peptides must be narrow in order to reduce the number of possible proteins. The number of proteins that yield peptide fragments within 1 Da of the experimentally determined mass value is fairly large; by contrast, the number of proteins that yield peptide fragments within 20 ppm of the measured m/z is remarkably small. In recent work reported by Mann and co-workers[33] a database search using mass limits of 30 ppm resulted in a unique protein match, whereas, searching with a 1 Da mass limit resulted in 163 proteins each having at least 10 matching peptides.

A serious caveat regarding database searching for protein identification is directly related to the issue of mass measurement accuracy. Although mass spectrometrists strive to achieve the highest level of mass measurement accuracy for the intact protein, this objective is not always of practical utility. For instance, accurate mass measurements can be obtained for proteins (see Fig. 1) but the practical uses of such data may be limited. For example, the protein being analyzed may have undergone post-translational modifications (see above), so the resulting measured M_r may differ from the calculated M_r contained in the database (calculated from cDNA). If the database is searched with a narrow M_r window, an incorrect identification may result. If, on the other hand, the database is searched with a larger M_r window, the protein may then be correctly identified! It is important to remember that the search is best performed using not only the M_r of the protein but also, more importantly, the accurate mass of the peptide digest fragments.

In addition to database searching for protein identification, accurate mass measurements of enzymatic digest fragments can be used in the identification of known proteins. By using multiple enzymes, overlapping fragments can be produced and used to confirm the sequence of recombinant proteins. This method can also

be used to identify and locate the site of post-translational modifications such as oxidized methionine or cysteine, glycosylation, phosphorylation or N-terminal acetylation. Accurate mass measurements can also be used to search for amino acid substitutions between protein isoforms. Often these modifications are substitutions of Asp for Asn or Glu for Gln, which result in a 1 Da mass shift.

Accurate mass measurements can aid in the correct assignment of digest fragment ions as illustrated below. Figure 9 contains a MALDI-DE/R-TOF mass spectrum of a trypsin digestion of equine cytochrome c. The inset shows an expansion of the ion at m/z 1633.620. A predicted tryptic fragment ion ($Ile^9–Lys^{22}$) has a mass (1633.820 Da) near the measured value. However, assigning the ion at m/z 1633.620 to the digest fragment ($Ile^9–Lys^{22}$) results in a mass error of 122 ppm. Conversely, assignment of the observed m/z 1633 peak to the digest fragment ($Cys^{14}–Lys^{22}$) with the heme group covalently bound to Cys^{14} and Cys^{17} results in a mass error of only 3 ppm. The assignment of the observed fragment to the heme containing peptide is further supported by comparing the observed isotopic distribution to the calculation distribution. The insets in Fig. 9 contain the isotope distribution of the observed fragment ion and the calculated isotopic distribution of the heme-containing digest fragment. The uncharacteristic isotope distribution is due to the iron-containing heme group.

CONCLUSIONS

The conversion of HRMS data to useful mass measurements requires the development of a method of mass calibration. Typically, the mass spectrometer is calibrated using a standard compound or mixture of compounds, and the analyte of interest is then analyzed using this calibration. This method is commonly referred to as an 'external calibration.' When more accurate mass measurements are required, the mass calibrant is introduced to the spectrometer along with the analyte, and the mass of the calibrant/analyte is acquired. This method of instrument calibration is referred to as an 'internal calibration.' The ion trap methods, FTICR and QIT, cannot accommodate an unlimited number and density of ions, owing to limitations imposed by coulombic interactions, thus internal calibration is only used for measurements that require the highest levels of accuracy. Marshall and co-workers[30] were able to overcome this limitation by using SWIFT and detecting a very narrow range of m/z values. A requirement of using SWIFT in FTICR is that the m/z of the calibrant used for internal calibration must also fall within the mass range of the analyte. For the analysis of complex mixtures that contain a large number of ions having different m/z ratios, this places considerable restrictions on the mass calibrant. For the analysis of peptide mixtures analyzed by MALDI, the limitations on ion number and density are less restrictive because only the $[M + H]^+$ ions of each analyte are formed and the m/z range that can be analyzed is increased. When using MALDI or ESI, the use of internal calibration is also limited by interferences in the ionization of mixtures if one compound, e.g. the calibrant, ionizes more easily than the other, e.g. the analyte.[78] McIver and co-workers[30] recently reported accurate mass measurements for peptide ions using MALDI combined with FTICR.[28] They demonstrate that a mass measurement accuracy of better than 10 ppm can be achieved under optimum instrument operating conditions when the observed frequency is cor-
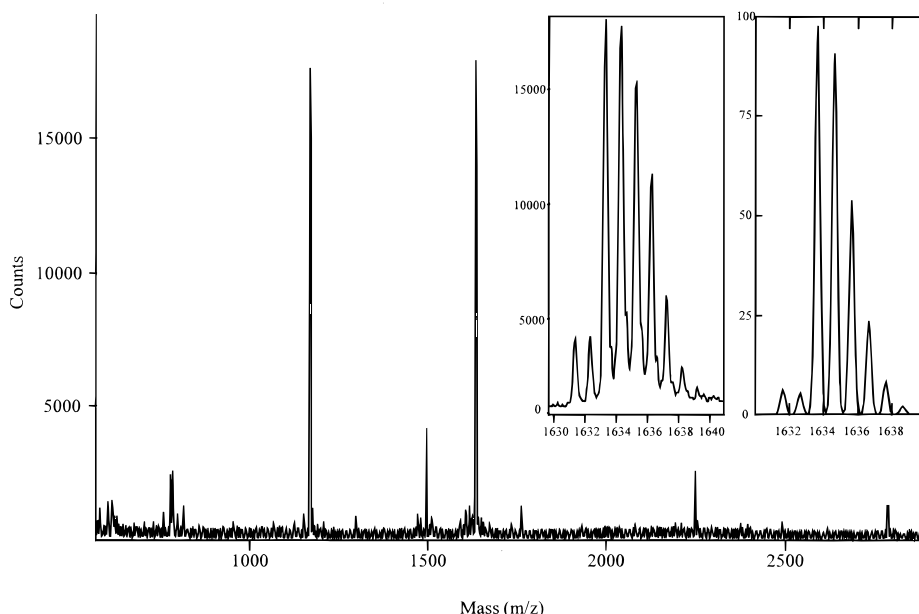
Figure 9. A MALDI-DE/R-TOF mass spectrum of a tryptic digest of equine cytochrome c. The insets show the isotope distribution of the ion observed at m/z 1633 and theoretical isotope distributions of the tryptic fragment $Cys^{14}–Lys^{22}$ with the heme group covalently bound.

rected for frequency shifts arising from electrostatic fields.

The principal limitation of accurate mass measurements by TOF is associated with the timing or jitter in firing the laser and initiating data acquisition, and obtaining accurate peak centroids for the available number of data points per peak. As illustrated in the previous section, the limitations of timing and jitter correspond to a mass measurement accuracy of <5 ppm for peptides of 1–5 kDa. The accuracy of the PPG data suggests that under optimum conditions one should be able to achieve a mass measurement accuracy of <1 ppm. The limitations in peak centroiding, e.g., an accuracy of 50 ps, do not appear to impose a serious limit to achieving such mass measurement accuracy, provided that the peak profile is defined by 4–5 data points.

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