7.6 Mass Spectrometry

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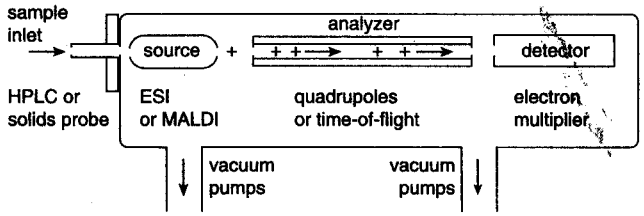
Mass spectrometry is an important tool in biochemical research. Scientists such as Fenn,[1] and Hillenkamp and Karas[2] have developed and established this technique into what it is today, a highly sensitive tool capable of analyzing small peptides as well as large proteins. Moreover, since adding electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) to the repertoire of research tools, the demand for this instrumentation has literally exploded. As a result the commercial availability of these instruments that offer picomole to femtomole sensitivity and that enable the direct analysis of biological fluids with a minimum amount of sample preparation has made the analysis of proteins and peptides routine.[3] Peptide mapping can be performed directly on a protein digest by MALDI, or by compiling the mass data obtained from individual peaks in liquid chromatography.

A mass spectrometer determines the molecular weight of chemical compounds by separating molecular ions according to their mass-to-charge ratio (m/z). The ions are generated by inducing either the loss or the gain of a charge, for example through electron ejection, protonation, or deprotonation. Once the ions are formed they can be separated according to mass and finally detected. As a result molecular weight information as well as structural characterization can be obtained. The basic components of a mass spectrometer: the sample inlet, ionization source, mass analyzer, and ion detector are illustrated in Figure 1. Once the sample is introduced into the instrument through the sample inlet it undergoes ionization in the ionization source and the charged molecules are then electrostatically propelled into the mass analyzer, which separates the ions according to their mass-to-charge ratio.[3] The ion detector then transmits its signal to a computer where the information is stored and processed.

Figure 1 Components of a Mass Spectrometer and the Names of Those Commonly Used to Perform the Function of Ionization (Electrospray or MALDI), Analysis (Quadrupoles or Time-of-Flight), and Detection (Electron Multiplier)



7.6.1 Ionization and Ionization Sources

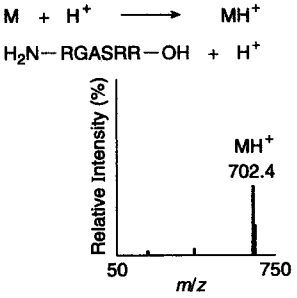
Electron ionization (EI) was the primary ionization source for mass analysis until the 1980s, limiting the chemist to the analysis of small molecules well below the mass range of common bioorganic compounds. This limitation motivated the development of the techniques commonly known as ESI,[1] MALDI,[2] and fast atom bombardment (FAB)[3,4] (Table 1). These ion sources allow for rapid and easy peptide analyses that previously required laborious sample preparation or were not possible with electron ionization. The mechanism of ionization these ion sources employ, which is somewhat responsible for their ability to generate stable molecular ions, is protonation and/or deprotonation.

Table 1 Ionization Techniques[1–4]

Ionization Technique	Acronym	Means of Ionization	Ref
electrospray ionization	ESI	evaporation of charged droplets	[1]
matrix-assisted laser desorption/ionization	MALDI	photon absorption/proton transfer	[2]
fast atom/ion bombardment	FAB	ion desorption/proton transfer	[3,4]

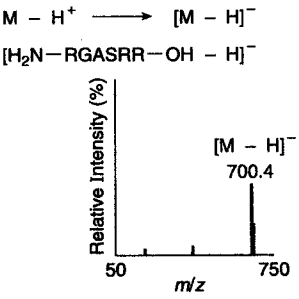
Protonation involves the addition of a proton to a molecule to produce a net positive charge of 1+ for every proton added (Figure 2). Positive charges tend to reside on the more basic residues of the molecule, such as amines, to form stable cations. A disadvantage of protonation is the covalent nature of the proton binding to the molecule which can initiate charge transfer and, in some cases, significant fragmentation.

Figure 2 The Mass Spectrum of a Peptide Obtained via Protonation



Deprotonation is the ejection of a proton from a molecule, resulting in a net negative charge of 1− for each proton ejected. This mechanism of ionization is very useful for acidic species, such as acidic peptides. With deprotonation, the net positive charge of 1− is achieved through the removal of a proton or even multiple negatively charged species (Figure 3).

Figure 3 The Mass Spectrum of a Peptide Obtained via Deprotonation



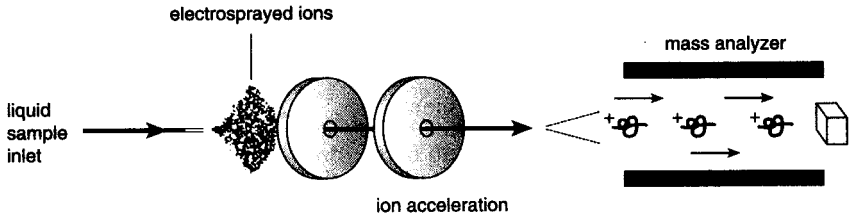
7.6.1.1 Electrospray Ionization

Electrospray ionization is a method used to produce gaseous ionized molecules from a liquid solution. This is done by creating a fine spray of highly charged droplets in the presence of a strong electric field. The sample solution is sprayed from a region of this field at the tip of a metal nozzle maintained at approximately 4000 V. The highly charged droplets are then electrostatically attracted to the mass spectrometer inlet where either dry gas, heat, or both are applied to the droplets before they enter the vacuum of the mass spectrometer, thus causing the solvent to evaporate from each droplet. As the droplet decreases in size, the electric field density on its surface increases. The mutual repulsion between like charges on this surface becomes so great that it exceeds the forces of surface tension, and ions begin to

for references see p 695

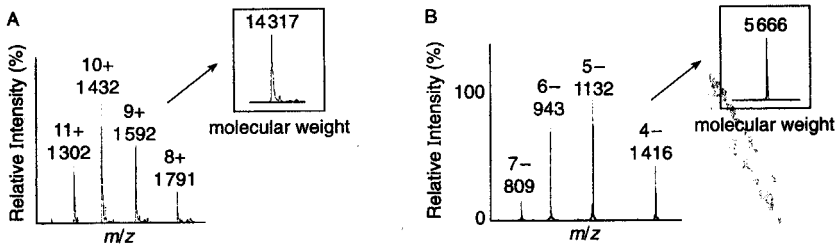
leave the droplet through what is known as a "Taylor cone". The ions are then directed into an orifice through electrostatic lenses leading to the mass analyzer (Figure 4). Since it is a continuous ionization method, ESI is suitable for using as an interface with modern separation techniques.

