

Mass Spectrometry

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ABSTRACT

Background

Mass spectrometry is a powerful analytical technique used to identify and quantify analytes using the mass-to-charge ratio (m/z) of ions generated from a sample. It is useful for the analysis of a wide range of clinically relevant analytes, including small molecules, proteins, and peptides. When mass spectrometry is coupled with either gas or liquid chromatographs, the resultant analyzers have expanded analytical capabilities with widespread clinical applications, including quantitation of analytes from myriad body tissues and fluids. In addition, because of its ability to identify and quantify proteins, mass spectrometry is widely used in the field of proteomics.

Content

This chapter describes the basic concepts and definitions of mass spectrometry. Techniques based on mass spectrometry require an ionization step wherein an ion is produced from neutral atoms or molecules. Electron impact and chemical

ionization (CI) are often used in gas chromatography–mass spectrometry. In liquid chromatography–mass spectrometry, electrospray ionization (ESI) and atmospheric pressure CI are the most commonly used techniques. In microbiology, a desorption/ionization technique termed MALDI (matrix-assisted laser desorption ionization) is employed. Each of these ionization techniques is described in detail, and advantages of the techniques are highlighted. Once molecules are ionized, resultant ions are analyzed using either beam type analyzers (eg, quadrupole, or time-of-flight [TOF]) or trapping mass analyzers (eg, ion trap). Mass analyzers also can be combined to form tandem mass spectrometers, which allow further expanding capabilities of the technique. Clinical applications of mass spectrometry are provided to illustrate the role of this technique in the analysis of clinically relevant analytes.

Mass spectrometry (MS) is a powerful qualitative and quantitative analytical technique that is used to identify and quantify a wide range of clinically relevant analytes. When coupled with gas or liquid chromatographs, mass spectrometers allow expansion of analytical capabilities to a variety of clinical applications. In addition, because of its ability to identify and quantify proteins, MS is a key analytical tool in the field of proteomics.

We begin this chapter with a discussion of the basic concepts and definitions of MS, followed by discussions of MS instrumentation and clinical applications, and we end the chapter with a discussion of logistic, operational, and quality issues. In this chapter it is impossible to cover all concepts in a field as vast as MS, even if focus is limited to clinical applications. The Clinical and Laboratory Standards Institute (CLSI) has published recommendations on clinical

MS that can serve as a good next step to study this topic and another gateway into the extensive literature on this topic.^{1,2}

BASIC CONCEPTS AND DEFINITIONS

MS is the branch of physical chemistry (often also considered a branch of analytical chemistry) that deals with all aspects of instrumentation and the applications of this technique. *Molecular mass* (sometimes referred to as *molecular weight*) is measured in *unified atomic mass units* (u), also known as the *dalton* (Da), equal to 1/12 of the atomic mass of the most abundant isotope of a carbon atom in its lowest energy state, defined as 12 Da. Although the term *atomic mass unit* (amu) has been regarded as equivalent to the Da, it is only approximately equal to the dalton and now is considered an obsolete unit; its use to refer to the dalton is strongly discouraged.

Most MS data are presented in units of mass-to-charge ratio, or m/z , where m is the molecular weight of the ion (in daltons) and z is the number of charges present on the measured molecule. For small molecules (<1000 Da) there is typically only a single charge and therefore the m/z value is the same as the mass of the molecular ion. However, when larger molecules such as proteins or peptides are

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measured, they typically carry multiple ionic charges and therefore the z value is an integer greater than 1. In these cases the m/z value will be a fraction of the mass of the ion.

All MS techniques require an initial *ionization* step in which an ion is produced from a neutral atom or molecule. Ions are formed in the ion source of the mass spectrometer. The development of versatile ionization techniques has allowed MS to become the excellent broad-spectrum analytical technique it is today; this was highlighted when, in 2002, John Fenn and Koichi Tanaka shared the Nobel Prize for their development of electrospray^{3,4} and laser desorption⁵⁻⁷ ionization, respectively. In the ion sources most commonly used with MS instruments in clinical chemistry, ionization in positive ion mode results from the addition of one (or more) protons to the basic sites on the molecule. This is referred to as protonation and leads to formation of a positively charged ion. The mass of the ion is greater than the mass of the uncharged neutral molecule by the added mass of one proton, approximately 1 Da, or multiples of a single proton mass (in case of multiply charged ions). Negatively charged ions (negative ion mode of MS operation) can be generated by the loss of a proton or addition of a negatively charged moiety (such as a hydroxyl group).

Ions may also be produced by removal of one or more electrons from a molecule using electron ionization (EI). This ionization method is historically the dominant ionization method used in MS (most commonly in gas chromatography–mass spectrometry [GC-MS] instruments) and is still used in some applications, but other ionization methods are now more frequently used in clinical laboratories. The removal of one electron produces a positively charged ion and reduces the mass by approximately 5×10^{-4} Da relative to the neutral molecule from which the ion is produced. This relatively small mass shift is often considered to be negligible and therefore ignored. More rarely, ions may be produced by addition of one electron to a molecule, producing a negative ion with mass approximately 5×10^{-4} Da greater than the neutral molecule from which the ion is produced. This small mass increment is also often considered to be negligible relative to the neutral molecule.

Ions formed in the ion source are separated according to m/z values in a mass analyzer. The term *mass analyzer* is in common use, although more correctly it would be termed an *m/z analyzer* given the fact that mass spectrometers separate ions according to m/z , not mass. This chapter will use the terms *mass analyzer* and *m/z analyzer* interchangeably.

While in a mass analyzer, ions may undergo *fragmentation*, whereby energy is imparted into the ionized analyte, causing internal bonds to break and resulting in the production of multiple independent unconnected chemical species. *Fragmentation can occur within different regions of the mass spectrometer and can occur due to the deliberate action of the operator or excessive energy imparted into the parent molecule as it is being ionized or passes through the vacuum region of the mass analyzer.* An unfragmented ion of the intact molecule is referred to as the *molecular ion*, whereas the species that occur on fragmentation of the molecular ion are called the *fragment ions*. *There is a certain ambiguity in the term molecular ion because in many cases the structure of the ion is not identical to the structure of the original neutral molecule (eg, differing by the addition or removal of one or more protons, so the term molecular ion must be thought of in terms of an unfragmented ion whose structure is closely related to, but not necessarily identical to, the original uncharged molecule.*

If the ionization of the analyte in the source produces little fragmentation, it is referred to as being *soft*, and the most abundant peak in the mass spectrum (the *base peak*) is often the molecular ion. If the ion source produces extensive fragmentation it is referred to as *hard* ionization, and the base peak in the resulting spectra may be one of the fragment ions. By convention, the base peak in a mass spectrum is assigned a relative abundance value of 100%.

Fragment ions that are formed in a separate dissociation cell (also known as the collision cell) inside a *tandem mass spectrometer* are known as *product ions*, and the technique is called *tandem mass spectrometry (MS/MS)*. Ions that give rise to product ions are known as precursor ions. A tandem mass spectrometer consists of two mass spectrometers operated in sequence (MS/MS in space) or a single mass spectrometer capable of sequential fragmentation and measurement of ions within a single region of space (MS/MS in time). Most commonly in the clinical diagnostic methods, precursor ions are dissociated into product ions between the two stages of m/z analysis (MS/MS in space).

