

[1] Molecular Weight Determination of Peptides and Proteins by ESI and MALDI

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Abstract

Several topics are covered, namely, general aspects important for mass determination of peptides and proteins, sample preparation for both ESI and MALDI, and various mass analyzers coupled to these ionization techniques. Finally, the discussion is carried out on peptide and protein mass analysis as related to accuracy and precision of mass determination for both ESI-MS and MALDI-MS.

Introduction

The techniques of electrospray/ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) have revolutionized biological mass spectrometry (MS). All state-of-the-art biochemistry and biology laboratories possess at least one of these ionization techniques and, in general, have access to both of them. Parallel to and motivated by the development of these techniques, a diminution of chromatographic separation techniques and purification techniques has taken place, and the success of ESI and MALDI in biochemistry and biology is also due to the possibility of direct or indirect coupling of the ionization techniques to appropriate separation and purification techniques. Both ionization techniques are applicable to peptides and proteins (Yates, 1998), DNA and RNA (Gross, 2000), glycoconjugates, and synthetic polymers (Nielen, 1999).

Considerable information can be derived from mass spectra of biological samples such as peptides and proteins. Besides the determination of the molecular mass M of a given compound (often named molecular weight M_r , which is not quite correct because no weight or force, respectively, is measured by MS) and the identification of proteins by accurate mass determination of their proteolytic fragments, mass spectrometry is capable of providing structural information (i.e., sequences) for peptides. The elucidation of post-translational modifications of peptides or proteins is an important branch of mass spectrometry. In combination with chromatography, known compounds can be determined quantitatively. Together with enzymatic degradation, the carbohydrate content of a glycoprotein

can be evaluated at least semiquantitatively. Furthermore, the quaternary structure of protein complexes, the interaction between proteins and ligands (Rogniaux *et al.*, 1999) or metal ions (Strupat *et al.*, 2000), as well as protein folding (Yao, 2005) can be studied by mass spectrometry.

In the rapidly expanding field of proteomics, high quality mass data—namely accurate mass determination—represent the key to unambiguous protein identification by peptide mass mapping and to determination of posttranslational modifications of proteins harvested from cells grown under different conditions (Stults, 2005). The accuracy of mass determination in peptide mass analysis obtained by ESI-MS and MALDI-MS has increased from less than $5 \cdot 10^{-4}$ or 500 ppm (0.5 u at 1000 u) 10 years ago to $0.5 - 2 \cdot 10^{-6}$ or 0.5 - 2 ppm (0.0005 - 0.002 u at 1000 u) at present (Senko, 2004). The striking advantage of an improved mass accuracy is the dramatic reduction of false hits in database interrogation for protein identity (Clauser *et al.*, 1999; Jensen *et al.*, 1996; Shevchenko *et al.*, 1996). It has to be noted, however, that such a high accuracy of mass determination is typically not achievable for analyte molecules of higher masses, such as proteins. This is due to both practical and fundamental reasons (see following paragraphs).

This paper is divided into several parts for ease of understanding. First, general aspects important for the understanding of the context are explained. This rather theoretical part is followed by an explanation of practical features, such as preparative steps and mass analyzers coupled to the ionization techniques, and, more importantly, results in a discussion of peptide and protein mass analysis with respect to accuracy and precision of mass determination for both ESI-MS and MALDI-MS. The performance of a Fourier transform ion cyclotron resonance (FTICR) mass analyzer with its ultra-high mass resolving power is discussed in a separate chapter.

Some General Aspects Important for Mass Determination of Peptides and Proteins

Isotopic Distribution of Peptide and Protein Signals

When discussing the topic of mass determination of biological molecules, one main aspect that should be kept in mind is the naturally occurring isotope distribution. It is necessary to distinguish between nominal mass, monoisotopic mass, and the average mass of a molecule (Yergey *et al.*, 1983). The nominal mass of a molecule is calculated by using the most abundant isotope without regard of mass defect/excess (i.e., H = 1, C = 12, N = 14, O = 16, etc.). The monoisotopic mass (M_{MONO}) of a molecule

again refers to the most abundant isotope, but the exact mass is used (i.e., $^1\text{H} = 1.007825$, $^{12}\text{C} = 12.000000$, $^{14}\text{N} = 14.003074$, $^{16}\text{O} = 15.994915$, $^{31}\text{P} = 30.973762$, $^{32}\text{S} = 31.972070$, etc.). The average mass (M_{av}) of a molecule is calculated from the average masses of the elements weighted for abundance (i.e., $\text{H} = 1.00794$, $\text{C} = 12.011$, $\text{N} = 14.00674$, $\text{O} = 15.9994$, $\text{P} = 30.97376$, $\text{S} = 32.066$, etc.) (Price, 1991; Winter, 1983; Yergey *et al.*, 1983). Mainly, due to the naturally occurring $^{12}\text{C}/^{13}\text{C}$ ratio (98.89% ^{12}C , 1.11% ^{13}C), biological molecules of mass 1000 units and larger show a considerable contribution of the ^{13}C isotope (see Fig. 1). The difference between monoisotopic mass and average mass increases with increasing mass and is larger for molecules containing a larger number of atoms with mass excess (such as $^1\text{H} = 1.007825$ for hydrogen) or a relatively abundant heavy isotope (Biemann, 1990). The difference is largest for peptides and proteins because of their relatively high content of carbon and hydrogen; the difference is less for carbohydrates and least for oligonucleotides because of their low carbon and hydrogen content and the large number of (mono-isotopic and mass-deficient) phosphorus atoms (Biemann, 1990).

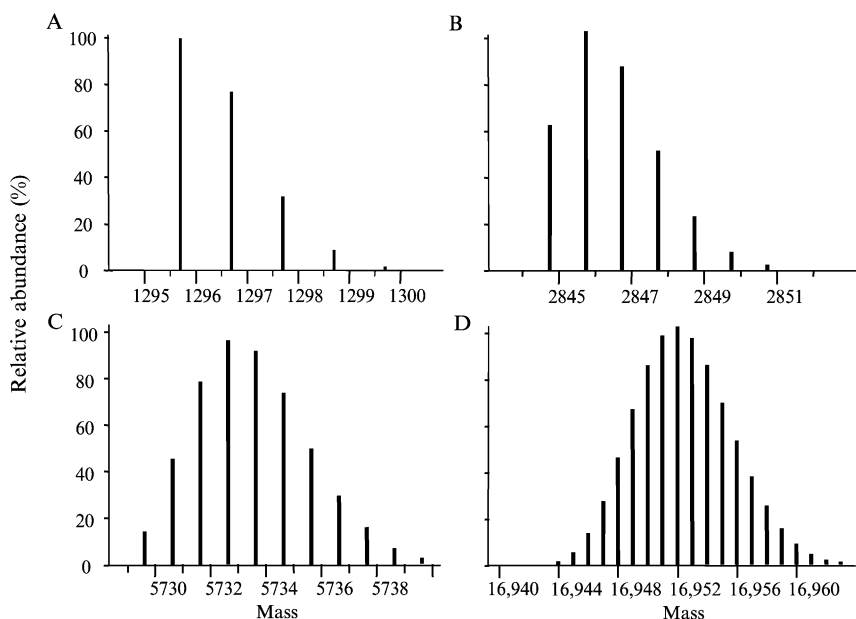


FIG. 1. Isotope distributions of selected peptides and one protein taking into account the naturally occurring $^{12}\text{C}/^{13}\text{C}$ ratio. (A) Angiotensin I, human ($M_{\text{MONO}} = 1295.6775$ u, $\text{C}_{62}\text{H}_{89}\text{N}_{17}\text{O}_{14}$). (B) Melittin, honeybee ($M_{\text{MONO}} = 2844.7542$ u, $\text{C}_{131}\text{H}_{229}\text{N}_{39}\text{O}_{31}$). (C) Insulin, bovine ($M_{\text{MONO}} = 5729.6009$ u, $\text{C}_{254}\text{H}_{377}\text{N}_{65}\text{O}_{75}\text{S}_6$). (D) apomyoglobin, horse ($M_{\text{MONO}} = 16940.9650$ u, $\text{C}_{769}\text{H}_{1212}\text{N}_{210}\text{O}_{218}\text{S}_2$).

In the mass range below 1500 u the monoisotopic mass of peptides is the most abundant mass with an asymmetry to larger masses. The isotope distribution loses its asymmetry with increasing mass. Particularly for proteins, the isotope distribution follows a Gaussian distribution (bell-shaped curve). For proteins above ≈ 15 ku, the abundance of the monoisotopic peak is negligible; for example, the monoisotopic peak of apomyoglobin (horse), a protein of mass 16951.49 u (average mass), is of low abundance (0.03%) and occurs at 16940.97 u, about 10.5 u beyond the average molecular mass of apomyoglobin. [Figure 1](#) gives the isotope distributions of angiotensin I, melittin, insulin, and apomyoglobin. Sum formulae and organisms are given together with monoisotopic masses and average (molecular) masses, respectively, in the legend for [Fig. 1](#). For apomyoglobin, (see [Fig. 1D](#)), the monoisotopic mass cannot be shown due to its low abundance.

Precision and Accuracy of Mass Determination

Precision of mass determination describes to what extent a mass measurement of a given compound can be reproduced. The precision is expressed by a statistic distribution (mean variation) of several independent mass determinations of a given analyte molecule; the average value of these independent mass measurements is given together with its standard deviation (σ_n). The accuracy of mass determination describes the accuracy of the measurement (i.e., the accuracy of mass determination expresses the deviation between the measured mass and the theoretical mass of the compound under investigation).

Both accuracy and precision are important characteristics of a mass spectrometric technique and account for its reliability. Achieving both high precision and high accuracy of mass determination are necessary to satisfy the demands on biological mass spectrometry, such as in protein identification by peptide mass mapping ([Yates, 1998](#)). Put simply, a high precision of mass determination allows reliance on a limited number of mass spectra of the same peptide map (high reproducibility of determined mass means a small standard deviation of measurement), while a high mass accuracy of mass determination results in a high probability of unambiguous identification of a protein by database interrogation using the masses of the peptide mass map. The higher the accuracy of mass determination of peptides in a peptide map, the fewer peptide masses are necessary for this approach.