Figure 4 The Electrospray Ionization Source Generates Ions via Charged Droplet Evaporation Followed by Electrostatic Direction of the Ions into a Mass Analyzer



Electrospray ionization is conducive to the formation of singly-charged small molecules, but is also well-known for producing multiply-charged species of larger molecules. This is an important feature since the mass spectrometer measures the *m/z*, making it possible to observe very large molecules with an instrument having a relatively small mass range. Figure 5 illustrates how the ions from proteins and large peptides are observed through the multiple-charging phenomenon, where each of the peaks can be associated with different charge states of the molecular ion. Fortunately, electrospray mass spectrometers are equipped with computer programs that facilitate these molecular weight calculations.

Figure 5 Examples of Data Generated on an Electrospray Ionization Mass Spectrometer. (a) Proteins Typically Produce Positive, Multiply Charged Ions and (b) Oligonucleotides Generate Negative, Multiply Charged Ions. Inset are the Computer-Generated Molecular Weight Spectra



The extent of multiple charging that occurs in ESI is a unique characteristic of the technique that enables an analyte's mass to be determined with great precision, as masses can be independently calculated from several different charge states. The multiple charging in ESI also permits the measurement of high molecular weight analytes, using conventional mass analyzers that are normally limited to the detection of ions with relatively low *m/z* ratios (< *m/z* 3000). For example, a 50 kDa protein will typically retain on the order of 30 to 50 charges in ESI, yielding multiply charged species with *m/z* ratios between 1000 and 2000 that are easily detected with quadrupole mass analyzers. Another advantage of ESI-MS is its compatibility as an interface with liquid chromatography (LC).[4]

Nanoelectrospray ionization (nanoESI), also known as nanospray, nanoflow electrospray, and micro-electrospray, is a low flow/high sensitivity approach to ESI. NanoESI[5] is a slight variation on ESI such that the spray needle has been made very small and is positioned close to the entrance of the vacuum of the mass spectrometer and the mass analyzer (Figure 6). This greatly reduces required sample amounts allowing nanoliter flow rates and femtomole sample consumption. The end result is increased efficiency since the flow rates for

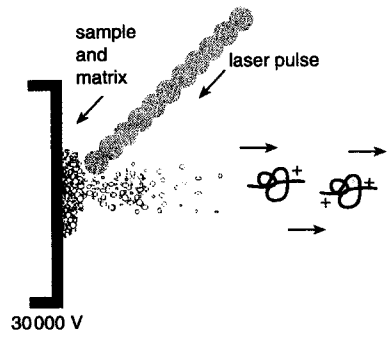
nanoESI sources are on the order of tens of nanoliters per minute. Another advantage of nanoESI is that the droplets formed are typically smaller than with normal ESI and thus nanoESI is more tolerant of salts and other impurities.

Figure 6 The Small Size Spray Needle for Nanoelectrospray

7.6.1.2 Matrix-Assisted Laser Desorption Ionization

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) permits the analysis of both low and high molecular weight compounds including carbohydrates,[6] lipids,[7] peptides,[8] proteins,[9] oligonucleotides,[10] as well as synthetic molecules and polymers[11] with high sensitivity. MALDI allows for the ionization and transfer of a sample from a condensed phase to the gas phase by first directing a pulsed laser beam to a sample suspended or dissolved in a matrix. This matrix plays a key role in the technique by absorbing the laser light energy and causing the matrix material to vaporize and the vaporized matrix carries some of the sample with it. Further, the matrix may play a role in the ionization of the analyte molecules once in the gas phase. Here, the charged molecules are directed by electrostatic lenses from the ionization source into the mass analyzer, while uncharged molecules will often react with the matrix or other molecules to produce charged species, transferred electrostatically into the mass analyzer. Once the molecules in the sample are vaporized, time-of-flight mass analysis is often used to separate the ions according to their mass-to-charge ratio (Figure 7).

Figure 7 Matrix-Assisted Laser Desorption/Ionization

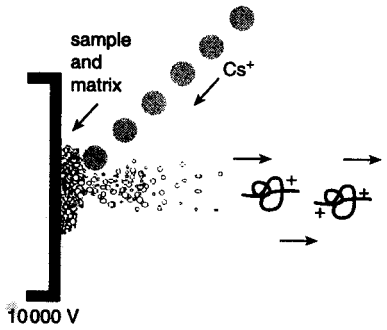


7.6.1.3 Fast Atom Bombardment

The fast atom bombardment ionization (FAB) technique is a soft ionization method, typically requiring the use of a direct insertion probe for sample introduction in which a high energy beam of Xe atoms, Cs^+ ions, or massive glycerol-NH_4^+ clusters sputter the sample and matrix from the probe surface (Figure 8).

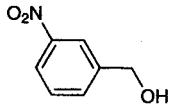
for references see p 695

Figure 8 Fast Atom Bombardment (FAB) Technique

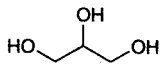


The FAB matrix is essentially a nonvolatile liquid material, such as those illustrated in Scheme 1, that serves to constantly replenish the surface with new sample as the incident ion beam bombards the surface. The matrix also serves to minimize sample damage from the high-energy particle beam by absorbing most of the incident energy and is believed to facilitate the ionization process. The spectrum produced often includes matrix peaks along with some fragments and a peak for the protonated or cationized (i.e., $M+Na^+$) molecular ion.

Scheme 1 Two Commonly Used FAB Matrices



3-nitrobenzyl alcohol



glycerol

A general comparison of ionization sources is given in Table 2.