A *mass spectrum* is represented by the relative abundance of each ion plotted as a function of m/z (Fig. 2.1). As mentioned earlier, for small molecules, usually each ion has

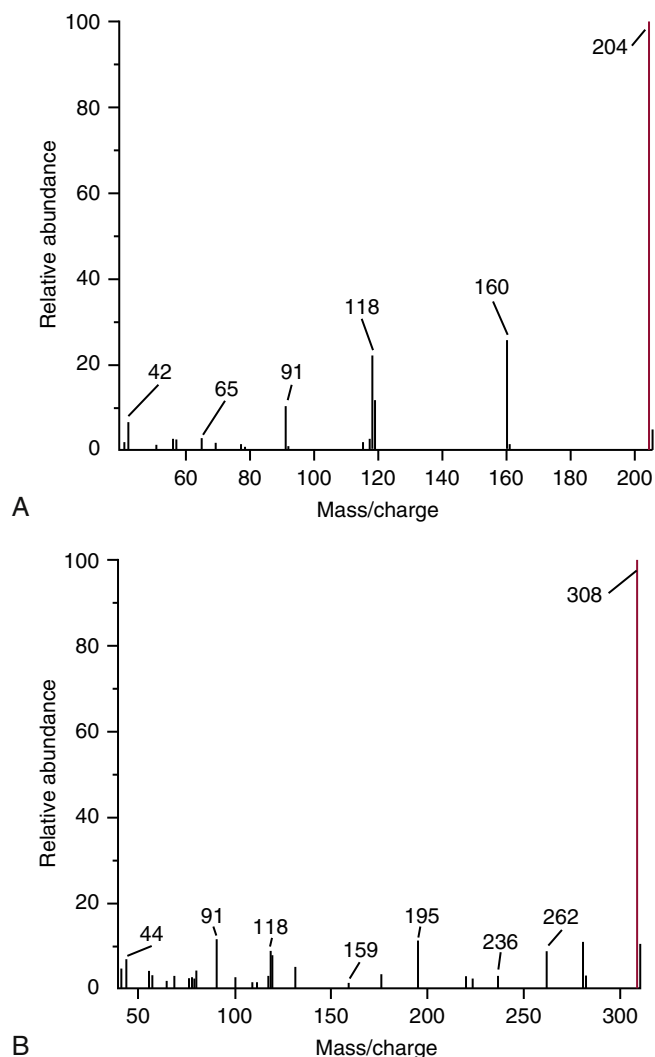


FIGURE 2.1 Mass spectrum of the pentafluoropropionyl (A) and carbethoxyhexafluorobutryl (B) derivatives of D -methamphetamine.

a single charge ($z = 1$); thus the m/z ratio is equal to the mass of the ion and is approximately 1 Da greater than the neutral molecule from which the ion is formed. However, in some cases, the charge may be represented by an integer number greater than 1, in which case the m/z ratio is not equal to the mass of the ion, but rather is some fraction of the mass of the ion.

An ion may be positively charged, in which case the number of electrons in the ion is less than the sum of the number of protons in all nuclei of the ion, or negatively charged, in which case the number of electrons is greater than the number of protons. By convention, in MS z is taken as an absolute value. For example, $z = 1$ for both Na^+ and Cl^- .

Chemical interferences as well as higher background noise are more common for analytes with m/z 200 to 500 than for m/z less than 200 and m/z greater than 500. Monitoring ions with higher m/z often results in lower limits of detection because of the lower background noise and lower occurrence of isomers and isobars of the targeted molecules (ie, superior signal to noise).

A peak in a mass spectrum can be characterized by its resolution $[(m/z)/(\Delta m/z)]$, where $\Delta m/z$ is the width of the mass spectral peak. This parameter characterizes the ability of a mass spectrometer to separate nearby masses from each other. Typically the width of the peak is measured at 50% of the height of the peak and is referred to as the full width half height (FWHH) or full width half maximum (FWHM) resolution. A second frequently encountered definition for resolution is 10% valley. It defines $\Delta m/z$ as the distance between two peaks of equal intensity, spaced so that the valley between the peaks is 10% of the peak height (Fig. 2.2). This is a more conservative definition than FWHM because for a given quoted resolution (eg, 2000) the peaks are narrower under the 10% valley definition, hence better separated. High resolution is a desirable property in MS because it can help reduce interferences from nearby peaks in the mass spectrum, thereby allowing it to achieve a higher specificity.

By setting the relative abundance of the base peak to 100% and therefore using the relative, rather than absolute, abundance of each ion fragment, instrument-dependent variability is minimized and the mass spectrum can be compared with mass spectra obtained on other instruments. Because fragmentation at specific bonds depends on their chemical nature and strength of the bonds, the mass spectrum can be interpreted in terms of the molecular

structure of the analyte. In some cases, the chemical structure of the analyte can be deduced or at least reconciled with features found in the mass spectrum. Computer-based libraries of mass spectra are also available to assist in identification of the analyte(s) based on fragmentation pattern. In some applications, the mass spectrum of an analyte may be matched against mass spectra in a database, thereby identifying the analyte by its mass spectral *fingerprint*. In general, an unknown is considered to be identified if the relative abundances of three or four ion fragments agree within $\pm 20\%$ of those from a reference compound and the relative abundances of the fragments, monoisotopic and isotopic ions of the molecular ion are in agreement with the relative abundances of the reference mass spectrum.

When interfaced to a liquid or gas chromatograph, the mass spectrometer functions as a powerful detector, providing structural information in real time on individual analytes as they elute from a chromatographic column. Depending on the operating characteristics of the mass spectrometer and the chromatographic peak width, multiple mass spectral scans can be acquired across the peak. The data also can be displayed as a function of time to yield a *total ion chromatogram* in which at each time point in the chromatogram the abundances of all ions in a mass spectrum are summed to constitute a single point in the ion chromatogram, regardless of m/z .

The mass spectrometer can be considered to be close to a universal detector because molecules of many identities may be ionized and then detected in a mass spectrometer. Furthermore there are different MS operation modes and different types of fragmentation that can be applied to provide different types of data, giving more information about the measured compound(s). Finally, the instrument data system can analyze and display the collected data in various manners, allowing the operator to selectively extract information from the acquired data.

For example, it is possible to display chromatograms of only preselected ions acquired during data acquisition—that is, representing data from only part of the mass spectrum. The resultant display of data is called an *extracted ion chromatogram*, displaying signal intensity plotted as a function of time; peak heights or peak areas can be integrated for use in quantitative analysis. Use of the extracted ion chromatogram allows selecting data corresponding to the analyte of interest, as identified by its m/z , while disregarding data corresponding to different m/z . With high-resolution instruments, specificity of analysis can be improved by use of narrow m/z windows for plotting extracted ion chromatograms. Such data processing results in a reduced number of overlapping chromatographic peaks from ions of nearby m/z thus improving the quantitative accuracy and the specificity (Fig. 2.3).