Precision and accuracy of mass determination of peptides (1 to 5 ku) using ESI and MALDI with state-of-the-art mass analyzers are in the low parts per million range. For example, insulin β -chain (3494.651 u) was measured by Edmondson and Russell with a precision of 7.3 ppm

(0.026 u) and an accuracy of -2.6 ppm (-0.009 u) using MALDI-MS and applying an internal mass calibration (see the following paragraphs) (Edmondson and Russell, 1996; Russell and Edmondson, 1997). The same authors describe how accurate mass measurement can aid correct assignment of proteolytic fragments while also taking into account patterns of isotope distributions: Patterns can be uncommon compared to simple peptides of the same size, such as if a prosthetic group is bound to the peptide. Russell and Edmondson (1997) report of MALDI mass spectra obtained for a proteolytic digestion of cytochrome *c* with its covalently bound heme group. The heme-containing tryptic fragment of cytochrome *c* (Cys14 – Lys22, m/z 1633.620) can clearly be assigned by the relative abundances of the resolved isotopes and, therefore, distinguished from another potentially occurring fragment (Ile9 – Lys22, m/z 1633.820). The prosthetic group contains iron, and the observed (uncommon) isotope distribution is due to the iron-containing heme group (Edmondson and Russell, 1996; Russell and Edmondson, 1997).

A frequently discussed challenge for peptide mass analyses is to distinguish, for example, between Lys and Gln in a tryptic fragment of typical mass (1000–5000 u) or in a small protein only from the determination of its absolute molecular mass (and not by acetylating the compounds and deducing the number of Lys by the mass differences of 42 u). Because Lys and Gln differ by 0.0364 u, an accuracy of 36.4 ppm at mass 1000 u is required, whereas an accuracy of 3.6 ppm is required at mass 10,000 u. There are many other examples for amino acid mass coincidences requiring high accuracy of mass determination to distinguish different amino acid compositions just from peptide mass.

Mass Resolution and Resolving Power

A peak width definition as well as a 10% valley definition exist (Price, 1991). The *peak width definition* considers a single peak in a mass spectrum made up of singly charged ions at mass m . The resolution R is expressed as $m/\Delta m$, where Δm is the width of the peak at a height that is a specific fraction of the maximum peak height. A common standard is the definition of resolution R based upon Δm being full width at half maximum (FWHM). Considering a signal at mass $m = 1000$ u and a peak width of this signal of $\Delta m = 0.5$ u FWHM, mass resolution is $R = m/\Delta m = 2000$. The *10% valley definition* considers two peaks of equal height in a mass spectrum at mass m and $(m - \Delta m)$ that are separated by a valley that at its lowest point is just 10% of the height of either peak. The resolution is then $R = m/\Delta m$. It is usually a function of m , and therefore $m/\Delta m$ should be given for a number of different values of m (Price, 1991).

The ability of a mass analyzer to distinguish between ions differing slightly in mass-to-charge ratio is expressed by its resolving power. The resolving power is characterized by the peak width (in mass units) for at least two points on the peak (50% and 5% of the maximum peak height) (Price, 1991). High resolving power of a mass analyzer is neither a necessary prerequisite for accurate mass determination nor a sufficient one because accurate mass determination depends also on well-defined calibration standards of known mass, correct calibration procedures (see the following paragraphs), and precise (i.e., reproducible) signal peak shapes. However, high mass resolution helps to obtain high mass accuracy if the latter conditions are optimized and adapted to the analytical problem. As a rough guideline, if mass resolution is $R = 10,000$ at mass 1000 u, that is the peak width $\Delta m = 0.1$ u FWHM, the achievable accuracy of mass determination is about or better than one-tenth of the peak width Δm . This means that the mass of an ion at mass 1000 u can be determined with an accuracy of at least 0.01 u at 1000 u (10 ppm).

Calibration of Mass Spectra

The calibration of mass spectra can be performed internally or externally (i.e., the calibration peptides or proteins can either be within the sample containing the analyte, or the calibration compound and the analyte can be measured separately from each other using the same instrument configuration and voltages). It should be noted that a given mass calibration is only valid for the mass range covered by the standards.

In general, high precision of mass determination is achievable for both MALDI and ESI because triggering of electronics, stability of power supplies (AC and DC voltages), and other such factors are reliable (precise). The achievable mass accuracy, therefore, depends strongly on the quality of standards used for mass calibration. In the mass range below 30 ku a number of homogeneous peptides and proteins of known mass exist that can be used for mass calibration. Table I gives an overview of commonly used standard peptides and proteins. The average mass of a peptide or protein is abbreviated by M_{av} , while the monoisotopic mass of a compound is abbreviated by M_{MONO} .

Due to a typically occurring sample heterogeneity of proteins increasing with mass (a fundamental problem) and a heterogeneity induced by impurities and/or by the ionization technique itself (a practical problem), the accuracy of mass determination is limited at high mass range. An intrinsic protein heterogeneity typical for large proteins (posttranslational modifications such as phosphorylation and glycosylation) and sample impurities also in the standards (such as sodium and potassium) do not allow

TABLE I
 MASSES, SUM FORMULAE, AND SWISSPROT REFERENCES OF SOME PEPTIDES AND PROTEINS
 FREQUENTLY USED FOR MASS CALIBRATION

Peptide/Protein	Sum formula	Monoisotopic mass, M_{MONO} Average mass, M_{av} , u	SwissProt access
Angiotensin II, human, free acid	$\text{C}_{50}\text{H}_{71}\text{N}_{13}\text{O}_{12}$	$M_{\text{MONO}} = 1045.5345$ $M_{\text{av}} = 1046.19$	P01019
Bradykinin, human, free acid	$\text{C}_{50}\text{H}_{73}\text{N}_{15}\text{O}_{11}$	$M_{\text{MONO}} = 1059.5614$ $M_{\text{av}} = 1060.22$	P01042
Angiotensin I, human, free acid	$\text{C}_{62}\text{H}_{89}\text{N}_{17}\text{O}_{14}$	$M_{\text{MONO}} = 1295.6775$ $M_{\text{av}} = 1296.50$	P01019
Substance P, human, free acid, ... Met-OH	$\text{C}_{63}\text{H}_{97}\text{N}_{17}\text{O}_{14}\text{S}_1$	$M_{\text{MONO}} = 1347.7122$ $M_{\text{av}} = 1348.81$	P20366
Substance P, human, ... Met-NH ₂	$\text{C}_{63}\text{H}_{98}\text{N}_{18}\text{O}_{13}\text{S}_1$	$M_{\text{MONO}} = 1346.7281$ $M_{\text{av}} = 1347.65$	P20366
Neurotensin, human, free acid	$\text{C}_{78}\text{H}_{121}\text{N}_{21}\text{O}_{20}$	$M_{\text{MONO}} = 1671.9097$ $M_{\text{av}} = 1672.95$	P30990
ACTH (CLIP), 18-39, human, free acid	$\text{C}_{112}\text{H}_{165}\text{N}_{27}\text{O}_{36}$	$M_{\text{MONO}} = 2464.1911$ $M_{\text{av}} = 2465.70$	P01189
Melittin, honeybee, ... Gln-NH ₂	$\text{C}_{131}\text{H}_{229}\text{N}_{39}\text{O}_{31}$	$M_{\text{MONO}} = 2844.754$ $M_{\text{av}} = 2846.50$	P01501
ACTH (CLIP), 1-39, human, free acid	$\text{C}_{207}\text{H}_{308}\text{N}_{56}\text{O}_{58}\text{S}$	$M_{\text{MONO}} = 4538.2594$ $M_{\text{av}} = 4541.13$	P01189
Insulin, bovine, β -chain, oxidized, sulphated C (R-SO ₃ H)	$\text{C}_{157}\text{H}_{232}\text{N}_{40}\text{O}_{47}\text{S}_2$	$M_{\text{MONO}} = 3493.6435$ $M_{\text{av}} = 3495.94$	P01317
Insulin, bovine	$\text{C}_{254}\text{H}_{377}\text{N}_{65}\text{O}_{75}\text{S}_6$	$M_{\text{MONO}} = 5729.6009$ $M_{\text{av}} = 5733.58$	P01317
Ubiquitin, bovine	$\text{C}_{378}\text{H}_{629}\text{N}_{105}\text{O}_{118}\text{S}_1$	$M_{\text{MONO}} = 8559.6167$ $M_{\text{av}} = 8564.86$	P02248
Lysozyme, hen egg, oxidized form	$\text{C}_{613}\text{H}_{951}\text{N}_{193}\text{O}_{185}\text{S}_{10}$	$M_{\text{MONO}} = 14295.8148$ $M_{\text{av}} = 14305.14$	P00698
Apomyoglobin, horse	$\text{C}_{769}\text{H}_{1212}\text{N}_{210}\text{O}_{218}\text{S}_2$	$M_{\text{MONO}} = 16940.9650$ $M_{\text{av}} = 16951.49$	P02188
Carbonic anhydrase, bovine ^a	$\text{C}_{1312}\text{H}_{1998}\text{N}_{358}\text{O}_{384}\text{S}_3$	$M_{\text{MONO}} = 29005.6750$ $M_{\text{av}} = 29023.65$	P00921

^a Assuming that amino acids 10, 100, and 101 assigned as Asx are actually Asp and that residue at position 13 designated Glx is actually Glu. See Senko, M. W., Beu, S. C., and McLafferty, F. W. (1994). *Anal. Chem.* **66**, 415.

reliance upon calibrations based on masses given in databases. Bovine serum albumin (BSA, 66 ku) used as a standard has often led to controversy due to its intrinsic sample heterogeneity (more than one sequence, ragged

ends) and its high affinity for cations, both causing an inaccurate mass determination.

Nevertheless, peptides and proteins used for mass calibration should be used in a highly purified form to produce clean, well-resolved peaks in the mass spectrum and should be freshly prepared to obtain optimal results. If available, recombinant proteins are recommended for mass calibration of proteins; however, it should be remembered that such proteins are still sensitive to oxidation and other modifications as well as proteolysis during storage in solution. The content of alkali cations or inorganic anions can also vary among suppliers and vials. Both modifications and adduct formation result in moving of the peak centroid and peak height, respectively. Especially in ESI mass analysis, a minor amount of salts and detergents can weaken protein ion intensities by adduct formation and can degrade spectra quality. Independent of these arguments, the question remains whether the protein under investigation behaves in the same way as the calibration protein with respect to both adduct formation and decay induced by the ionization technique (for the latter, see upcoming text).