Table 2 General Comparison of Ionization Sources

Characteristics	Ionization Source			
	Electrospray Ionization (ESI)	NanoESI	MALDI	FAB
Typical Mass Range	70 000	70 000	300 000	8000
Matrix Interference	none	none	yes	yes
Degradation	none	none	possible photo-degradation & matrix reactions	matrix reactions & some thermal degradation
Ability to Analyze Complex Mixtures	somewhat limited	somewhat limited but better than ESI	excellent	Somewhat amenable
LC/MS Capability	excellent	OK, but low flow rates can present a problem	very limited	very limited
Sensitivity	high femtomole to low picomole	high zeptomole to low femtomole	low to high femtomole	nanomole
Salt Tolerance	low (low millimolar)	moderate (low–mid millimolar)	moderate (low millimolar)	high (to 0.01 M)
Other	multiple charging useful but significant suppression can occur with mixturessoft ionization = low fragmentation	multiple charging useful but significant suppression can occur with mixturessoft ionization = low fragmentation	matrix background can be problem for low mass ionssoft ionization = low fragmentation	solubility with matrix required soft ionization = low fragmentation

7.6.2 The Mass Analyzer

Once ions are formed the mass analyzer is primarily responsible for providing information on the mass of an ion. It accomplishes this by separating and measuring the ions according to their different mass-to-charge ratios. This concept is the most important thing to understand and remember about mass analyzers; they measure the *m/z* ratio, not the mass! This is often a point of confusion, especially in electrospray because if an ion is multiply charged, the *m/z* will be significantly less than the actual mass (e.g., a peptide ion, $C_{37}H_{68}N_{16}O_{14}^{2+}$, *m/z* 488.3, has a mass of 976.5 daltons). Since multiple charging is especially common with electrospray ionization that yields numerous peaks corresponding to the same species, one must keep in mind the importance of the *m/z* ratio.

The first mass analyzers, developed in the early 1900s, employed magnetic fields to separate ions according to their *m/z*. Modern analyzers, whose designs include variations on the early magnetic methods, have advanced to offer higher accuracy, sensitivity, mass range, and an ability to give structural information. Just as ionization techniques have evolved, mass analyzers have also changed to meet the demands for observing larger molecules with mass accuracy of the order of ±0.1 to 0.0001%. In addition, the sensitivity required (picomole to femtomole) for many biological applications has been obtained with electrospray and MALDI ionization sources, in part due to the high transmission efficiency of the analyzers.

The general characteristics of a mass analyzer are accuracy, resolving power, mass range, and tandem analysis capabilities.

Accuracy is the ability of the analyzer to provide accurate *m/z* information and is largely dependent on an instrument's stability. For example, an instrument with 0.01% accuracy can provide information on a 1000 Da peptide to ±0.1 Da or a 10 000 Da protein to ±1.0 Da. The accuracy will of course vary, sometimes dramatically, from analyzer to analyzer.

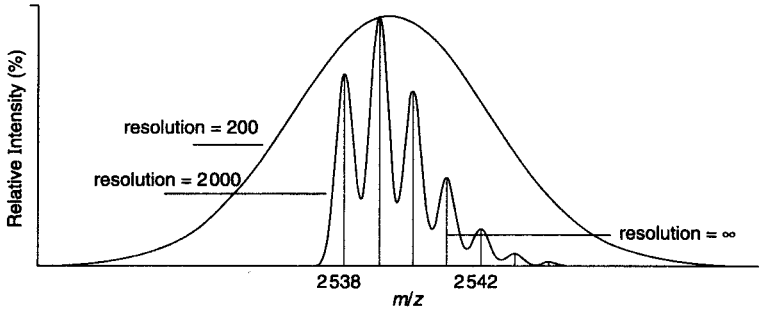
Resolving Power or Resolution is the ability of a mass spectrometer to distinguish between ions of different mass-to-charge ratios such that greater resolution corresponds directly to the increased ability to differentiate ions. For example, a mass spectrometer with a resolution of 500 can distinguish between ions of *m/z* = 500 and 501. The most common definition of resolution is given by the following equation:

$$\text{Resolution} = M/\Delta M$$

where M is the *m/z* of an ion and ΔM is the full-width at half-maximum peak. For example, a peak with *m/z* 500 and a full-width at a half maximum of 1 has a resolution of M/ΔM = 500/1.0 = 500.

The analyzer's resolving power does, to some extent, determine how much accuracy can be obtained with a particular instrument. The average mass of a molecule is calculated using the weighted average mass of all isotopes from each constituent element of the molecule. The monoisotopic mass is calculated by using the mass of the elemental isotope having the greatest abundance for each constituent element. Many instruments cannot resolve between the isotopes and will give only the average mass, yet sometimes the resolution is so low that it may be difficult to determine even the average mass with reasonable accuracy. Higher resolution allows separation of ions according to individual isotopes, or simply produces a very narrow peak allowing for greater accuracy. An example of how resolution affects the observed mass for a compound having a molecular formula $C_{101}H_{145}N_{34}O_{44}$ is shown in Figure 9.

Figure 9 The Effect of Resolution upon Mass Accuracy. The Overlaid Spectra Were Calculated for the Same Molecular Formula $C_{101}H_{145}N_{34}O_{44}$, at Resolutions of 200, 2000, and Infinity[3][a]



[a] Notice that at higher resolution the accuracy is increased because the center of the peak can be more accurately identified.

Mass range is the *m/z* range of the mass analyzer. For example, quadrupole analyzers typically only scan up to *m/z* 3000, magnetic sector analyzers can go to *m/z* 10 000, and time-of-flight analyzers have virtually unlimited *m/z*.

Tandem analysis is the ability with which the analyzer can separate an ion from other ions of different *m/z*, perform collision-induced dissociation on that ion, and analyze the respective fragmentation ions. The process is called tandem analysis because subsequent analyses are performed either by a consecutive analyzer, such as a triple quadrupole mass analyzer or they are performed with the same analyzer, such as an ion trap which isolates the ion of interest, fragments it, and analyzes the fragment ions all within the same ion cell. Typically, tandem MS experiments are performed by generating the ion of interest and selecting it with the first analyzer. The ion is then collided with inert gas molecules such as argon or helium, and the fragments generated by the collision are analyzed. The information obtained can be used to sequence peptides, carbohydrates, small oligonucleotides, and lipids.