Sample preparation is critical to successful MS, particularly when dealing with complex matrices, such as are commonly encountered in clinical chemistry. This typically involves one or more of the following steps: (1) protein precipitation followed by centrifugation or filtration, (2) solid-phase extraction, (3) liquid-liquid extraction, (4) affinity enrichment, or (5) *derivatization* (see Chapter 3).

Derivatization is the process of chemically modifying the target compound(s) to be more favorably analyzed by MS. Derivatization usually involves the addition of some well-defined functional group. The goals of derivatization vary, depending on the application, but typically include (1) increased volatility, (2) greater thermal stability, (3) modified chromatographic properties, (4) greater ionization efficiency, (5) favorable fragmentation properties, or a combination of these.

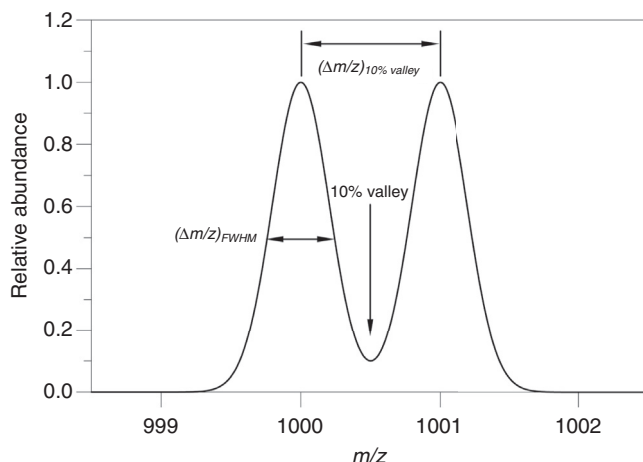


FIGURE 2.2 Parameters used to define resolution in mass spectrometry. FWHM, Full width half maximum.

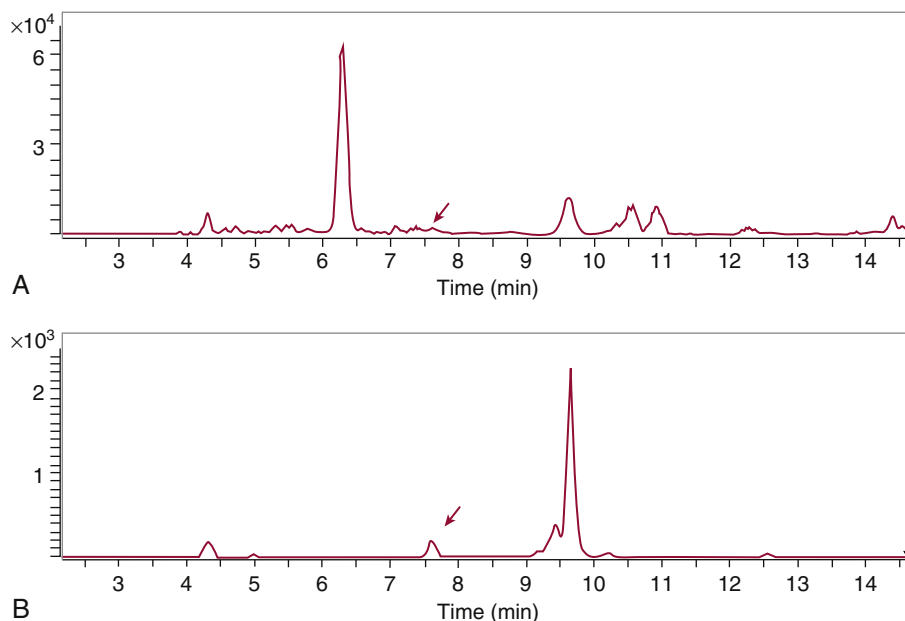


FIGURE 2.3 Extracted ion chromatograms for a peptide ion of m/z 761.3718 using a window of 1 Da (A) and 0.0076 Da (B). Arrows point on peak that is completely hidden in the chemical background noise while resolved from the noise using mass extraction window of 0.0076 Da.

Analysis by MS can be used to target specific known compounds (targeted analysis) or seek to identify one or more unknown compounds in a sample (screening). When only one or a few targeted analytes are of interest for quantitative analysis and their mass spectrum is known, the mass spectrometer is set to monitor only those ions of interest. This selective detection technique is known as *selected ion monitoring* (SIM). Because SIM focuses on a limited number of ions, more data points are collected for the selected m/z , which results in better, more precise measurements. The SIM data acquisition increases the signal-to-noise ratio for the analyte of interest, improves the lower limit of detection, and enables more accurate quantitation. One drawback of SIM is that it is based around measurement of a nominal analyte mass. Most biological samples are highly complex, and thus it is not unexpected to find multiple compounds with very close or identical masses in the matrix. In those cases, chromatography can aid in separation of these isobars; however, they still can affect a SIM result should the isobar not be separated completely from the analyte to be measured. By using a triple-quadrupole mass analyzer, a method known as selected reaction monitoring (SRM) (or generalized for the analysis of many ions at the same time, multiple reaction monitoring [MRM]) can be used to help alleviate such potential issues. This is where the first quadrupole instrument is set to transmit the m/z of the molecular ion, the analyte is caused to fragment in the second quadrupole and the third quadrupole is set to transmit the m/z of one or more known fragment ions from the analyte. In this manner data similar to those gathered by SIM can be produced but with added specificity from the structural information gathered by the use of the fragment ion as a gatekeeper. A more detailed description of MRM is given in the section of this chapter on tandem mass spectrometers.

Screening methods (used here in the analytical chemistry sense, not to be confused with screening in a clinical or medical sense) are less common in clinical chemistry than the analysis of target compounds; the main task for screening methods is qualitative identification of unknowns in a sample.

In most cases this reduces to the problem of matching chromatographic retention time and fragment ion patterns, that is, mass and abundance patterns of either fragment ions generated in the source of a single-stage mass spectrometer or product ions from a collision cell in a tandem mass spectrometer.

A chemical element may be composed of a single or multiple isotopes. Each isotope of an element has the same number of protons in its nucleus but different numbers of neutrons. For example, naturally occurring carbon is composed primarily of two isotopes: ^{12}C , whose nuclei contain six protons and six neutrons, and ^{13}C , whose nuclei contain six protons and seven neutrons. (Here we ignore ^{14}C , which is generally of negligible abundance compared to the other two isotopes.) The natural abundance of ^{12}C is approximately 98.9%, and the natural abundance of ^{13}C is approximately 1.1%. Some elements, such as arsenic, have only a single isotope in the naturally occurring state, whereas other elements, such as tin, may have as many as 10 naturally occurring isotopes.