Although it is preferable to use peptides or proteins to calibrate peptide or protein signals to obtain high mass accuracy, mixtures of inorganic salts, such as CsI/NaI, can be used as calibration compounds in ESI. Signals from these calibration compounds are not affected by the effects previously discussed and allow for determination of a precise peak centroid because these salts are monoisotopic. It should be noted, however, that the use of inorganic salts often leads to source contamination, especially in ESI under flow rates in the microliters per minute range, and internal mass calibration is not recommended due to the cation formation already discussed.

Factors Affecting Mass Resolution and Mass Accuracy

Besides the problem of intrinsic mass heterogeneity, a signal heterogeneity can be induced by the ionization technique itself (a practical problem): adduct ion formation (e.g., cations, solvents, or matrix molecules getting attached to the analyte molecule) or small neutral loss (loss of H₂O or NH₃) from the calibration compound or from the compound of interest are the most important factors that can deteriorate mass calibration in MALDI-MS and ESI-MS. Adduct ion formation and small neutral loss can cause severe problems if mass resolution is insufficient and accurate signal assignment is no longer possible. The former leads to a shift of the peak centroid toward higher masses, while the latter leads toward lower masses. In addition, adduct ion formation and small neutral loss can cause particularly severe problems when the calibration compound

and the compound of interest behave differently in adduct ion formation or decay.

In this context, explaining sample purification seems to be appropriate because purified calibration standards and analyte molecules are required for best results. Typically, sample cleanup procedures are required prior to mass analysis to obtain high mass accuracy. Samples must be freed from sodium and potassium as much as possible; often—in protein identification by peptide mass mapping—sample cleanup is essential to get rid of detergents and other biochemical additives that were required for proteolytic degradation in steps prior to mass analysis. MALDI-MS is known to be more tolerant toward common impurities than ESI-MS using flow rates in the microliters per minute range. In comparison to ESI, nanoelectrospray is significantly more tolerant toward common contaminants than ESI due to the formation of smaller, more highly charged droplets undergoing fissions at earlier stages (Juraschek *et al.*, 1999). For both the MALDI-MS and ESI-MS techniques, sample cleanup procedures were established, including cation or anion exchange procedures and purification using reversed phase surfaces, which are on a microliter scale and are easy to use (Gobom *et al.*, 1999; Kussmann *et al.*, 1997).

Several other parameters can influence the achievable mass resolution of a given system; these parameters include sample preparation, purity of the ion source, density of ions (e.g., in the mass separating device, such as an ion trap or FTICR), and other such factors. The detection system can also limit mass resolution; for example, the postacceleration of large mass ions onto a conversion dynode followed by a secondary electron multiplier produces a variety of (nonresolved) secondary ions as well as electrons, and mass dispersion leads to a significant peak broadening (Spengler *et al.*, 1990). Multichannel plate detectors (MCP) are therefore typically used for both ESI and MALDI mass analysis (Baldwin, 2005), and the conversion dynode approach appears superior only for very high m/z values produced by MALDI.

ESI-MS

In this section, the results and discussion are based mainly on quadrupole mass filters and orthogonal acceleration TOF (oa-TOF) mass analyzers for electrospray/ionization-mass spectrometry (ESI-MS) applications.

ESI: Sample Preparation

The general idea of an ESI sample preparation is to dissolve the analyte molecules (peptides or proteins) in a 1/1 (v/v) water/methanol or water/acetonitrile mixture, typically containing 0.5–1% acetic or formic acid

(*denaturing conditions*). As a rough rule, the sample concentration is in the 10^{-6} M range (1 pmol/ μ l). The sample is continuously injected into an electric field via a metal capillary (Fenn *et al.*, 1989) (atmospheric pressure ESI) with flow rates of 1–5 μ l per minute or via a metal-coated glass capillary (Wilm and Mann, 1994, 1996) (nanoelectrospray source) with flow rates in the low nanoliters per minute range. The electric field generates a mist of highly charged droplets containing analyte molecules. The droplets move down a potential gradient ΔU and a pressure gradient Δp —which both finally liberate analyte molecules from solvent molecules—toward the mass analyzer (Kearle, 2000).

While the preparation conditions just described are appropriate for the determination of molecular masses of single peptide or protein chains, ESI–MS is being used more for the mass analysis of noncovalently bound complexes, such as multimeric proteins or protein/ligand complexes (Strupat *et al.*, 2000). To guarantee a complex in solution, mild solvent conditions must be chosen. Typically, the multimeric protein or protein/ligand complex is dissolved in an aqueous, buffered solution adjusted to appropriate pH-values (*native conditions*). Many noncovalently bound complexes are dissolved in and measured directly from aqueous solutions containing, for example, 5–25 mM NH_4Ac with pH values between 5 and 8.5, depending on the analyte molecules under investigation (Loo, 1997; Smith *et al.*, 1997; Strupat *et al.*, 2000; Yao, 2005).

Mass Analyzers

The continuity of the spray in electrospray/ionization enables a scanning device, such as the quadrupole mass filter, to be conveniently used as a mass analyzer (Fenn *et al.*, 1989). Quadrupole mass filters (scanning filters) coupled to ESI sources, therefore, were the workhorses for ESI for many years in both off-line and on-line couplings (liquid chromatography [LC] or capillary electrophoresis [EC]–ESI–MS) approaches (Fenn *et al.*, 1989; Voyksner, 1997).

Triple quadrupole instruments offer the opportunity to obtain tandem MS (MS/MS) data; the first quadrupole serves as precursor ion selector, followed by a collision cell (hexapole in rf-only mode) in which collision-induced dissociation (CID) between analyte molecules and Argon (low-energy collision) are performed. The collision cell is followed by another quadrupole mass filter that separates the fragment ions by the m/z ratio. The coupling of ESI sources to other scanning devices, such as ion traps (van Berkel *et al.*, 1990) and magnetic sectors (Meng *et al.*, 1990a,b), which are also able to perform MS/MS measurements, is described in the literature.

The coupling of ESI sources to (pulsed) time-of-flight (TOF) analyzers in so-called orthogonal acceleration TOF setups (oa-TOF) is routinely

used after the introduction of the oa-TOF by the groups of Guilhaus (Dawson and Guilhaus, 1989) and Dodonow (Dodonow, 1991). A recently published review by Guilhaus *et al.* reports on the principle instrumentation and different applications of the oa-TOF instrument (Guilhaus *et al.*, 2000). The main advantages of an oa-TOF over a quadrupole are the higher mass resolution, a higher achievable mass range, and a higher sensitivity (Guilhaus *et al.*, 2000). The higher sensitivity is achieved by the orthogonal accelerator, which is a highly efficient device for sampling ions from an ion beam into a TOF mass analyzer. A higher mass range is of interest because proteins or noncovalent complexes measured under native conditions (see previous paragraphs) require a high mass-to-charge range of the mass analyzer. The excellent performance of the oa-TOF mass analyzer for very high masses (noncovalent complexes) was first realized by the Manitoba group (Chernushevich *et al.*, 1999; Werner, 2005) and by Robinson *et al.* (Rostom and Robinson, 1999; Yao, 2005).

A further development of the oa-TOF instrumentation was made by the introduction of a hybrid instrument (quadrupole-TOF combinations, Q-TOF or qQ-TOF (Chernushevich *et al.*, 1999) that enabled MS/MS applications (Werner, 2005). ESI-produced ions are transferred to an analytical quadrupole, where a specific precursor ion is selected. The selected ion can be fragmented in the succeeding collision cell. Mass analysis of the fragment ions is performed in the adjacent oa-TOF.

Appearance of ESI-MS

ESI-MS generates highly charged ion species of peptides or proteins. An ESI mass spectrum is characterized by a number of signals that each differ by one charge. Such a distribution of charge states is typically produced by multiple protonation (positive ion mode) or deprotonation (negative ion mode) of the species, but cation formation is also known to occur. An ion signal of a species of mass M produced by n -fold protonation (mass of a proton m_{H}) has the mass $M + n \cdot m_{\text{H}}$; this signal is assigned as $(M + nH)^{n+}$ or M^{n+} (positive ion mode, $n \in \mathbf{N}$) in the mass spectrum. Note that the signal occurs at mass-to-charge ratios $m/z = (M + n \cdot m_{\text{H}})/n$, ($n \in \mathbf{N}$). Depending on the purity of the sample, undesired though typically less pronounced, sodium and potassium adduct ions, such as $(M + nH + mNa)^{(m+n)+}$ ($n + m > 0$, $n \in \mathbf{Z}$, $m \in \mathbf{N}$), also appear. An ion signal of an n -fold deprotonated species (of mass M) possesses a mass $M - n \cdot m_{\text{H}}$, which is assigned as $(M - nH)^{n-}$ or M^{n-} in the mass spectrum (negative ion mode).

Dissolving peptides and proteins under denaturing conditions (organic solvent plus water containing acid), as already described, results in a more

or less pronounced unfolding of the analyte molecule in solution. The degree of unfolding depends on the features of the individual analyte; unfolding is typically limited for proteins with intramolecular bonds (disulfide bonds). Acetic or formic acid in the solvent ensures protonation of basic amino acids, and unfolding of the analyte molecule by organic solvent increases the number of achievable basic amino acids. As a rough rule, the number of basic amino acids determines the number of charges observed in positive ESI-MS.

Peptides are typically observed with two to five protons, $(M + 2H)^{2+}$, \dots , $(M + 5H)^{5+}$, in the mass spectrum depending on the mass of the peptide and the number of its basic residues. The m/z ratios are typically between $m/z = 300$ and $m/z = 1500$. ESI-MS data of an equimolar mixture of angiotensin I, substance P, and neurotensin (1 pmol/ μ l each peptide, all peptides of human source) are shown in Fig. 2. The triply charged species $(M + 3H)^{3+}$ are observed predominantly for these three peptides; angiotensin I is also observed as fourfold-charged species ($m/z = 325$). The charge state information observed for the three peptides, therefore, is in agreement with the number of basic amino acids plus one charge for the N terminus. Angiotensin I has four likely protonation sites, while substance P and neurotensin have three potential protonation sites (i.e., the sum of basic amino acid residues (Lys, Arg, His) plus one protonation site at the amino terminus). Data shown in Fig. 2 were obtained using an ESI-*oa*-TOF mass analyzer (Mariner, Applied Biosystems, Framingham, MA, www.appliedbiosystems.com) equipped with a nanoelectrospray source. Typically, a mass resolution of $R = 6000$ FWHM for each peptide is observed as shown in the insets of Fig. 2. If the triply charged species of angiotensin I and neurotensin are used for calibration of the mass scale $300 \leq m/z \leq 1000$, then the mass of substance P is determined with an accuracy of ≈ 10 ppm or 10^{-5} (Fig. 2, internal calibration). Using the calibrated mass spectrum for further mass analysis in subsequent spectra (external calibration), the accuracy of mass determination is still better than 100 ppm. For peptides, common mass analyzers enable the resolution of the corresponding isotope distribution (Fig. 2). To resolve adjacent isotopes is of particular interest because it allows the assignment of the charge state of an ion only by the spacing between adjacent signals. The spacing between the monoisotopic peak and the peak including one ^{13}C isotope equals to $(\text{charge state})^{-1}$. In the example shown in Fig. 2, the spacing between adjacent signals of angiotensin equals to $m/z = 0.3$; this corresponds to a charge state of 3. Knowing the charge state of a given peptide signal (see the following paragraphs) enables the determination of the monoisotopic mass of a peptide M_{MONO} .