7.6.3 Ionization Method and Mass Platform Configurations

Having examined ionization sources and mass analyzers as separate components of a mass spectrometer in Sections 7.6.1–7.6.2, we can now turn to the most common ways these two components are interfaced.

ESI with Quadrupole and a Quadrupole Ion Trap

Quadrupole mass analyzers have shown new utility in their capacity to interface with electrospray ionization. This interface has three primary advantages. First, quadrupoles are tolerant of relatively high pressures ($\sim 5 \times 10^{-5}$ Torr), which makes them well suited to electrospray ionization since the ions are produced under atmospheric pressure conditions. Secondly, quadrupoles are now capable of routinely analyzing up to an *m/z* of 3000, which is useful because electrospray ionization of proteins and other biomolecules commonly produces a charge distribution in that range. Finally, the relatively low cost of quadrupole mass spectrometers makes them attractive as electrospray analyzers. Considering these mutually beneficial features of electrospray and quadrupoles, it is not surprising that most of the successful commercial electrospray instruments thus far have been coupled with quadrupole mass analyzers.

The quadrupole ion trap mass analyzer was developed at the same time as the quadrupole mass analyzer. The physics behind both of these analyzers are very similar. Ion traps also allow one to perform multiple collision-induced dissociation (CID) experiments (also known as tandem mass analysis events) which represent a valuable tool for obtaining sequence information. Quadrupole ion traps, primarily utilized with the electrospray ionization of biomolecules, typically consist of a ring electrode and two hyperbolic endcap electrodes. After applying radio frequency and direct current voltages, the motion of the ions trapped by the electric field allows them to be trapped or ejected from the ion trap. In the normal mode the radio frequency is scanned to higher voltages, and the trapped ions with the lowest *m/z* are ejected to a detector through small holes in the endcap. As the radio frequency is scanned further, higher *m/z* ratios are ejected and detected.

MALDI with Time-of-Flight

A time-of-flight (TOF) analyzer[12] is one of the simplest mass analyzing devices and is commonly used with MALDI ionization. Time-of-flight analysis is based on accelerating a set of ions to a detector with the same amount of energy. As the ions have the same energy, yet a different mass, they reach the detector at different times. The arrival time of an ion at the detector is dependent upon the mass, charge, and kinetic energy of the ion. Since kinetic energy (KE) is equal to $^1/_2 mv^2$, or velocity $v = (2KE/m)^{1/2}$, ions will travel a given distance, d, within a time, t, where t is dependent on their *m/z*. The process is analogous to a pitcher throwing a golf ball and a basketball at a catcher with the same amount of energy. The golf ball will reach the catcher faster because it has a smaller mass and therefore a greater velocity. In mass spectrometry, the smaller ions reach the detector first because of their greater velocity and the larger ions take longer, thus the analyzer is called time-of-flight. Due to the greatly improved resolving power that has been developed for TOF analyzers over the past decade, along with the advantage of having virtually no upper mass limitation, the MALDI-TOF interface now offers good accuracy, excellent mass range, as well as its inherent high sensitivity.

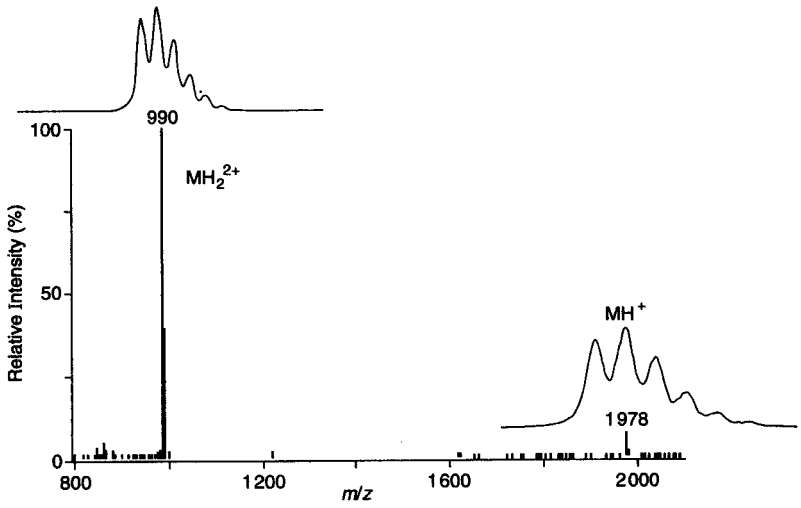
7.6.4 Peptide and Protein Analysis

7.6.4.1 By Electrospray

The electrospray ionization of peptides and proteins involves the addition of a proton or multiple protons. Sample preparation is achieved by dissolving the sample in a protic volatile solvent system (H_2O, MeOH, and $MeCN/H_2O$) that is relatively homogeneous and less than 5 mM of salt. An important feature of most electrospray mass spectrometers is their ability to generate and observe multiply charged species. This multiple charging makes it possible to observe large proteins with mass analyzers that have a relatively small mass range (such as quadrupoles). In addition, observing multiple peaks for the same peptide allows one to extract multiple molecular weight calculations from a single spectrum. Thus, one can average these values and obtain a very accurate molecular weight.

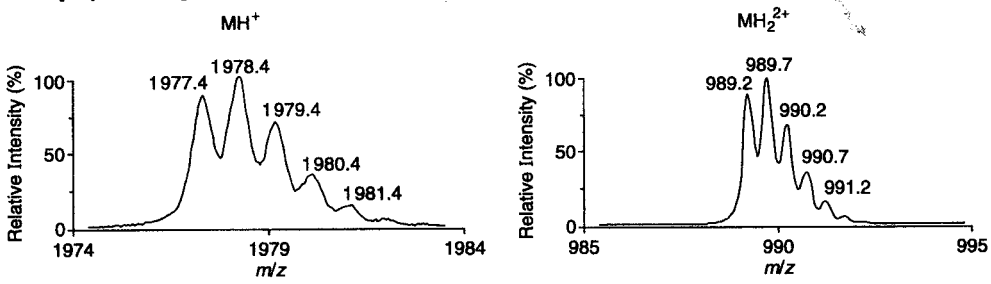
The question often arises, especially concerning the analysis of peptides and proteins, how is the charge state of the observed ion determined? The answer is straightforward. The spectrum in Figure 10 below shows two peaks, one at *m/z* 1978 and another at *m/z* 990.