When a compound is made of multiple atoms the isotope pattern is a convolution of the isotope patterns of the individual atoms. To illustrate with a simple example, carbon monoxide (CO) has the following combinations of isotopes $^{12}\text{C}^{16}\text{O}$ (molecular weight 28), $^{13}\text{C}^{16}\text{O}$ (molecular weight 29), $^{12}\text{C}^{17}\text{O}$ (molecular weight 29), $^{12}\text{C}^{18}\text{O}$ (molecular weight 30), and $^{13}\text{C}^{18}\text{O}$ (molecular weight 31).

Nitrogen (N_2) is isobaric with CO; that is, it has nearly the same mass. However, the accurate masses of the isotope peaks of isobars may differ. For example, the monoisotopic mass of $^{12}\text{C}^{16}\text{O}^+$ (the isotopic peak composed of the most abundant atomic isotopes) has an accurate mass of 27.9944 Da, whereas the isobaric mass 28 Da peak of N_2^+ has an accurate molecular mass of 28.0056 Da. The small difference in masses of isobars can be used to infer the chemical formula of a compound or to confirm the identity of a target compound. This technique requires a mass analyzer capable of mass accuracy of a few parts per million, is limited to compounds of a few hundred Da or less, and it is not capable of discriminating between isobars of the same chemical formula.

Compounds that have the same chemical formulas but different chemical structures are also isobars and might therefore be referred to as strict isobars because they have exactly the same mass. Succinic acid and methylmalonic acid provide an example of a pair of compounds that are strict isobars because they have the same chemical formula ($C_4H_6O_4$). Unlike isobars that have nearly the same masses but different accurate masses, strict isobars cannot be separated or distinguished by MS alone, although they often can be separated if MS is combined with a separation method or if tandem MS is applied.

Isotopic information also can be used to identify a compound in a different way. Using CO^+ and N_2^+ as examples, the first three isotopic peaks of CO^+ have a relative abundance pattern of 0.986, 0.011, and 0.002, whereas the first three isotope peaks of N_2^+ has a pattern of 0.993, 0.007, and 0.000. Differences in the isotopic pattern can be used to infer the chemical formula of an unknown or to confirm chemical identity of a target compound. This technique requires accurate measurement of relative isotopic peak abundances and is sometimes used in conjunction with accurate mass measurements, particularly when using TOF mass spectrometers.

A distinct advantage of the mass spectrometer is that it can distinguish between ions of the same chemical formula that have different masses because of the different isotopic composition. To illustrate with a trivial and simple example, $^{12}C^{16}O^+$ has a different mass than $^{12}C^{18}O^+$, and these two forms can be separated and detected in a mass spectrometer. One can take advantage of this fact by using artificially labeled forms of a target analyte. The labeling consists of substitution of a less common isotope for one or more of the atoms in the analyte, for example, substituting 2H for 1H , ^{13}C for ^{12}C , or ^{15}N for ^{14}N . The substituted molecule is prepared artificially and added to the sample as an internal standard, which behaves nearly identically to the native compounds during sample preparation and chromatographic separation. In this respect, ^{13}C or ^{15}N is generally preferred over 2H labeling because 2H -labeled compounds sometimes exhibit chromatographic shifts compared to unlabeled compounds, whereas ^{13}C - or ^{15}N -labeled compounds generally do not. A quantitative analysis can then be carried out by comparison of the signal from

the native compound versus the artificially added labeled version of the compound spiked into the sample.

An internal standard should be selected to have a sufficient number of isotopic ions so that no naturally occurring isotopes (such as 2H or ^{13}C) of the analyte of interest would significantly contribute to the signal of the internal standard. For the methamphetamine derivatives shown in Figure 2.4, A, an internal standard with at least three 2H or ^{13}C atoms is preferred, because contribution of the natural abundance of these isotopes to the molecular ion $[(M + 3)^+]$ would be $\approx 0.1\%$. The position of the stable isotope atoms within the molecule and the number of isotopic ions within the structure is also important for adequate performance of the methods.⁸ For example, the m/z 204 ion for methamphetamine represents the aliphatic portion of the molecule (loss of the aromatic ring). If three deuterium atoms were located on the aromatic ring of the pentafluoropropionyl derivative of methamphetamine, the native and the isotope-labeled molecules would both yield the m/z 204 ion. This m/z 204 ion would therefore fail to distinguish the native compound from the isotope-labeled compound and would therefore not be useful as an internal standard. On the other hand, if 2H labeling were to occur in the aliphatic portion of the molecule, the fragment ion analogous to the m/z 204 fragment ion would be labeled, producing a higher m/z than 204 (eg, 207), and the ion could be useful as an internal standard. The same comments apply to the compound illustrated in Figure 2.4, B.

These concepts of internal standard selection must be modified slightly when applied to tandem MS. For example, it is possible for a native compound and the internal standard to have product ions of the same m/z , because they can be distinguished by their differing precursor ion masses.

When using hydrogen (2H) labeling the stable isotope must be located on atoms from which it will not be exchangeable with hydrogen atoms in solution or in gas phase (in the ion source). For example, deuterium labeling of an acidic hydrogen position would be useless because the 2H would easily exchange with protons in the matrix, making the original labeling moot. Certain other labeling positions also must be avoided. The hydrogen atoms in the constituent

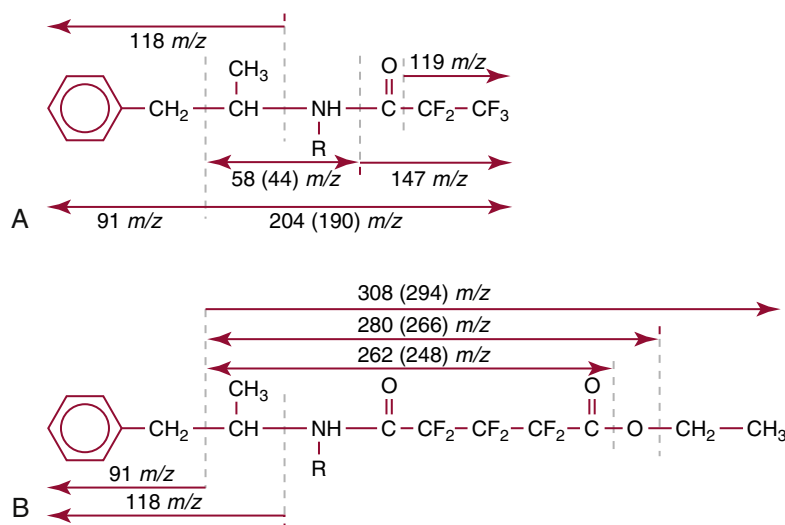


FIGURE 2.4 Fragmentation patterns for the pentafluoropropionyl (A) and carbethoxyhexafluorobutyryl (B) derivatives of methamphetamine ($R = CH_3$) and amphetamine ($R = H$; masses in parentheses). Compare the predicted masses with the spectrum shown in Figure 2.1. Note that for the pentafluoropropionyl derivative, only one ion [204 (190) m/z] is characteristic of the aliphatic portion of the molecule.

groups of alcohols, amines, amides, and thiols all may readily exchange with hydrogen ions in an aqueous matrix.