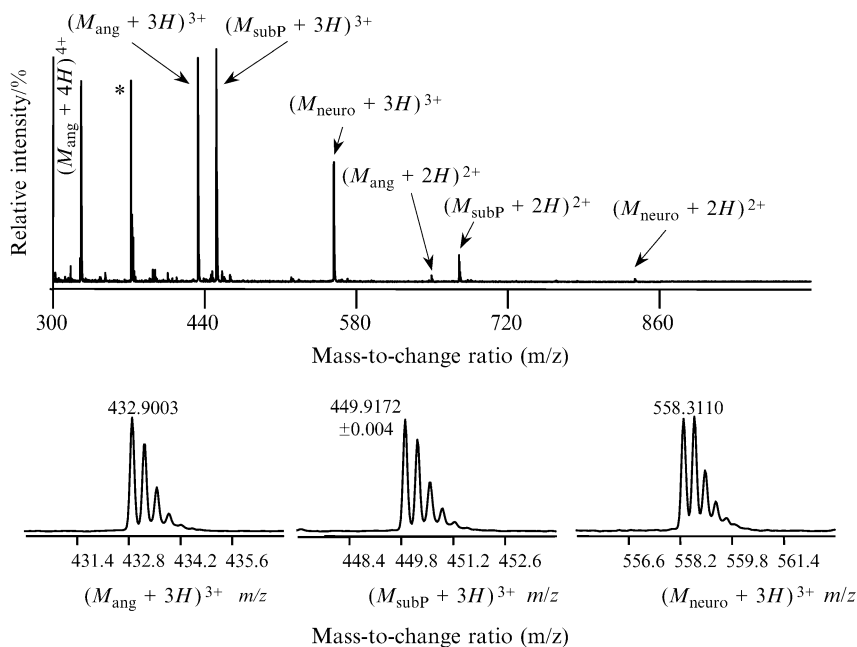


FIG. 2. ESI (nanoelectrospray) mass spectrum of three peptides: equimolar mixture of angiotensin I (M_{ang}), substance P (M_{subP} , Met-NH₂), and neurotensin (M_{neuro}) ([angiotensin] = [substance P] = [neurotensin] = 1 μM ; oa-TOF mass analyzer). The triply charged species of the three peptides are enlarged, and the monoisotopic signals are assigned. Note that the spacing between adjacent isotopes ($m/z = 0.3$) equals to the (charge state)⁻¹. Calibrating the spectrum internally with angiotensin and neurotensin as calibration peptides, the mass of substance P can be measured with a precision better than ± 10 ppm. The peak labeled with the symbol * is due to an impurity (no peptide).

For proteins, a broader charge state distribution and more highly charged species are observed. As a rough guide, one charge per 1000 u is observed using denaturing solvent conditions, and the mass-to-charge range covered by the distribution is between $m/z = 500$ and $m/z = 2000$. The width of this charge state distribution is often about half that of the highest charge state (Smith *et al.*, 1990). For a protein such as myoglobin (apo-form, horse, 16951.49 u) (Zaia *et al.*, 1992), signals will appear between $m/z = 680$ and $m/z = 1700$ with 24 ($m/z = 679.06$, $(M + 25H)^{25+}$) to 10 ($m/z = 1696.15$, $(M + 10H)^{12+}$) charges (see Fig. 3).

The average mass M_{av} of the molecule is “reconstructed” from the ESI mass spectrum while taking into account the assumptions that (i) adjacent

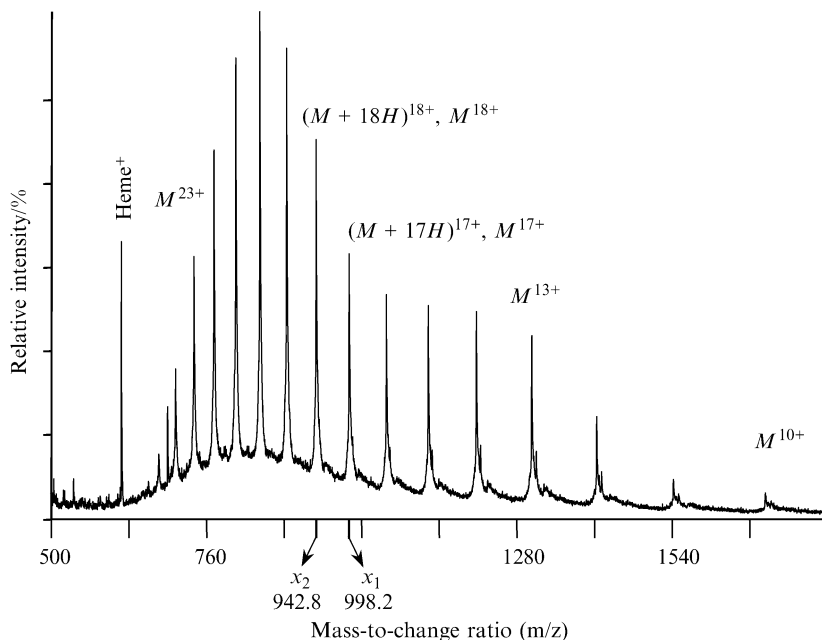


FIG. 3. ESI (nanoelectrospray) mass spectrum of apomyoglobin (horse heart) measured from a water/acetonitrile mixture containing 1% formic acid ([apomyoglobin] = 2 μM ; oa-TOF mass analyzer). Some charge states are assigned. For x_1 and x_2 , see text.

peaks differ by one charge and (ii) charging is due to the adduction of the same species (e.g., proton H^+). The basic equations for this reconstruction are given below although average masses or monoisotopic masses of analyte molecules are calculated easily by appropriate software provided with the mass spectrometer. The procedure described in the upcoming paragraphs is slightly simplified because it takes into account protonated ion species only; a more complete description is given by the “averaging algorithm procedure” described by Mann and coworkers that also takes into account cations as adducts and generalizes for negative ion mode (Mann *et al.*, 1989).

For reconstruction of the molecular mass of a protein, any two adjacent peaks are sufficient to determine the molecular mass of a species (Edmonds, 1990; Mann *et al.*, 1989); in this context, the redundancy of mass and charge state information contained in ESI mass spectra is notable. Referring to Fig. 2, the mass-to-charge ratios $m/z = x_1$ and $m/z = x_2$ are two adjacent members of an ion series obtained by protonation ($m_{\text{H}} = 1.00794$ u). The m/z ratios of the two ions can be expressed by the following:

$$x_1 = (M + z \cdot m_{\text{H}})/z. \quad (1)$$

$$x_2 = (M + (z + 1) \cdot m_{\text{H}})/(z + 1). \quad (2)$$

The factors in equations (1) and (2) can be defined as:

M : molecular mass of the analyte molecule, M_{av} or M_{MONO}

x_1 : m/z ratio of the analyte molecule with z protons

x_2 : m/z ratio of the analyte molecule with $(z + 1)$ protons

Equations (1) and (2) require that x_1 is greater than x_2 . Combining these equations and solving them for charge z allows the determination of the charge state z of the ion signal at $m/z = x_1$, as shown in the following equation:

$$z = x_2 - m_{\text{H}}/x_1 - x_2. \quad (3)$$

The number of charges z of the ion signal of $m/z = x_1$ allows the determination of the molecular mass M of the analyte molecule:

$$M = z \cdot x_1 - z \cdot m_{\text{H}} = (z + 1) \cdot x_2 - (z + 1) \cdot m_{\text{H}} \quad (4)$$

Taking the spectrum of Fig. 3 as an example, two adjacent charge states might be considered: $x_1 = 998.2$ and $x_2 = 942.8$; the charge state of x_1 is easily calculated to $z = 17$ from these numbers. The molecular mass of apomyoglobin (16951.49 u) can be determined with a precision of better than ± 50 ppm or $\pm 5 \cdot 10^{-5}$ (± 0.85 u) using external mass calibration.

Common mass analyzers (such as quadrupole mass filters or oa-TOF-mass analyzers) enable the observation of the envelope of the isotope distribution of proteins as in the case of apomyoglobin (Fig. 3), but these analyzers do not allow resolution of certain isotopes of the distribution (see Fig. 1). Therefore, the values of peak height or, better yet, of peak centroid (averaging over the peak area) of a given charge state are taken into account, and the experimentally determined value is the average mass, M_{av} .

Calibration of Mass Spectra

Denaturing Conditions. A standard mixture containing some peptides is typically used for mass calibration of peptides as analyte molecules. Due to the complexity of an ESI mass spectrum and of peptide mixtures (a number of different charge states show up for each peptide), external mass calibration might be preferred; that is, the standard mixture is run, the mass spectrum is then calibrated with the corresponding monoisotopic masses of the peptides, and, finally, the sample of interest is run under the same instrumental conditions. A similar procedure is performed for proteins. Apomyoglobin from horse heart is used as a calibration

protein in many applications because it is well-defined (one amino acid sequence, no heterogeneity due to posttranslational modifications) and because it results in a wide charge state distribution (see Fig. 3). Bourell and colleagues report a mass determination accuracy of less than or equal to 200 ppm for purified antibody fragments of 100 ku that are engineered in a recombinant manner using a triple quadrupole mass analyzer and performing peak height measurement (Bourell *et al.*, 1994). Such an accuracy will reflect whether correct translation and proper posttranslational modification of the proteins are achieved (Bourell *et al.*, 1994).