Figure 10 Electrospray Mass Spectrum of a Peptide Having a Molecular Weight of 1977 Daltons[3]



How does the investigator know there are not two separate peptides? Since most electrospray spectrometers have good resolution, it is often possible to look at the isotopic distribution of singly and doubly charged ions. By looking at the isotopic distribution of the ions MH^+ and MH^{2+} (Figure 11) we can readily see that the isotopes are separated by one mass unit at *m/z* 1978 and $^1/_2$ mass unit at *m/z* 990. Each pattern therefore corresponds to the 1+ and 2+ charge states, respectively. This is due to the fact that the mass spectrometer measures the mass-to-charge ratio. Therefore, as the calculation demonstrates, for the 2+ charge state the isotopes are separated by $^1/_2$ mass unit, at 3+ the isotopes are separated by $^1/_3$ mass unit, and at 4+ the isotopes are separated by $^1/_4$ mass unit. Unfortunately, the resolving power of the common quadrupole electrospray mass spectrometers only allow for distinguishing between singly and doubly charged species.

Figure 11 The Isotopic Pattern of a Peptide with Singly and Doubly Charged Molecular Ions Obtained from an Electrospray Quadrupole Mass Spectrometer[3]



(molecular mass + no. of protons)/charge = mass-to-charge ratio

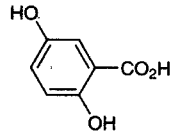
singly charged	doubly charged
(1976.4 + 1.0)/1 = 1977.4	(1976.4 + 2.0)/2 = 989.2
(1977.4 + 1.0)/1 = 1978.4	(1977.4 + 2.0)/2 = 989.7
(1978.4 + 1.0)/1 = 1979.4	(1978.4 + 2.0)/2 = 990.2
(1979.4 + 1.0)/1 = 1980.4	(1979.4 + 2.0)/2 = 990.7
(1980.4 + 1.0)/1 = 1981.4	(1980.4 + 2.0)/2 = 991.2

Fortunately, as larger peptides and proteins are analyzed a distribution of ions is obtained. Even though we cannot look at the individual charge states from the isotopic pattern (because the resolving power of the instruments are too low) we can deduce the charge state by looking at two consecutive peaks in the mass spectrum [m/z and $m/(z+1)$]. All commercial instruments now allow for these calculations to be carried out very easily.

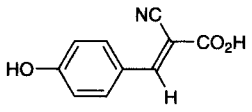
7.6.4.2 By Matrix-Assisted Laser Desorption

MALDI mass analysis of peptides and proteins is typically accomplished with a time-of-flight analyzer having resolving capabilities up to 2000. MALDI is a useful technique for these types of MS analysis for three reasons. The first is speed: the use of multi-sample probes (arranged on a single plate) allows one to prepare and run many samples simultaneously. Secondly, MALDI is very sensitive. Typical accuracies range from ±0.5 to 0.01% depending upon the presence of an internal standard, the type of instrument being used, and the selection of matrix material (Scheme 2). Lastly, MALDI is relatively tolerant of heterogeneous samples. Included here is the ability to measure peptides and other compounds in the presence of salt and the ability to analyze complex mixtures. MALDI can also be applied to the analysis of peptides generated from digests of a protein, a procedure used for peptide mapping.

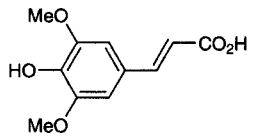
Scheme 2 Commonly Used MALDI Matrices



2,5-dihydroxybenzoic acid
peptides and small proteins



2-cyano-3-(4-hydroxyphenyl)acrylic acid
peptides and glycopeptides



3-(4-hydroxy-3,5-dimethoxyphenyl)acrylic acid
(sinapinic acid)
peptides and proteins

Many of the advantages that MALDI offers for peptide analysis are equally applicable to proteins. Protein analysis is similar to peptide analysis, in which ionization usually occurs through the addition of one, two, or three protons. However, since proteins are significantly bigger than peptides, ion detection is typically less efficient. Therefore, while peptides are measured at the femtomole or even attomole level with MALDI, proteins are usually measured at the high femtomole to low picomole level.

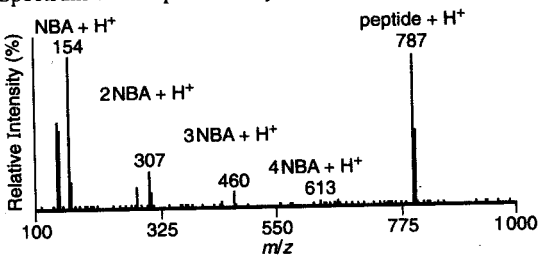
Sample Preparation and Matrix Recommendations for MALDI; Typical Procedure:
Pipet 0.5 μL of sample to the sample plate. Preferable concentration ~ 1pmol·μL^{-1}. Mix the sample and matrix[a] by drawing in and out of the pipette. Allow to air dry and acquire spectra.
[a]For peptides, small proteins, and most other compounds: a sat. soln of 2-cyano-3-(4-hydroxyphenyl)acrylic acid in 0.1% TFA in H_2O/MeCN (1:1) is used. This matrix requires about half the laser energy of other matrices.
[a]For proteins and other large molecules: sinapinic acid is substituted for the matrix in the above procedure and sample concentration is increased if necessary.
[a]For less polar compounds or small molecules: a 2,5-dihydroxybenzoic acid sat. soln in H_2O/MeCN (1:1) is used. 2,5-Dihydroxybenzoic acid works well as a matrix because it produces only a minimal amount of interference in the low molecular weight range.
[a]For oligonucleotides: 2,3-dihydropyridine-2-carboxylic acid or 2,4,6-trihydroxyacetophenone matrix in H_2O/MeCN (1:1) is used. These must be combined in equal parts with about 30 mM ammonium citrate to work. Sample concentrations may need to be higher than those used for peptides and laser energy may have to be increased.