A technique of quantitative analysis of compounds relative to their isotopic analogs added to the samples at known or fixed concentration is called *isotope dilution analysis* or isotope dilution mass spectrometry (IDMS). The IDMS technique has been used to develop definitive methods for a number of clinically relevant analytes, including drugs of abuse and disease markers.

MS is often referred to as a highly sensitive technique. *Sensitivity* is a somewhat problematic term because it is used in two different ways. In an official definition^{9,10} it means the slope of a calibration curve (or more generally, a change in signal vs the change in concentration), but far more commonly it is used to signify the ability to detect or quantify an analyte at very low concentration; that is, a highly sensitive technique would be able to detect or quantify a very low concentration of the target analyte.

INSTRUMENTATION

A mass spectrometer consists of (1) an ion source, (2) a vacuum system, (3) a mass analyzer, (4) a detector, and (5) a computer (Fig. 2.5).

Ion Source

Many approaches have been used to form ions in both high-vacuum and near-atmospheric pressure conditions. EI and CI are ionization techniques used when gas-phase molecules are introduced directly into an ion source operated at very low pressure, often from a gas chromatograph. In other analyses, such as high-performance liquid chromatography–mass spectrometry (HPLC-MS), ESI, *atmospheric pressure chemical ionization* (APCI), and *atmospheric pressure photoionization* (APPI) ion sources are often used.^{11–14} Ionization in these

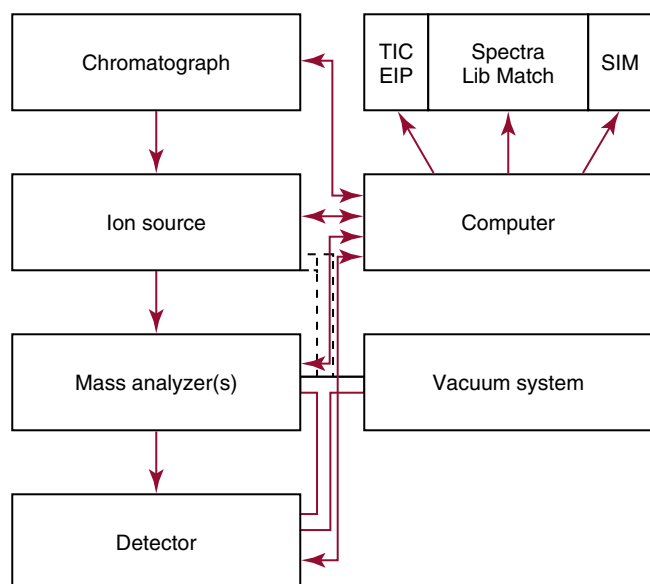
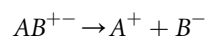


FIGURE 2.5 Block diagram of the components of a chromatograph–mass spectrometer system. The mass analyzer and the detector are always under vacuum. The ion source may be under vacuum or under near-atmospheric pressure conditions, depending on the ionization mode. The computer system is an integral part of data acquisition and output. *EIP*, Extracted ion profile; *SIM*, selected ion monitoring; *TIC*, total ion current.

three ion sources takes place at atmospheric pressure. Other ionization techniques include (1) *inductively coupled plasma* (ICP), (2) MALDI (see Chapter 4), (3) *atmospheric pressure matrix-assisted laser desorption ionization*, and others. This chapter will limit its discussion primarily to ion sources of interest to clinical applications of MS. The CLSI documents C-50A and C-62A contain recommendations for matching the capabilities of different ion source technologies to various application classes.^{1,2}

Electron Ionization

In EI, gas-phase molecules are bombarded by electrons emitted from a heated filament and attracted to a collector electrode (Fig. 2.6). To prevent filament oxidation, as well as to minimize scattering of the electron beam, this process must occur in a vacuum. EI is typically performed using electrons with a kinetic energy of 70 eV; collision of electrons having such energy with most organic molecules results in formation of *radical* cations, that is, a structure that is both a positively charged ion and a radical.¹⁵ A radical is a molecule or ion with an unpaired electron. The radical ion then often undergoes unimolecular rearrangement and dissociation to produce a cation and an uncharged radical:



Positive ions are drawn out of the ionization chamber by an electrical field. The cations are then electrostatically focused and introduced into the mass analyzer. EI is primarily used as an ion source in GC-MS. Because the same ion energy (70 eV) is used in commercial instruments using EI, and because the fragmentation pattern is only weakly dependent on small deviations from 70 eV, fragmentation patterns observed using an EI source are reasonably reproducible among the GC-MS instruments. The fragmentation pattern

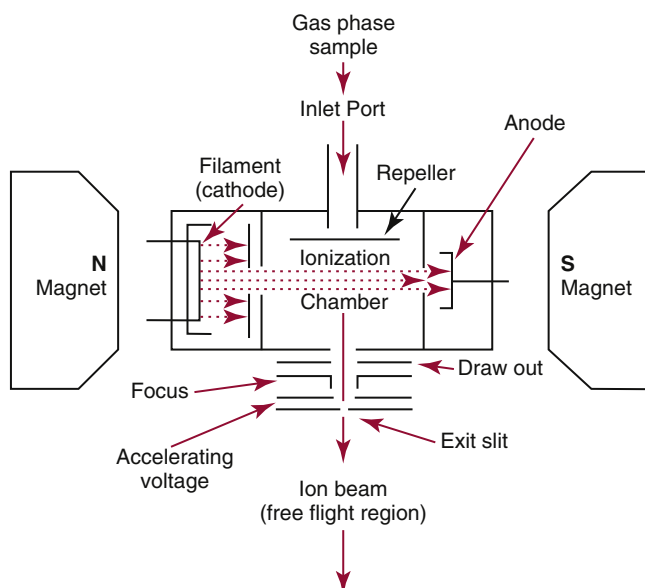


FIGURE 2.6 Electron impact ion source. The small magnets are used to collimate a dense electron beam, which is drawn from a heated filament placed at a negative potential. The electron beam is positioned in front of a repeller, which is at a slightly positive potential compared with the ion source. The repeller sends any positively charged fragment ions toward the opening at the front of the ion source. The accelerating plates strongly attract the positively charged fragment ions.

is therefore often used as a fingerprint to identify compounds by matching mass spectra of unknowns to the entries in the mass spectral libraries.

Chemical Ionization

CI is a soft ionization technique in which a proton is transferred to, or abstracted from, a gas-phase molecule by a reagent gas molecule such as methane, ammonia (NH_3), isobutane, or water. The reagent gas is supplied into a CI ion source at a pressure of about 0.1 torr. (Note: For virtually all practical purposes, torr is equivalent to millimeters of mercury and is the more customary term used in the field of MS). An electron beam produces reactive species through a series of ion-molecule reactions, intermediate species (such as methonium $[\text{CH}_5^+]$ if methane is the CI reagent gas); further ion-molecule reactions can cause analyte ions to become charged, usually via attachment of a proton. In most cases, relatively little fragmentation occurs, and for the majority of the molecules, only molecular ions (in the form of a protonated version of the neutral molecule) are observed in the mass spectra; the lack of fragmentation enhances sensitivity of detection. Negative ion electron capture CI has become popular for quantification of drugs such as benzodiazepines. Negative ions are formed when thermalized electrons are captured by electronegative functional groups, such as chlorine or fluorine atoms within structure of the molecule. Negative ion CI often leads to very low limits of detection.