Native Conditions. For the analysis of noncovalently bound multimeric proteins or protein-ligand complexes, samples are dissolved in aqueous, buffered solutions (see previous paragraphs) with the aim of preserving the (native) solution state of the complex. If compounds are mass analyzed under these conditions, mass calibration is typically a little more tedious because charge state distributions are narrower and shifted to higher m/z values and because well-defined standards that give intense signals at high m/z - values are rare. In addition, the quality of ion signals may be degraded with respect to mass resolution due to unresolved solvent and/or buffer molecules (e.g., acetate, ammonia, water) attached to the protonated ion species (Potier *et al.*, 1997; Rogniaux *et al.*, 1999). Lysozyme (14305.14 u, chicken egg white), when dissolved in pure water and measured from a 5 μM solution, produces nicely resolved peaks and a relatively wide charge state distribution from $z = 4$ to $z = 11$, which covers the mass range between $1300 < m/z < 3600$. It is, therefore, a reasonable calibration protein for medium-sized proteins measured under native conditions.

The noncovalent complex between the protein apomyoglobin and its heme group (616 u) is observed (myoglobin) if the protein is dissolved in an aqueous buffered solution (5–10 mM NH_4Ac). The intact complex is observed largely with two charge states at $m/z = 1953$, $(M + 9H)^{9+}$, and $m/z = 2197$, $(M + 8H)^{8+}$ (as shown in Fig. 4). Emphasized are the differences in the width of charge state distribution, most abundant charge states, and last, but not least, the determined molecular mass of apomyoglobin (16951 u, Fig. 3, denaturing conditions) and myoglobin (17568 u, Fig. 4, native conditions).

MALDI-MS

In this section, results and discussion are based mainly on axial acceleration (aa-TOF mass analyzers) for matrix-assisted laser desorption/ionization–mass spectrometry (MALDI–MS) applications (Gross and Strupat, 1998). The general idea of a MALDI sample preparation is to mix a solution of analyte molecules of relatively low concentration with a

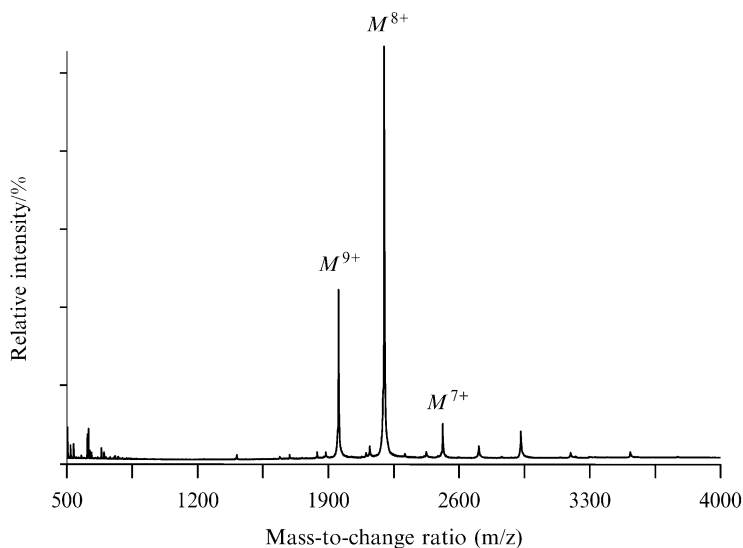


FIG. 4. ESI (nanoelectrospray) mass spectrum of myoglobin (horse heart) measured from an aqueous, buffered solution (10 mM NH_4Ac , pH 7) ($[\text{myoglobin}] = 10 \mu\text{M}$; oa-TOF mass analyzer). The noncovalent complex between the protein chain and the prosthetic group (heme) survives the transfer from the liquid to the gas phase.

so-called matrix solution of a relatively high concentration. The matrix—typically a small organic molecule—has mainly three tasks: **(i)** to strongly and resonantly absorb the irradiated laser wavelength, **(ii)** to force separation of analyte molecules from each other (matrix isolation), and **(iii)** to help or to initiate analyte ionization (Karas and Hillenkamp, 1988; Karas *et al.*, 1985). The matrix finally enables a desorption independent of the individual features of the investigated analyte molecule. The requirements for the matrix are as follows: First, to absorb the laser light strongly and resonantly, the matrix compound is an aromatic system in the case of ultraviolet-MALDI (UV-MALDI, electronic excitation) or an aromatic or aliphatic system in the case of infrared-MALDI (IR-MALDI, rotational-vibration excitation). Specific matrix compounds can be derived by various functional groups ($-\text{OH}$, $-\text{NH}_2$, OCH_3 , etc.) to accommodate the absorption of the matrix to the irradiating laser wavelength. Second, the required analyte separation (matrix isolation) is achieved by a large molar excess of the matrix and a molar matrix-to-analyte ratio between 10^3 and 10^6 in the final sample preparation. Therefore, the matrix concentration is $\approx 10^{-1} M$,

while the analyte concentration is in the range between 10^{-5} M and 10^{-7} M, depending on the size of the analyte molecule and its purity. In other words, a peptide or protein concentration of 0.1 g/l (10^{-4} M for mass 1000 u and 10^{-6} M for mass 100,000 u) is sufficient for a successful MALDI mass analysis. Third, it is assumed that the choice of the matrix plays an important role in analyte ionization, and a proton transfer from the electronically excited matrix compound to the analyte molecules in the expanding plume might be responsible for ionization; however, a model taking into account the different physical properties of UV- and IR-MALDI has yet to be explored (Ehring *et al.*, 1992; Karas *et al.*, 2000; Zenobi and Knochenmuss, 1999).

MALDI Sample Preparation

Two different main sample preparation techniques should be distinguished and can lead to very different sample morphologies, which influence the achievable precision and accuracy of mass determination. The fast evaporation or thin layer preparation introduced by Vorm *et al.* (Vorm and Mann, 1994; Vorm *et al.*, 1994) results in a very homogeneous sample morphology. This preparation technique is suited to matrix compounds that are almost water insoluble, such as α -cyano-4-hydroxy cinnamic acid (ACCA) (Beavis *et al.*, 1992). The MALDI sample is prepared by first producing a thin matrix layer on the target and afterward spotting the analyte solution on top of this matrix layer. For this purpose, a saturated matrix solution dissolved in acetone is spread over the target; the solvent evaporates quickly, leaving a thin, dry matrix layer behind. The analyte solution (preferentially in a slightly acidic solution containing 0.1% trifluoroacetic acid or TFA) is added on top of the matrix layer that is not dissolved completely by the solvent. Whether this results in analyte incorporation into the ACCA matrix crystals or the analyte molecules are only attached to the matrix surface is still debated (Horneffer *et al.*, 1999). The thin layer preparation technique using the ACCA matrix is preferentially used for peptide mass mapping with the aim of protein identification. The ACCA matrix is not a matrix of choice for high mass compounds, such as proteins analyzed in a reflector TOF, because the ACCA matrix tends to induce a considerable amount of metastable fragmentation of analyte molecules (Karas *et al.*, 1995).

The dried droplet preparation is best suited for water-soluble matrix compounds, such as 2,5-dihydroxybenzoic acid (2,5-DHB), or is best suited for mixtures with 2-hydroxy-5-methoxy benzoic acid (DHBs) (Karas *et al.*, 1993), 3-hydroxy picolinic acid (both UV-MALDI), or succinic acid (IR-MALDI) and results in a more heterogeneous sample morphology.

The MALDI sample is prepared by mixing analyte and matrix solution directly on the target and air-drying the sample. The cocrystallization of matrix and analyte results in matrix crystals with dimensions of the 100- μm range or larger. Crystals can tower into the acceleration region of the ion source, which limits precision of mass determination, if mass spectra are taken from different spots. Typically a dried droplet preparation leads to a more or less pronounced hot spot phenomenon (i.e., signal intensity can differ quite dramatically within one given sample preparation). This is especially pronounced for the 3-high-performance addressing (HPA) matrix.

Several helpful hints about sample preparation, including sample purification steps on a microliter scale prior to matrix incubation and on-target reactions such as dithiothreitol (DTT)-reduction, are described in the literature (Gobom *et al.*, 1999; Kussmann *et al.*, 1997).

Last, but not least, some comment about the liquid IR matrix glycerol seems appropriate. Glycerol acts as an IR matrix due to its three hydroxyl groups and can be used at a laser wavelength of 3 μm as well as of 10.6 μm (Berkenkamp *et al.*, 1997; Menzel *et al.*, 1999). Glycerol has the advantage of also being a liquid in a vacuum, and no cocrystallization with the analyte molecules occurs. The laser beam irradiates the liquid sample and always finds a “healed” surface from which analyte molecules can be desorbed. In practice, the observed ions—singly to more highly charged monomeric ions or singly charged oligomers of the analyte molecules—depend on the molar matrix-to-analyte ratio chosen (Berlenpamp, 2000; Berkenkamp *et al.*, 1997; Menzel *et al.*, 1999). Molar matrix-to-analyte ratios are between 10^4 and 10^7 . The lower the molar glycerol excess, the more pronounced are the oligomeric states (most likely gas-phase induced) and the less pronounced become doubly or triply charged ions of the analyte molecule.

Mass Analyzers. The laser-pulsed MALDI source is most suitable for TOF mass analyzers. Therefore, TOF mass analyzers in an axial acceleration (aa-TOF) geometry were the first to be employed in this ionization technique (Hillenkamp *et al.*, 1991). Peptide mass mapping is straightforward and the ease of interpretation of MALDI mass spectra (see *appearance of mass spectra*) often avoids the need to couple to the chromatographic separation of peptides prior to mass analysis.

A state-of-the-art MALDI-TOF instrument is equipped with both a linear port (ion source, field-free drift region, and detector are in a linear row) and a reflector port (which divides the field-free drift region by an electrostatic mirror that compensates for energy deficits of ions of the same m/z ratio) and, most importantly, with the possibility for delayed ion extraction in the MALDI source. In particular, the introduction of delayed ion extraction has improved the quality of MALDI mass spectra

significantly in terms of mass resolution R (Brown and Lennon, 1995; Colby *et al.*, 1994; Vestal *et al.*, 1995; Whittall and Li, 1995). Basically, delayed ion extraction (DE–MALDI) compensates for the initial velocity distribution (Beavis and Chait, 1991) of MALDI-produced ions (Juhasz *et al.*, 1997).

Mass resolution of more than $R = 10,000$ is achievable for peptides in the mass range up to 5000 u (Vestal *et al.*, 1995), and DE–MALDI-produced ions of a peptide mass map can be determined with an accuracy of 10 to 50 ppm (0.01 u to 0.05 u in 1000 u) or better. Such a high mass accuracy dramatically increases the specificity of database interrogation, and identification of proteins can be achieved unambiguously if at least five peptide masses are determined with better than 50 ppm accuracy (Clauser *et al.*, 1999; Jensen *et al.* 1996; Shevchenko *et al.*, 1996).