7.6.4.3 By Fast Atom Bombardment

The application of FAB to peptide analysis has two general advantages: it typically generates accurate molecular weight information due to the mass analyzers to which it is interfaced, and it offers rapid analysis. Given that FAB is not commonly applied to the analysis of proteins due to its limited mass range, routinely of the order of 5000 to 8000 Da, and relatively poor sensitivity (typically requiring >500 pmol), we will only briefly focus on the sample preparation and analysis procedures for peptides.

FAB peptide preparation is accomplished by dissolving a relatively pure (>70%) peptide sample into a 3-nitrobenzyl alcohol (NBA) matrix. There are many matrices used in FAB analyses, however, 3-nitrobenzyl alcohol has become a standard for FAB peptide analysis (Figure 12). The peaks associated with the NBA matrix can make the spectra more cumbersome to interpret. However, to the experienced mass spectroscopist the matrix ions can act as reference ions and are a useful indicator of accuracy. Rarely are the NBA matrix ions observed above *m/z* 1000.

Figure 12 The FAB Mass Spectrum of a Peptide Analyzed in the NBA Matrix



The sample can be prepared in a cosolvent, such as 5–50% acetic acid in water, acetonitrile/water, chloroform, methanol, and trifluoroacetic acid (0.1 to 25%), at a concentration of approximately 1 to 5 $mg \cdot mL^{-1}$. Sample solubility in this cosolvent is crucial because it acts as a medium between the sample and the matrix. The peptide solution (1–4 μL) is dissolved directly into the matrix on the probe tip (a flat round surface approximately 2–5 mm in diameter). When large amounts of sample (hundreds of micrograms) are available, the peptide can be added directly into the matrix, followed by the addition of a cosolvent such as trifluoroacetic acid (TFA) which, in addition to sample matrix solubility, can also facilitate protonation.

FAB – Sample Preparation; Typical Procedure:
Place 2–5 μL of NBA directly on the probe. Dissolve sample (~5 $\mu g \cdot \mu L^{-1}$) in 0.1% TFA in H_2O/MeCN (1:1). Add 1–4 μL of sample soln to the matrix. Wait ca. 1 min for solvent to evaporate. Acquire spectra.

7.6.5 Sequencing of Peptides and Proteins

Mass spectrometry has developed a symbiotic relationship with other technologies, such as the widely used Edman degradation techniques, in determining protein structure. Having played a central role in the discovery of unique protein modifications, mass determination is also becoming more important in obtaining total sequence information. This section discusses the different methods for obtaining sequence information on peptides and proteins, including tandem mass spectrometry and protein ladder sequencing.

Sequencing with MS/MS

In general, mass spectrometry yields structural information on peptides and proteins by collision-induced dissociation, in which precursor (or parent) ions are subjected to collisions and the *m/z* of the resulting ions is measured. In order to obtain sequence information, fragments of an ion must be produced that reflect structural features of the original compound. Fortunately, most peptides are linear molecules, which allows for relatively straightforward interpretation of the fragmentation data. The process is initiated by converting some of the kinetic energy from the peptide ion into vibrational energy. This is achieved by introducing the selected ion, usually an $(M+H)^+$ or $(M+nH)^{n+}$ ion, into a collision cell where it collides with neutral Ar, Xe, or He molecules. The resulting fragment ions are then mass analyzed. More specifically, this application of tandem mass spectrometry allows for a heterogeneous solution of peptides to be analyzed, and then by filtering the ion of interest into the collision cell, structural information can be derived on each peptide from a complex mixture.

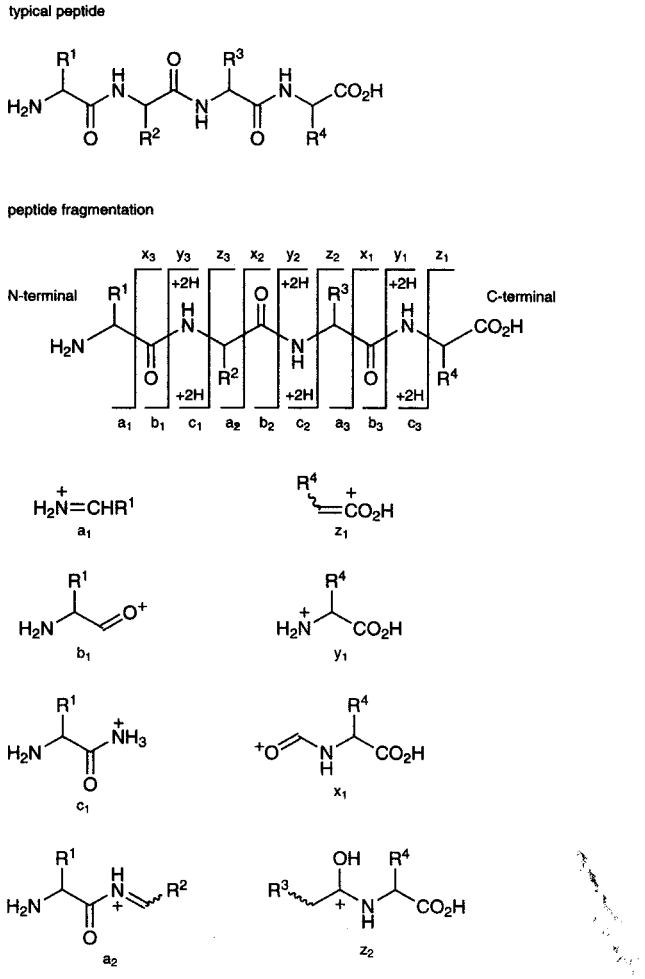
Sequence information can be obtained for peptides with molecular weights up to 2500 Da. Collision-induced dissociation of larger peptides reveal at least partial sequence information that will often suffice to solve a particular problem. The collision-induced dissociation method has been particularly useful on peptides from proteolytic digests, from which MS/MS data on different peptides can help identify the structure of a digested protein.