Electrospray Ionization

ESI is a soft ionization technique in which a sample is ionized at atmospheric pressure before introduction into the mass analyzer.^{16,17} An effluent from a separation device, typically an HPLC, is passed through a narrow metal or fused silica capillary to which a 1- to 5-kV voltage has been applied (Fig. 2.7, A). The partial charge separation between the liquid

and the capillary results in instability in the liquid that in turn results in expulsion of charged droplets from a Taylor cone, which forms at the tip of the capillary (Fig. 2.8). In many variations of ESI a coaxial nebulizing gas aids in nebulization and helps direct the charged droplets toward a counter-electrode as well as speeding up the evaporative process. As droplets evaporate while migrating through the atmospheric pressure region, they expel smaller droplets as the charge-to-volume ratio exceeds the Raleigh instability limit. The adducts of the molecules (with solvent molecules, NH_3 , etc.) are desolvated to form bare ions that are typically formed in the ionization process. However, other ionization products are sometimes observed, such as metal ion adducts, or ions formed by redox processes. Ions then pass through a sampling cone and one or more extraction cones (skimmers) before entering the high-vacuum region of the mass analyzer.

One feature of ESI is the production of multiple charged ions, particularly from peptides and proteins. It is common to observe approximately one charge for every 10 to 15 amino acid residues in a protein. For example, for a molecule of mass 20,000, 20 charges supplied by the addition of 20 protons would be detected at m/z approximately 1000 [or more correctly, m/z $1001 = (20,000 + 20)/20$]. This phenomenon greatly extends the accessible mass range of an instrument. It is frequently observed that a distribution of charges occurs; in cases of multiple charged molecules, one usually observes a series of peaks, with each peak corresponding to a different number of charges. Multiply charged ions are also observed for nucleic acid polymers, particularly when ESI mass spectra are acquired in negative ion mode.

It should be noted that Figure 2.7 is a simplified illustration of the probe being directed toward the sampling cone of the mass detector. To enhance performance and minimize contamination of the mass analyzer, modern hardware configurations have offset the probe and/or the

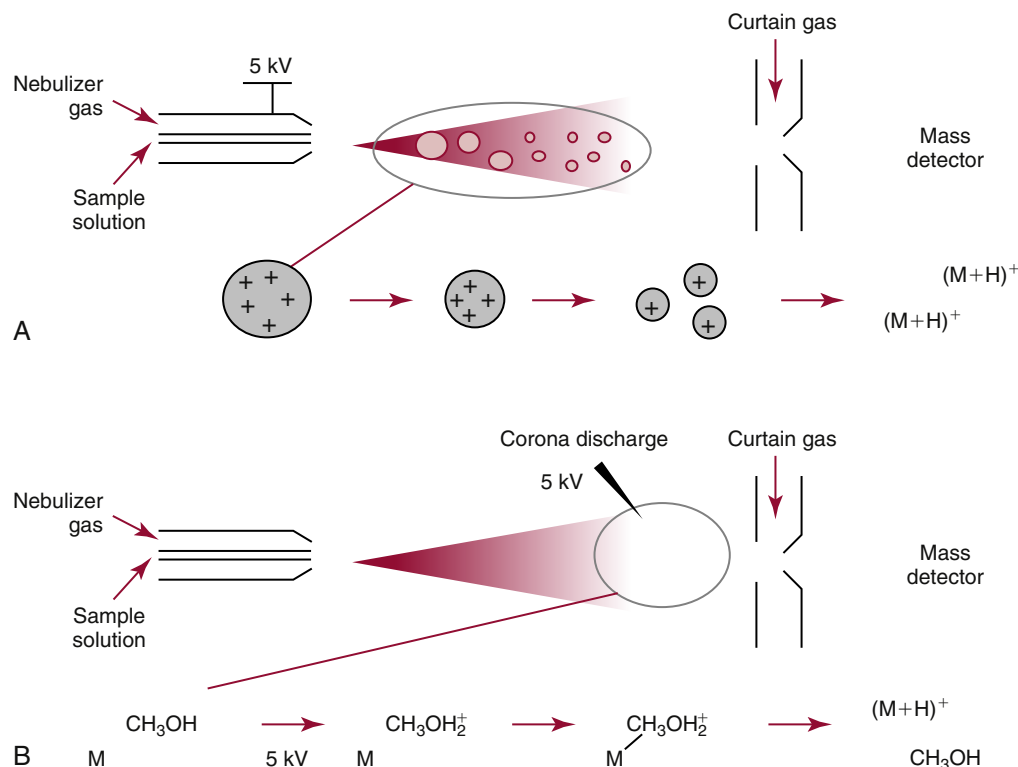


FIGURE 2.7 Schematics of (A) electrospray and (B) atmospheric pressure chemical ionization sources. Note the different points where ionization occurs, as described in the text.

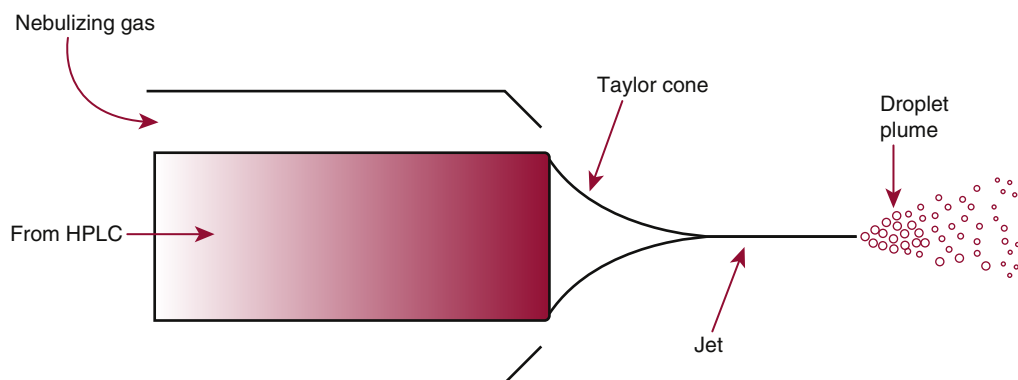


FIGURE 2.8 Simplified conceptual schematic of electrospray ion source showing Taylor cone. *HPLC*, High-performance liquid chromatography. (Published with permission from Eclipse Business Media Ltd. Reprinted from *MS Solutions*, Issue 7 [2010]. <http://www.sepscience.com/Information/Archive/MS-Solutions/235-/MS-Solutions-7-Adjusting-Electrospray-Voltage-for-Optimum-Results.>)

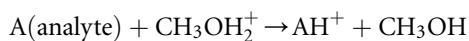
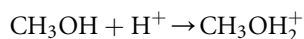
mass detector relative to the sampling cone; in some instruments the spray is orthogonal to the sampling cone.