In contrast to the first conclusions made after the introduction of the technique—that MALDI-produced ions would be extremely stable and that no fragment ions would be observed—postsource decay (PSD) analysis allows investigation and identification of structural fragment ions of peptides up to 3000 u that result from decay taking place in the field-free drift region of the mass spectrometer after leaving the ion source (Kaufmann *et al.*, 1996; Spengler *et al.*, 1992). The complexity of PSD spectra, the relatively low abundance of fragment ions, and, most importantly, the limited mass accuracy still make PSD-based peptide sequencing for protein identification difficult, at least for high-throughput analysis (Spengler, 1997).

A MALDI source has been coupled to an oa-TOF arrangement (Krutchinsky *et al.*, 1998) and to a qQ-TOF setup (Krutchinsky, 1998; Loboda, 1999). The oa-TOF geometry substantially decouples the desorption process of MALDI (ions with large initial velocity distribution) from the subsequent mass analysis in the TOF. This facilitates mass calibration for both MS and MS/MS applications. MALDI-produced ions are cooled in the collisional damping interface (q) and transferred to the analytical quadrupole (Q), which is operated to transmit the ions to the oa-TOF (peptide mass map) or a precursor ion is selected that is fragmented in the succeeding collision cell (MS/MS) (Werner, 2005). Details on the design and performance of the qQ-TOF equipped with a MALDI-source have been published by Loboda *et al.* (2000). The power of this instrumentation and its promising impact on proteomics by MALDI-produced ions were described recently by Shevchenko *et al.* (2000). With high mass resolution R ($R = 10000$ FWHM) and high mass accuracy (10 ppm) of both, MS and MS/MS spectra are provided by this approach to peptide mass analysis (Shevchenko *et al.*, 2000).

MALDI sources have also been coupled to other mass analyzers, such as ion traps (Doroshenko *et al.*, 1992; Qin *et al.*, 1996), magnetic sectors

(Hill *et al.*, 1991), and double-focusing instruments (combined with an oa-TOF) (Bateman *et al.*, 1995), allowing the performance of MS and MS/MS applications.

Appearance of MALDI Mass Spectra

When accumulating spectra in the positive ion mode, single protonation of peptide and protein species is the most frequent mechanism. The most abundant ion signal is, therefore, assigned as $(M + H)^+$ or M^+ . Depending on sample purity, less pronounced sodium and potassium adduct ions $(M + Na)^+$ or $(M - H + Na + K)^+$ occur and broaden the signal to the higher mass side. This becomes a severe problem with increasing mass of the protein, because cation adducts cannot be resolved any more from the protonated species.

The singly charged (protonated) ion species M^+ is accompanied by less intense doubly and more highly charged ions (M^{n+}) and some still less abundant singly or more highly charged (most likely) gas phase-induced oligomers (mM^{n+}) of the species. The fact that MALDI produces predominantly singly charged ions significantly facilitates mass spectra interpretation compared to what is possible with ESI if more than one species is present in the sample. Even in the analysis of mixtures, signals corresponding to the same analyte molecule are easy to assign, making MALDI mass analysis most straightforward for peptide mass mapping (Clauser *et al.*, 1999; Jensen *et al.*, 1996; Shevchenko *et al.*, 1996).

In peptide mass analysis and positive ion mode, signals corresponding to singly charged protonated species $(M + H)^+$ are almost the only signals obtained. The information in Fig. 5 shows the same peptide mixture as shown in Fig. 2, but the data in Fig. 5 result from using an ACCA matrix prepared as a thin layer analyzed by MALDI-MS; angiotensin I, substance P, and neurotensin are added on top of the dried matrix layer. Each peptide has a concentration of 1 μM . Interestingly, only singly charged species appear in the mass spectrum (as protonated species and, although less abundant, as sodium and potassium attached species) together with matrix signals. Signals corresponding to dimers ($2M^+$) and doubly charged species (M^{2+}) are not observed. This finding is very typical for the mass analysis of peptides by MALDI-MS.

With increasing mass, more highly charged signals, such as $(M + 2H)^{2+}$ or $(M + 3H)^{3+}$, and singly or doubly charged oligomers, such as $(2M + H)^+$ or $(3M + 2H)^{2+}$, may show up in the mass spectrum. To provide an example of a medium-sized protein, Fig. 6 shows apomyoglobin (3 μM) prepared with DHBs matrix (Bahr *et al.*, 1997). The inset shows the singly charged ion species; a peak width at half maximum of $\Delta m = 9.6$ u is

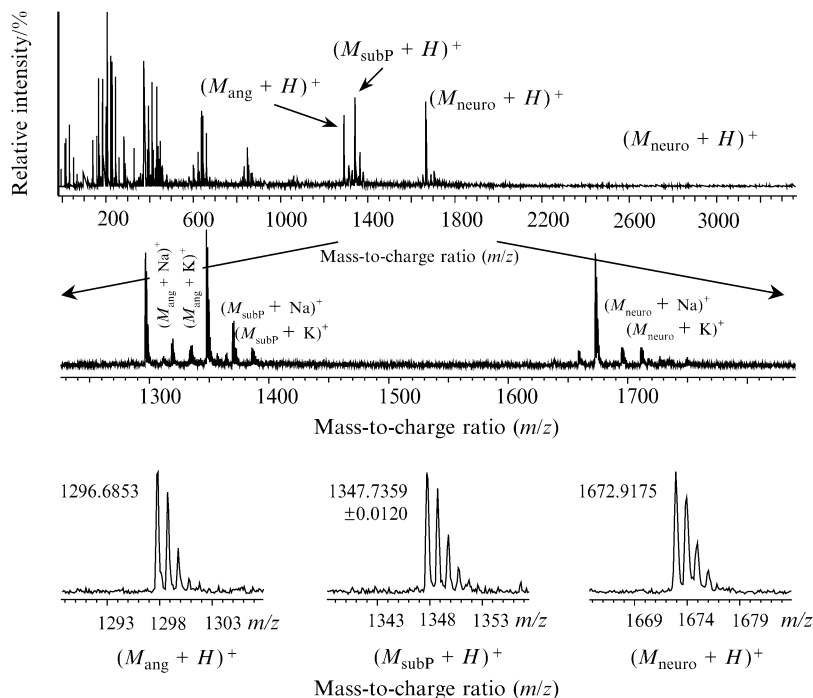


FIG. 5. Delayed extraction UV-MALDI mass spectrum of three peptides: equimolar mixture of angiotensin I (M_{ang}), substance P (M_{subP}), and neurotensin (M_{neuro}) ([angiotensin] = [substance P] = [neurotensin] = $1 \mu\text{M}$). ACCA thin-layer preparation; aa-TOF mass analyzer. The singly charged species of the three peptides are enlarged, and the monoisotopic signals are assigned. Calibrating the spectrum internally with angiotensin and neurotensin as calibration peptides, the mass of substance P can be measured with a precision better than ± 10 ppm.

obtained (this corresponds to a mass resolution of $R = m/\Delta m = 1765$), which is still broader than the envelope of the isotope distribution of apomyoglobin (see Fig. 1). A mass resolution of $R = 3500$ would be required to obtain the envelope of the isotope distribution of apomyoglobin (Bahr *et al.*, 1997).

Figure 7 shows various data, taken from the literature, obtained by UV- and IR-DE-MALDI of the four compounds simulated in Fig. 1. The agreement of the isotopic distributions of angiotensin I (Fig. 7A, mass resolution $R = m/\Delta m = 8600$), melittin (Fig. 7B, mass resolution $R = m/\Delta m = 9500$), and insulin all obtained by UV-DE-MALDI (Fig. 7C, mass resolution $R = m/\Delta m = 12500$) is obvious. For apomyoglobin obtained by UV-DE-MALDI (Fig. 7D), a mass resolution of $R = m/\Delta m = 1765$ is obtained (Bahr *et al.*, 1997).

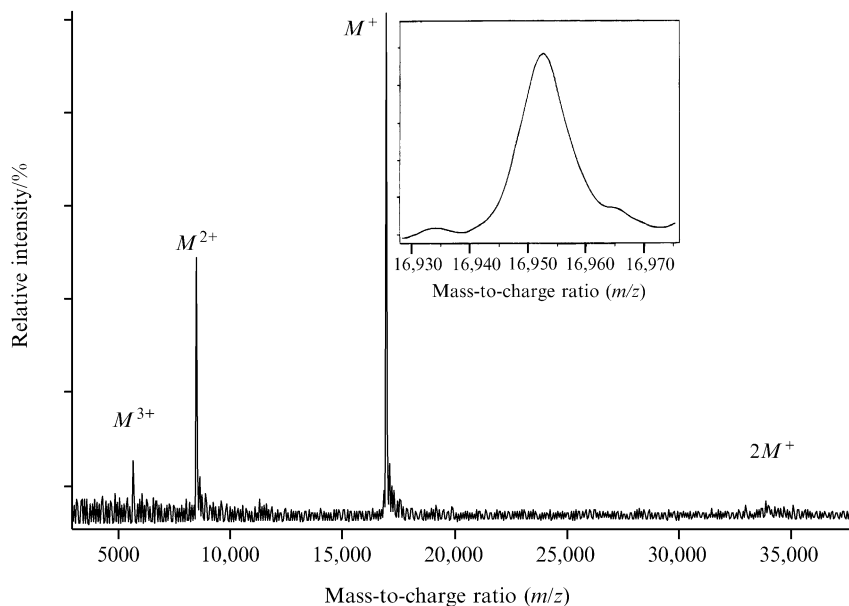


FIG. 6. Delayed extraction UV-MALDI mass spectrum of apomyoglobin (horse heart) ([apomyoglobin] = $3 \mu\text{M}$; aa-TOF mass analyzer). Use of DHBs matrix. Singly, doubly, and triply protonated species are shown together with a very low abundant dimer, and the singly charged species is enlarged. (Refer to acknowledgments).