Collision-induced dissociation can be accomplished with a variety of instruments, including tandem double-focusing magnetic sectors, triple quadrupoles, quadrupole ion traps, and Fourier transform-ion cyclotron resonance. The quadrupole ion trap combined with electrospray is currently the most common means of generating structural data, being capable of high sensitivity, producing a reasonable amount of fragmentation information, and being relatively inexpensive.

From the discussion thus far, it is apparent that fragmentation is a vital step in sequencing peptides. The fragment ions produced in this process can be separated into two classes. One class retains the charge on the N-terminal while cleavage is observed from the C-terminal (Figure 13). This fragmentation can occur at three different positions, each of which is sequence-designated as types a_n, b_n, and c_n. The second class of fragment ions generated from the N-terminal retains the charge on the C-terminal, while cleavage is observed from the N-terminal. Like the first class, this fragmentation can occur at three different positions, types x_n, y_n, and z_n.

Certain limitations for complete sequence information with tandem mass analysis do exist. In determining the amino acid sequence of a peptide, a complete ion series could be used except that neither leucine and isoleucine, nor lysine and glutamine can be differentiated because they have the same mass. As a complete ion series is not usually observed, the information from both the N- and C-terminal ions must be used to help determine the entire sequence.

Figure 13 Peptide Fragmentation Results in the Ions Shown. Collision-Induced Dissociation Spectra Often Result in the Dominant Fragmentation at the Amide Bonds in the Polyamide Backbone, Producing Ions of the Type b or y



It may seem difficult to identify a particular ion series in a spectrum, however there are several rules that can be used to determine the identity of peaks belonging to any ion of the backbone fragmentation (a_n, b_n, c_n, x_n, y_n, and z_n). First, backbone fragmentation can differ in *m/z* by only one of the 18 mass values associated with the amino acids and secondly, fragments a_n, b_n, and y_n generally form a continuous series.

Protein Ladder Sequencing

Protein ladder sequencing is a MALDI-based sequencing approach that consists of a two-step process. The first step is ladder-generating chemistry or enzymology; the controlled generation of sequence-defining peptide fragments, with each fragment differing from the next by one amino acid. The second step is MALDI mass analysis of the resulting protein-sequencing ladder. Each amino acid is identified from the mass difference between successive peaks, and the position in the data set defines the sequence of the original peptide chain. Protein ladder sequencing is currently being developed as a routine alternative to Edman sequencing and has been most useful for C-terminal sequencing when used in conjunction with carboxypeptidase enzymes.

7.6.6 Liquid Chromatography-Mass Spectrometry

Throughout the 1990s, tremendous progress has been made in the analysis of peptides and proteins by liquid chromatography-mass spectrometry (LC-MS). This two-dimensional analytical technique effectively couples the separation performance of liquid chromatography with the state-of-the-art detection, selectivity, and sensitivity of MS. The increased application of this technique for peptide and protein analysis is mostly a result of improvements in column chemistry, rendering a variety of commercially available novel columns and of technological advances achieved in interfacing the two analytical techniques, namely ESI and atmospheric pressure chemical ionization.[13,14] The utilization of ESI and atmospheric pressure chemical ionization as ionization sources has made the analysis of peptides and proteins routine at the low picomole levels with analytical columns, and sub picomole levels via small-bore columns. Mass spectrometry can serve as a general detector at full scan mode, a selective detector by monitoring single ions, and as a means to elucidate structure when utilizing tandem analysis. As a result of the inherent multicharging accompanied by atmospheric pressure ionization-mass spectrometry, the *m/z* charge ratios of large macromolecules such as proteins and proteins are well within the instrumental limits of commercially available quadrupoles platforms.

Chromatographic Conditions

The routine analysis of peptides and proteins using HPLC has been aided by advances in stationary phases particularly suited for the work. Most applications today are carried out via reversed-phase columns [e.g., octasilylated (C8) and octadecylsilylated (C18) materials] that have been deactivated (endcapped) to improve peak shape and overall sample throughput. The popularity of reversed-phase chromatography is primarily a result of its chromatographic characteristics, such as column efficiency and short analysis times, and compatible mobile phases consisting of volatile organic solvents specifically suited to currently available ionization interfaces. Other media typically used for routine analysis include size exclusion, ion exchange, and affinity column phases.

The mobile phase typically consists of an organic solvent such as methanol or acetonitrile diluted with aqueous buffer, normally applied via gradient-pump programming. Buffers are added to aid the chromatography, particularly as a means to increase solute retention and to improve peak shape. The use of volatile buffers that are compatible with LC-MS are also desirable as a means for enhancing atmospheric pressure chemical ionization. They include acetic acid, trifluoroacetic acid, formic acid, ammonium hydroxide, ammonium acetate, and triethylamine. The nonvolatile portion of the buffer must also be ionizable in the mode used (e.g., $H_2PO_4^-$ in the negative mode). Buffer concentrations should be optimized for chromatographic characteristics and detector sensitivity, and should be lower than 10 mM for ESI and 100 mM for atmospheric pressure chemical ionization. The gradient is typically programmed over the range of 0–90% organic phase, with the run time proportional to number of constituents in the sample matrix.