ESI tends to be an efficient ion source for polar compounds or for molecules that are present as ions in solution, which includes a majority of biomolecules. ESI, along with APCI, allows an effective interface between a liquid chromatograph and a mass spectrometer. ESI and APCI have become the most widely used ion sources in mass spectrometers used in clinical laboratories.

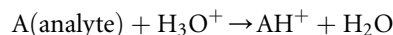
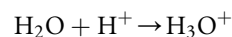
As already mentioned, electrospray is considered a soft ionization source. However, it is also possible to generate fragment ions before mass analysis using a technique known as nozzle-skimmer dissociation,¹⁸ infrared multiphoton dissociation,¹⁹ and thermally induced dissociation.²⁰ In these methods, ions are heated before entering the mass analyzer, and this causes ions to dissociate into fragment ions. In nozzle-skimmer dissociation, a higher than normal voltage gradient is applied in the first low-pressure region of the electrospray interface, resulting in collisional heating of the ions. In infrared multiphoton dissociation, ions are subjected to an intense bombardment of infrared photons, resulting in heating of the ions, followed by dissociation. In thermally induced dissociation, ions are activated and dissociated by excess heating of the gas used to transport ions from atmospheric pressure to the vacuum system of the mass spectrometer.

Atmospheric Pressure Chemical Ionization

APCI is similar to ESI in the sense that ionization takes place at atmospheric pressure, involves nebulization and desolvation, and uses the same design of the ion extraction cone as ESI. The major difference lies in the mode of ionization. In APCI, no high voltage is applied to the inlet capillary. Instead, the mobile phase from the separation device gets evaporated and the vapor passes by a needle with applied current.²¹ This process generates a corona discharge. Somewhat analogously to the processes occurring in a CI source, ions generated by the corona discharge undergo variety of ion-molecule reactions such as the following:



or



Because solvent molecules from the evaporated mobile phase (eg, water, methanol, acetonitrile) are present in the vapor in excess relative to the sample constituents, they are predominantly ionized early in the ion molecule cascade of reactions and then act as a reagent gas that reacts secondarily to ionize analyte molecules (see Fig. 2.7, B). The products of these secondary reactions may contain clusters of solvent and analyte molecules, and thus a heated transfer tube or a countercurrent flow of a curtain gas, such as nitrogen, is used to decluster the ions. As with ESI, APCI is a soft ionization technique that produces relatively little fragmentation. However, unlike ESI, APCI uses much higher heat and this can cause pyrolysis of the compound leading to loss of metabolically induced modifications to targeted compounds (glucuronidation, glutathionylation, etc.) and therefore can cause issues with analyses (in the methods that do not chromatographically resolve peaks of unconjugated and conjugated targeted molecules). On the other hand, in methods using ESI at lower temperature, those modifications tend to remain intact. When compared with EI, the mass spectra produced by APCI, ESI, and other soft ionization techniques typically have fewer fragments and are less useful for analyte identification by mass spectral fingerprinting. However, because the ion current is concentrated into a single mass spectral peak (or relatively few mass spectral peaks), APCI and other soft ionization sources are well matched to the requirements of tandem MS (discussed later) and are well suited for quantitative analysis. APCI and ESI are the most commonly used ion sources in quantitative analysis. However, in the case of nonpolar compounds such as steroids and some drug molecules, APCI is often a more efficient ion source than ESI.

Atmospheric Pressure Photoionization

APPI is a relatively new and less frequently used ion source in clinical chemistry that provides a complementary ionization approach to ESI or APCI. The physical configuration of an APPI source is similar to that for APCI, but an ultraviolet

photon flux (typically Krypton lamp that emits photons at 10 eV) is used instead of a corona discharge needle to generate ions in the gas phase.^{22–24} In APPI, an ionizable dopant, such as toluene or acetone, is often infused coaxially to the nebulizer to provide a source of ions that participate in charge or proton transfer to analyte molecules, thus increasing the efficiency of analyte ionization. APPI has a similar range of application to APCI and could be more useful than APCI for compounds of very low polarity, such as some steroids.

Inductively Coupled Plasma

ICP, as ESI, APCI, and APPI, is an atmospheric pressure ionization method. However, unlike most atmospheric pressure ionization methods, which are soft (ie, producing little fragmentation), ICP is the ultimate in hard ionization, typically leading to complete atomization of the sample during ionization. Consequently, its primary use is for elemental analysis. In the clinical laboratory, it is particularly useful for trace element analysis in tissues or body fluids. ICP is extremely sensitive (eg, parts per trillion limits of detection) and is capable of extremely wide dynamic ranges.

After sample preparation, which generally includes the addition of an internal standard such as yttrium and sometimes includes an acid digestion step, the sample is introduced into the ion source, usually via a nebulizer fed by a peristaltic pump. The nebulized sample is transmitted into hot plasma generated at atmospheric pressure by inductively coupling power into the plasma using a high-powered, radiofrequency (RF) generator (Fig. 2.9).²⁵ The temperature of the plasma is typically 6000 to 10,000 K (comparable to the temperature of the surface of the sun). Sample is introduced in the plasma, and ions are transmitted to the mass analyzer through a series of differential pumping stages. The atmospheric sampling apparatus is conceptually similar to that of other atmospheric pressure ion sources, such as electrospray, except that the device must withstand the extremely high temperatures generated by the plasma.

ICP-MS is comparatively free from most interference. However, some interfering species can be extremely troublesome. Most interfering species are small polyatomic ions

formed in the torch via ion-molecule reactions. For example, argon oxide (ArO^+) interferes with iron at m/z 56. One solution to this problem is to use a reaction cell, which consists of a moderate-pressure gas region in front of the m/z analyzer,²⁶ with a reactant gas, such as NH_3 , bled into the reaction cell. The reactant gas reacts with polyatomic interferences and removes them before introduction into the m/z analyzer. A related technique uses a nonreactive collision gas, which removes interferences using collisions, relying on differences in collision cross-sections between polyatomic ions and atomic ions. Another approach to removing interferences of the same nominal mass is to use a high-resolution mass spectrometer, which is capable of resolving species with similar nominal mass.²⁶ For example, the masses of ArO^+ and $^{56}\text{Fe}^+$ differ by 0.022 Da—a difference that may be resolved using a high-resolution mass spectrometer.

Matrix-Assisted Laser Desorption Ionization

MALDI is another type of soft ionization method that typically produces singly charged ions. MALDI and related techniques rely on energy transfer processes from a pulsed laser beam to the sample for ion generation. In most cases, the analyte is dissolved in a solution containing a solid phase matrix, a small molecular weight UV-absorbing compound, and this solution is placed on a target and dried. A pulsed laser irradiates the dried spots, triggering ablation, and desorption of the sample and matrix material; ions produced in the process are accelerated and introduced into the mass analyzer (Fig. 2.10). In other cases a layer of the solid matrix is deposited on the target and allowed to crystallize, and then the sample applied on top. The sample causes the top portion of the matrix to solubilize and mix with the sample before recrystallizing. In this way the sample is maintained in the very outer layer of the matrix, and this can help with enhancing the sensitivity and reducing the background noise. A related technique includes atmospheric pressure matrix-assisted laser desorption/ionization, in which the MALDI process occurs at atmospheric pressure rather than reduced pressure (see Chapter 4).