Calibration of Peptide Mass Spectra

For the most frequent application—peptide mass mapping by UV-DE-MALDI-MS—a standard containing peptides is used for external mass calibration, or the calibration is performed internally using autolysis products of the enzyme (e.g., 2163.057 u, autolysis product of trypsin) and matrix signals (Jensen *et al.*, 1996). The ACCA matrix, using a thin-layer preparation technique, is often used for this kind of analysis because it introduces a more homogeneous sample morphology than matrices better suited to dried droplet preparation, such as the 2,5-DHB or DHBs matrices. Therefore, the ACCA matrix is best suited for rapid mass finger printing.

To determine how accurately masses of peptides can be measured over a wide mass range, Takach *et al.* (1997) investigated a peptide mixture containing 12 standard peptides in the mass range between 900 u and 3700 u. Mass resolution of each peptide signal is between $R = 7500$ and $R = 10,000$ in the reported UV-MALDI mass measurements. Using two of the peptides (904.4681 u and 2465.1989 u) to calibrate the mass scale

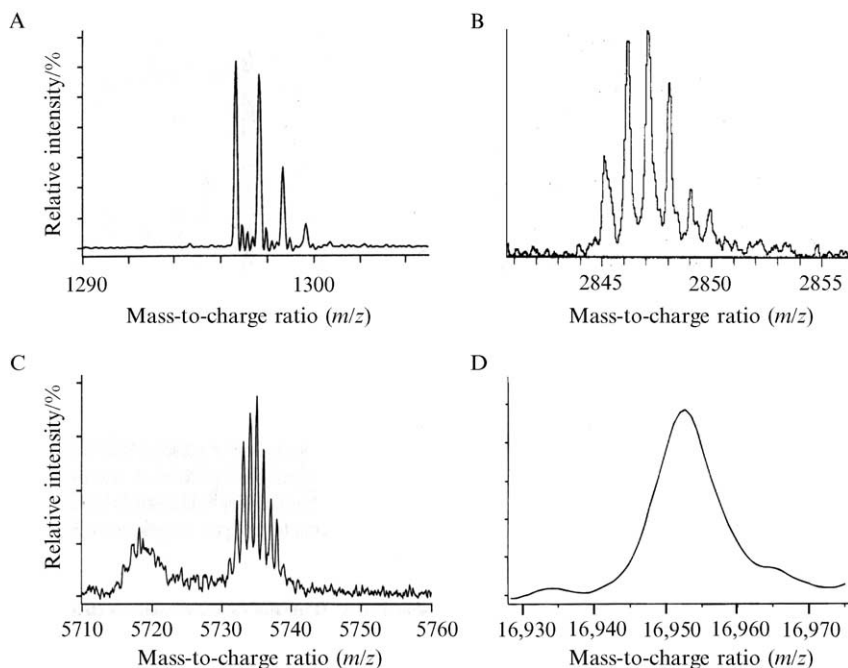


FIG. 7. Delayed extraction MALDI mass spectra of peptide/protein. (A) Angiotensin I (human), ACCA matrix. (B) Melittin (honeybee), succinic acid. (C) Insulin (bovine), sinapic acid matrix. (D) Apomyoglobin (horse), DHBs matrix. The singly protonated species are shown ($[\text{peptide/protein}] = 1 - 5 \mu\text{M}$; axial-TOF as mass analyzer). For graphs A and C, the spectra are according to [Vestal *et al.* \(1995\)](#); for graph B, the spectra are according to [Berkenkamp *et al.* \(1997\)](#); and for graph D, the spectra are according to [Bahr *et al.* \(1997\)](#). Reprinted with permission from John Wiley & Sons, New York. Graph B.

internally results in mass errors of less than 6 ppm for all peptides in a single mass spectrum ([Takach *et al.*, 1997](#)).

High accuracy of mass determination in peptide mass analysis allows one to distinguish between Gln and Lys in peptides on the basis of peptide mass. This is demonstrated by [Takach *et al.* \(1997\)](#) for the renin inhibitor K10 (1318.6737 u) and the renin inhibitor Q10 (1318.6773 u), respectively. The mass accuracy required to distinguish the chosen peptide masses is 27.5 ppm; the experimental results differ from the theoretical values by 1.5 ppm or less, using internal mass calibration ([Takach *et al.*, 1997](#)).

Mass calibration can be performed externally; that is, the standard is run, the mass spectrum is then calibrated with the corresponding mono-isotopic masses, and, finally, the sample of interest is run under the same

instrumental conditions. Consequences and limitations with respect to mass accuracy resulting from external mass calibration of MALDI data are mainly due to the heterogeneity of sample preparation both from spot-to-spot and from preparation-to-preparation. Russell and Edmondson discuss the influence of mass resolution and peak shapes on accurate mass assignment in the mass analysis of peptides; they have obtained an accuracy better than 5 ppm for internal and 10 to 15 ppm for external mass calibration in the mass range between 1 and 4 ku while achieving a mass resolution of $R = 10,000 - 15,000$ in the mass range of peptides (Edmondson and Russell, 1996; Russell and Edmondson, 1997).

Calibration of Protein Mass Spectra

In protein mass analysis, precision and accuracy of mass determination drops to values of 100 to 1000 ppm when using static ion extraction. Beavis and Chait reported results of UV-MALDI mass analysis of proteins in the mass range up to 30 ku and determined the mass of bovine pancreatic trypsinogen to 23980.3 ± 2.6 u, which is equal to a precision of 110 ppm and an accuracy of -29 ppm; the mass of protease subtilisin Carlsberg (*Bacillus subtilis*) was measured to (27288.2 ± 1.7) u, which is equal to a precision of 62 ppm and an accuracy of -7.3 ppm (Beavis and Chait, 1990). However, both the accuracy and precision drop down for proteins with increasing mass due to their intrinsic heterogeneity and their decay and adduct formation. For monoclonal antibodies (150 ku), Siegel *et al.* (1991) demonstrated that a precision between 100 and 700 ppm is achievable using nicotinic acid as UV-MALDI matrix.

Due to the introduction of delayed ion extraction and the higher mass resolution thereby obtained, precision of mass determination of proteins above 20 ku has been increased to ± 50 ppm for successive measurements from a given spot and is still ± 200 ppm for several spots in between one preparation of a DHBs matrix rim, as shown by Bahr *et al.* (1997). The accuracy of mass determination is in the 100 ppm range for proteins exceeding 25,000 u. The best conditions were explored for weak extraction field strengths and long delay times (Bahr *et al.*, 1997). By far the best results with respect to matrix choice were obtained using a DHBs matrix; the strength of this matrix for high mass compounds is due to the fact that it does not transfer much energy into the analyte molecules (as the ACCA matrix does, for example) and therefore prevents or reduces decay of analyte molecules in the field-free drift region of the mass analyzer. The softness of the DHBs matrix (Karas *et al.*, 1993) compensates for the heterogeneous sample morphology obtained using this type of matrix. Bahr *et al.* (1997) investigated α -amylase (from

Bacillus amyloliquefaciens) by DE-MALDI-MS using the DHBs matrix. A sharp peak of the singly protonated species is obtained and is accompanied by matrix adducts attached to the protein (see Fig. 8). From the peak shape obtained it is obvious that several adducts contribute to the shoulder at the high mass side of the peak. The average mass of α -amylase was determined from six measurements on different spots performing an external calibration using bovine carbonic anhydrase as a calibration protein (M^+ , $2M^+$). A mass of 54850 ± 9 u was determined for α -amylase, which is in very good agreement with literature data of 54851 u (accuracy -18 ppm, precision ± 164 ppm).

There may be several reasons why mass accuracy and precision of DE-MALDI data are still limited for proteins above 50 ku (Bahr *et al.*, 1997). These reasons include an increase in protein heterogeneity with increasing mass (covalent modifications such as glycosylation or phosphorylation, ragged ends, etc.), ion formation between analyte molecules and

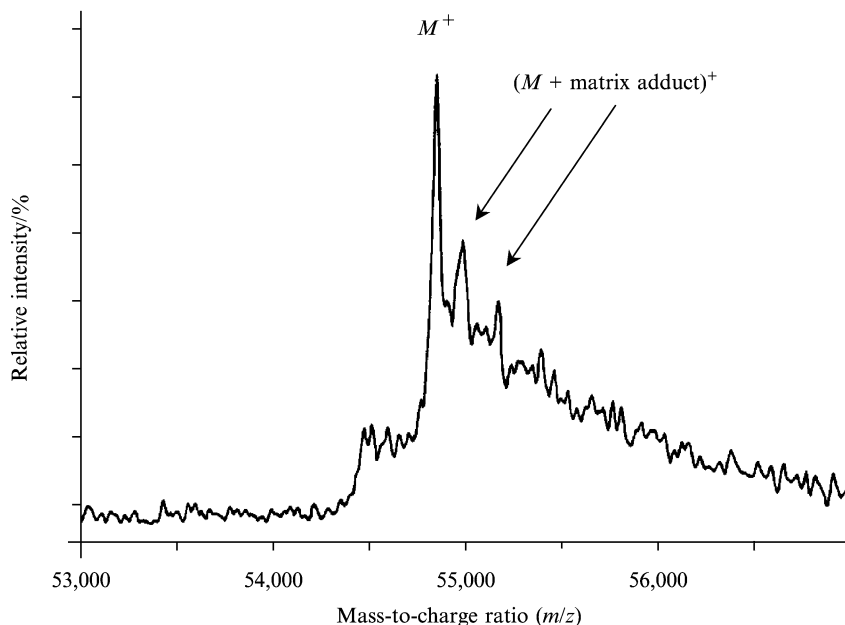


FIG. 8. Delayed extraction MALDI mass spectrum of α -amylase (*B. amyloliquefaciens*) (DHBs matrix; [α -amylase] = $3 \mu\text{M}$). Use of aa-TOF mass analyzer. The singly protonated species is shown together with matrix adducts attached to the molecular ion. Spectrum according to Bahr *et al.* (1997). Reprinted with permission from John Wiley & Sons, New York.

matrix molecules (matrix adducts), and an increasing contribution of the initial energy and energy distribution with increasing analyte mass (Bahr *et al.*, 1997).

Calibration of Mass Spectra Using IR-MALDI

In static extraction using a reflectron TOF mass analyzer, the precision of mass determination of IR-MALDI is typically 400 to 700 ppm for molecular masses up to 150 ku using solid matrices, while the precision is a little better, at 200 to 500 ppm, using the liquid matrix glycerol. Using solid matrices precision is mainly limited by the need for frequent changes of desorption location, as is typical for IR-MALDI. Precision in IR-MALDI is, therefore, one order of magnitude worse for analyte molecules below 30 kDa using static extraction compared to UV-MALDI, while it is better by a factor of 2 for larger analyte molecules. This finding might be explained by the typically better mass resolution of IR-MALDI ion signals compared to UV-MALDI ion signals of high mass compounds, which is a consequence of the lower yield of metastable fragmentation in IR-MALDI compared to UV-MALDI. For proteins up to 40 ku, mass accuracy is about 100 to 500 ppm, while it is limited to 1000 to 5000 ppm for analytes exceeding 40 ku (Berkenkamp *et al.*, 1997).