7.6.6.1 Liquid Chromatography-Electrospray Ionization-Mass Spectrometry

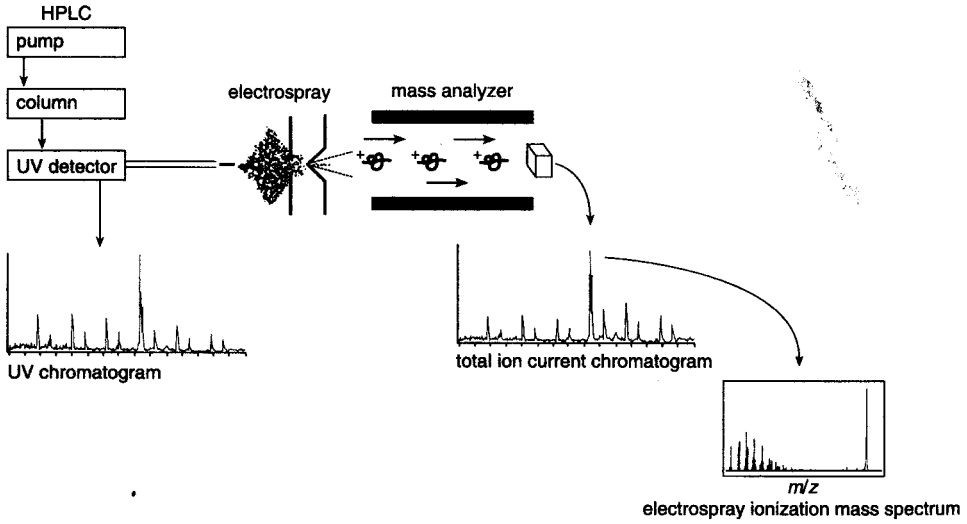
In order for mass spectrometry to be used as an effective tool in LC, the analyte must be ionized in the interface region (source) since MS can only detect charged species. Consequently, the role of the ionization source is twofold: (1) it is responsible for interfacing the chromatographic instrument with the detector and (2) it provides an effective and efficient means for sample ionization. It should be pointed out that source ionization efficiency plays a significant role in sample sensitivity for LC-MS analysis. The application of LC is ideally

for references see p 695

suited for the separation of nonvolatile and thermally labile compounds such as peptides and proteins. As a result, the ionization method should be efficient for the same compounds. Desorption ionization methods MALDI and FAB, while useful for peptides and proteins, are not suitable as a LC interface. However, since ESI and atmospheric pressure chemical ionization generate ions directly from a solution phase such as acetonitrile/water, they are the most successful LC interfaces used today.

Since the early 1990s, electrospray has been utilized routinely as a means for sample introduction in the field of mass spectrometry. An advantage of ESI is that ions are formed directly from solution which is usually an aqueous or aqueous/organic solvent system, a feature that has established the technique as a convenient mass detector for LC. While in the past, attempts to couple liquid chromatography with mass spectrometry resulted in limited success, ESI has made LC-MS routine. ESI also allows for MS analysis at relatively high LC flow rates ($1.0\,mL \cdot min^{-1}$) and high mass accuracy (±0.01%), adding a new dimension to the capabilities of LC characterization. The use of ESI as a detector for LC was one of its first obvious applications. Figure 14 illustrates how mass spectrometry is interfaced to liquid chromatography by ESI. Most instrument configurations utilize narrow-bore (ca. 0.005 inches) PEEK tubing to transfer HPLC effluent to the MS. For chromatographic methods that require high flow rates, such as rapid analysis of random libraries of peptides, a flow splitter is positioned between the LC and MS to divert a portion of the effluent directly to waste. This ESI ionization source is specifically tailored to molecules that are capable of supporting multiple charges, such as peptides and proteins. In addition, samples also compatible with the source include, but are not limited to, ions such as quaternary amines and sulfate conjugates, and heterocyclic compounds such as benzodiazepines. Nonpolar analytes are not very compatible with ESI and are more likely to ionize in atmospheric pressure chemical ionization sources.

Figure 14 Interfacing Liquid Chromatography with Electrospray Ionization Mass Spectrometry



Sample Preparation and Procedures for LC-MS; Typical Procedure:
Configure the LC instrument with a 5-μL injection loop (or autosampler); a precolumn (for endogenous samples); a column (ca. 2.1×30 mm, 3.5 μm particle size C18 column); two solvent reservoirs, Reservoir A: 0.05% TFA in H_2O and Reservoir B: 95% MeCN and 5% a soln of 0.05% TFA in H_2O; a mobile phase gradient (e.g., 5% A for 3 min, increase to 90% A over 8 min, hold at 90% A for 3 min, at $0.5\,mL \cdot min^{-1}$ flow rate); and an MS inlet flow appropriate for the mass spectrometer. Dissolve sample (preferably $1\,pmol \cdot \mu L^{-1}$) in 20% MeCN with 0.05% TFA. Inject 10 μL of sample into LC-MS and aquire the spectra.

7.6.6.2 Liquid Chromatography-Atmospheric Pressure-Chemical Ionization-Mass Spectrometry

The application of atmospheric pressure-chemical ionization has gained wide acceptance in bioanalysis. Similar to electrospray, the liquid effluent of atmospheric pressure chemical ionization is introduced directly into the ionization source. However, the similarity stops there; the atmospheric pressure chemical ionization source contains a heated vaporizer which facilitates rapid desolvation/vaporization of the droplets. Vaporized sample molecules are carried through an ion-molecule reaction region at atmospheric pressure. The ionization occurs through a corona discharge creating reagent ions from the solvent vapor. Chemical ionization of sample molecules is very efficient at atmospheric pressure due to the high collision frequency. Proton transfer (for protonation MH^+ reactions) occur in the positive mode, and either electron transfer or proton transfer (proton loss, $[M-H]^-$ in the negative mode). The moderating influence of the solvent clusters on the reagent ions, and of the high gas pressure, reduces fragmentation during ionization and results in primarily molecular ions. This ionization technique is more useful for a wider variety of samples (polar to nonpolar) than the ESI method.

7.6.6.3 Liquid Chromatography-Tandem Mass Spectrometry

Since LC-MS yields only one kind of ion (normally $[M+H]^+$), experiments with LC-MS/MS in the ion source must be carried out if increased detector specificity or structural information is needed. Furthermore, it is always possible in LC-MS to observe co-eluting compounds superimposed in the mass spectrum, making quantification and interpretation difficult. The technique of LC-MS/MS therefore offers distinct advantages over LC-MS by improving detection selectivity and sensitivity and by generating and acquiring ion fragmentation information to be used toward structural identification. For improved detector selectivity and sensitivity for peptides and proteins, typical protocols call for monitoring the parent ion (e.g., $[MH]^+$) signal simultaneously with a known ion fragment signal. This procedure significantly reduces the possibility of observing interference peaks present in complex mixtures.

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