Mass calibration of high mass compounds in IR-MALDI can be performed conveniently by using lysozyme (chicken egg white) desorbed from glycerol matrix. Depending on the mass range of interest, slightly different molar lysozyme-glycerol ratios are prepared, resulting in more or less pronounced oligomeric signals of lysozyme of the type $(nM + H)^+$ up to 200,000 u. This performance of IR-MALDI using a glycerol matrix permits calibration of higher mass ranges; however, high accuracy of mass determination is still desirable: lysozyme was used to calibrate two different chondroitinase enzymes that digest a polysaccharide part of the eye's proteoglycan. The molecular masses of these two enzymes were calculated from their c-DNA-derived sequences; chondroitinase I has a molecular mass of 112,508 u, while chondroitinase II has a molecular mass of 111,713 u. IR-MALDI determined the molecular masses with an accuracy of -1600 ppm (-185 u) using static ion extraction (Berlenpamp, 2000; Kelleher *et al.*, 1997).

Using delayed extraction, the achievable accuracy of mass determination of a mixture of peptides was tested for succinic acid matrix (2.94 μm) (Berkenkamp *et al.*, 1997), glycerol matrix (2.94 μm) (Berkenkamp *et al.*, 1997), and fumaric acid (10.6 μm) (Menzel *et al.*, 1999). All three matrices resulted in an accuracy of 10 ppm or better using internal mass calibration. With this respect, accuracy of mass determination of peptides achieved by

IR–MALDI–MS is in excellent agreement with values obtained by UV–MALDI–MS and ESI–MS.

FTICR Mass Analyzers Coupled to ESI and MALDI

The underlying physical principle of mass determination by a FTICR mass analyzer is the relationship between the cyclotron frequency (ν_c) of an ion in a magnetic field of the magnetic field induction (B) and the mass-to-charge ratio (m/z) of the ion: $\nu_c \propto (m/z)^{-1}$ (Marshall and Grosshans, 1991). The accuracy of mass determination by FTICR mass analyzers is potentially ultra-high because frequencies can be measured more accurately than any other physical property. Mass calibration is performed by the determination of the cyclotron frequency of a calibration peptide or protein. The cyclotron frequency of the analyte compound under investigation is compared to that of the calibration molecule. As a rough guide, mass accuracy performing an external mass calibration is about 10 ppm or better (Li *et al.*, 1994), while applying mass calibration is better by a factor of 3 (Wu *et al.*, 1995). The masses of the most abundant isotopes of the two chondroitinases (112,508 u and 111,713 u) mentioned earlier could be determined with an accuracy of ± 3 u (± 2.6 ppm, external mass calibration) by Kelleher *et al.* (1997).

Although ESI is the more frequently used ionization technique employed with FTICR mass analyzers, MALDI can also be applied with this kind of mass analyzer. The challenge to couple MALDI-produced ions to FTICR mass analyzers lies in the relatively broad mass-independent velocity distribution of MALDI ions (Beavis and Chait, 1991). The kinetic energy dependent on velocity distribution makes an efficient trapping of ions in the FTICR cell more difficult (Hettich and Buchanan, 1991; Li *et al.*, 1996).

The striking feature of FTICR mass analyzers is their ultra-high mass resolution ($R > 10^5$ at 1000 u). The mass resolution increases with the applied magnetic field induction B , which motivates the performance of experiments with higher and higher values of B . The high mass resolution achievable with FTICR mass analyzers allows the determination of the charge state of high molecular compounds, such as proteins, directly from one single charge state: the distance between isotopic signals is now also resolved for larger compounds (compare to Fig. 2 and Fig. 3), and adjacent ion signals differing by one mass unit (1 u) have a distance of (charge state) $^{-1}$.

This feature greatly simplifies the mass assignment of an ion in ESI (Beu *et al.*, 1993; Senko *et al.*, 1996; Speir *et al.*, 1995). Figure 9 shows the

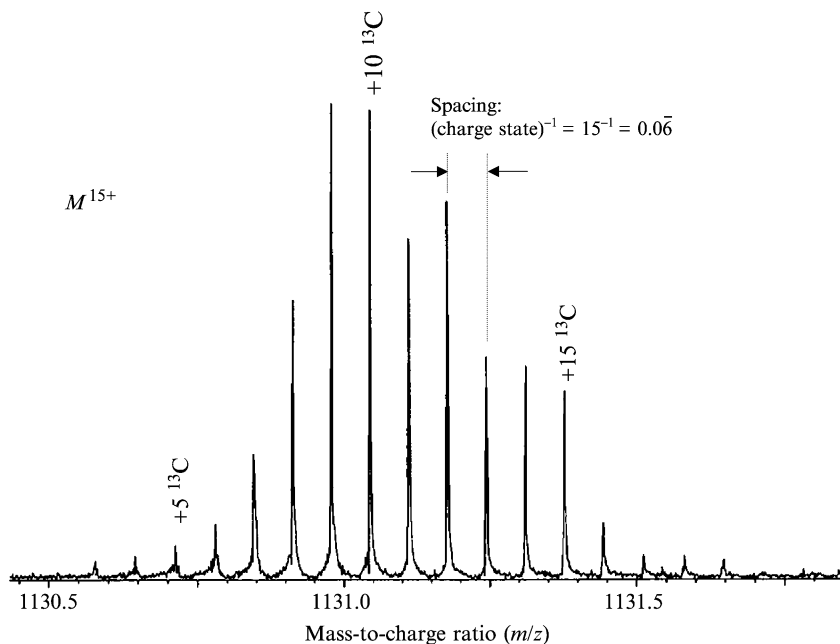


FIG. 9. Inset of an ESI mass spectrum of the 15-fold charged apomyoglobin (horse heart), m/z 1131.1, $(M + 15H)^{15+}$, using an FT-ICR mass analyzer. Adjacent isotopes of the distribution can be distinguished; the spacing correlates to $(\text{charge state})^{-1}$. Figure according to [Beu *et al.* \(1993\)](#). Reprinted with permission from Elsevier Science.

signal of the 15-fold charged apomyoglobin, $(M + 15H)^{15+}$, obtained by an FTICR mass analyzer (data taken from the literature). Mass resolving power is $9 \cdot 10^5$ ([Beu *et al.*, 1993](#)). The irregular shape of the isotopic distribution is due to a small ion population because a few thousand ions only contribute to the entire isotope profile shown in [Fig. 9 \(Beu *et al.*, 1993\)](#).

Because ions can be stored in the trap, MS^n investigations can be performed in an FTICR analyzer using a variety of different approaches, such as SORI-CAD ([Senko *et al.*, 1994](#)), BIRD ([Price *et al.*, 1996](#)), IR-MPD ([Little *et al.*, 1994](#)), and ECD ([Zubarev *et al.*, 1998](#)), which are applicable to multiply charged ions obtained by an ESI source ([Williams, 1998](#)). The possibilities and features mentioned in the previous paragraphs make ESI-FTICR-MS an important, valuable tool in biological mass spectrometry and, moreover, in life sciences.

Addendum

Mass Analyzers

A variety of different analyzers or combinations of analyzers (hybrid instruments) have been applied very successfully for both ESI and MALDI ionization techniques, and most of them are commercially available. These include quadrupole time-of-flight instruments available with exchangeable ion sources, making them applicable to a large spectrum of analytical questions (Chernushevich *et al.*, 2001). The TOF–TOF instrument equipped with a MALDI source and a collision cell for high energy collisions allows the identification of up to 10,000 proteins per day (Medzihradzky *et al.*, 2000). Another approach to identifying proteins via a sequencing of specific peptides of MALDI-induced PSD products is opened by the LIFT technology realized on an aa-reflectron TOF; PSD-spectra can be accumulated much faster because all fragment ions are found for one given reflectron potential (La Rotta, 2001). In addition, a hybrid instrument that is a combination of a linear ion trap followed by a FTICR mass spectrometer has also been introduced. The LTQ–FT is capable of detecting fragment ions in the linear trap (low mass resolution and accuracy) of a given precursor, while (in parallel) the corresponding precursor is detected with high mass resolution and ultra-high mass accuracy (0.5–2 ppm) (Olsen and Mann, 2004). The future will show how these analyzers will answer the variety of analytical questions in life sciences with respect to factors such as speed, sensitivity, dynamic range, and mass accuracy.

MALDI Sample Preparation

When addressing the question whether analyte molecules should be incorporated into the solid matrix to enable a successful desorption/ionization event of analyte molecules by MALDI, it should be mentioned that results indicate the necessity of a chemisorption of analyte molecules (i.e., only partly incorporated) into the matrix surface to enable MALDI. A physisorption (a simple deposition of analyte molecules onto the matrix surface) is not sufficient (Gluckmann, 2001; Horneffer, 2001).

Acknowledgments

ESI mass spectra of the peptide mixture, of apomyoglobin and myoglobin, were accumulated using a Mariner instrument (oa-TOF) equipped with a nanoelectrospray source. The instrument was kindly given to chapter author Kerstin Strupat for research work; this support by Applied Biosystems is highly appreciated. Strupat wants to thank Dr. Jonathan

Butler (Laboratory of Molecular Biology, Cambridge, United Kingdom) for proofreading the manuscript and helping with the English language. The MALDI mass spectrum of apomyoglobin (Fig. 6) was kindly donated by Dr. Ute Bahr and Dr. Michael Karas from the University of Frankfurt, Germany. Dr. Michael Mormann (Institute for Medical Physics and Biophysics, Biomedical Analytics, University of Münster) is also thanked for the discussion about FTICR instrumentation.

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[2] Batch Introduction Techniques

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Abstract

Mass spectrometry (MS) is widely used as a rapid tool for peptide profiling and protein identification. However, the success of the method is compromised by dirty and contaminated samples. Moreover, analysis from a small sample volume with a relative high concentration is usually required. In this chapter, different microscale sample preparation methods are discussed for off-line, matrix-assisted laser desorption/ionization (MALDI) and nanoelectrospray ionization (nanoESI) MS analysis.