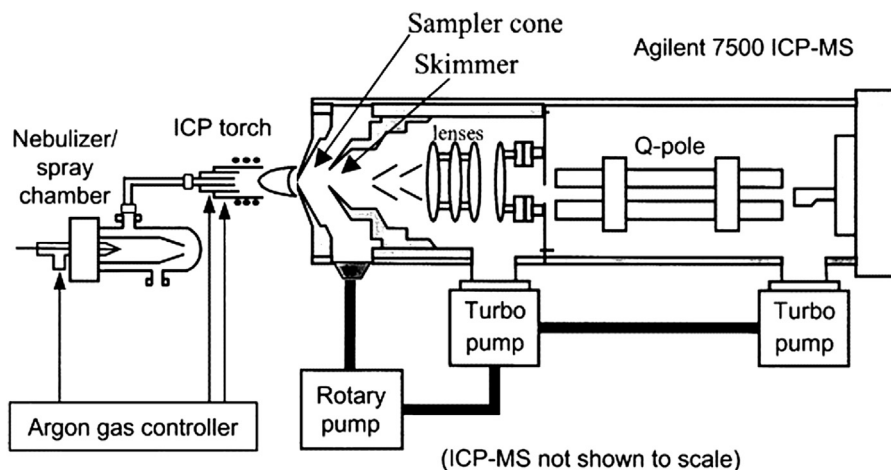


FIGURE 2.9 Simplified conceptual schematic of inductively coupled plasma–mass spectrometer (ICP-MS). Q-pole, Quadrupole. (From Kannamkumarath S, Wrobel K, Wrobel K, et al. Capillary electrophoresis–inductively coupled plasma-mass spectrometry: an attractive complementary technique for elemental speciation analysis. *J Chromatogr A* 2002;975:245–266.)

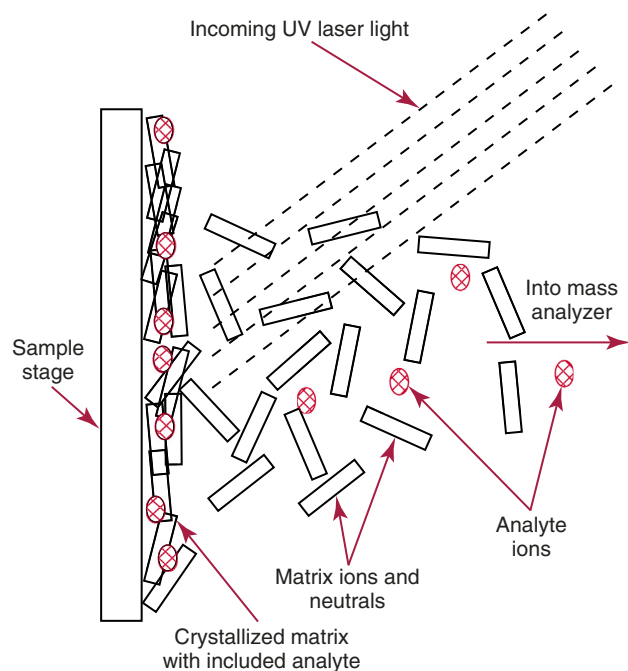


FIGURE 2.10 A generic view of the process of matrix-assisted laser desorption ionization. Co-crystallized matrix and analyte molecules are irradiated with an ultraviolet (*UV*) laser. The laser vaporizes the matrix, producing a plume of matrix ions, analyte ions, and neutrals. Gas-phase ions are directed into a mass analyzer.

Ionization Methods of Potential Interest

Desorption electrospray ionization (DESI)²⁷ and direct analysis in real time (DART)²⁸ are two relatively new ionization methods that generate ions from surfaces at atmospheric pressure. Most applications of DESI and DART to date have been directed toward minimal sample preparation. Paper spray MS is another emerging ionization method of potential interest. It is essentially a version of electrospray MS, but the spray is generated at the point of a triangular cut in a solvent-wetted piece of paper rather than from a capillary. It has the feature of easily integrating paper chromatography with MS.²⁹ It also has been used for rapid measurement of therapeutic drugs from dried blood spots without the need for complex sample preparation and separation.³⁰

Ionization Methods of Historical Interest

The older literature includes several ionization methods or sample introduction methods that, although promising, or even widely used at one time, hold little interest for current practice in clinical chemistry. Nevertheless, it is useful to be aware of them because they may still be referred to in the literature from time to time. Some of these include (1) *fast atom bombardment*, (2) *thermospray*, (3) *direct liquid introduction*, (4) *plasma desorption*, (5) *field ionization*, (6) *field desorption*, (7) *secondary ion mass spectrometry*, and (8) *laser desorption*. ESI and APCI ion sources have largely rendered these techniques obsolete.

Vacuum System

With the exception of certain ion trap mass spectrometers, ion separation in any of the mass analyzers requires that

the ions do not collide with other molecules during their interaction with magnetic or electric fields. This requires the use of a vacuum from 10^{-3} to 10^{-9} torr, depending on mass analyzer type. The length of the ion path in the analyzer must be less than the mean free path length, unless collisions play a role in mass analysis.

Fourier transform ion cyclotron resonance (FT-ICR) requires the lowest pressure (10^{-9} torr). The quadrupole ion trap (QIT) tolerates the highest pressure (10^{-3} to 10^{-5} torr), a pressure range in which some collisions occur between ions and background gas. Routine quality assurance checks for vacuum leaks should include evaluation of the presence of air and water in the mass spectra.

Efficient high-vacuum pumps generally do not operate well near atmospheric pressure. Thus the vacuum system must have a positive displacement (mechanical) vacuum pump to evacuate the system to a pressure at which the high-vacuum pumps are effective. Mechanical pumps require routine maintenance, such as ballasting and replacing the pump oil.

Although diffusion pumps are the least expensive and most reliable high-vacuum pump, they are rarely used outside of some very specialized mass spectrometers that typically are not used in the clinical laboratory. Cryopumps are another class of pumps that are sometimes used in specialized mass spectrometers but not in the clinical laboratory. In modern instruments, the most common high-vacuum pumps are turbomolecular (often referred to as “turbo”) pumps; they have largely replaced diffusion pumps and cryopumps because they are more convenient to use. A key consideration in the design of the vacuum system is pumping speed. The ability of the pump to maintain the vacuum by removing any gas (or solvent vapor) that enters the system determines the maximum flow rate of gas introduced into the mass spectrometer. In general, higher pump capacities are associated with lower detection limits because noise arising from the gas background is reduced.

Mass Analyzers, Tandem Mass Spectrometers, and Ion Detectors

The term *mass spectrometry* is somewhat a misnomer because mass spectrometers do not measure molecular mass, but rather they measure the mass-to-charge ratio. This fact is fundamental to the physical operating principles of mass spectrometers and consequently affects all aspects of instrumentation design, instrument operation, and interpretation of results. The symbol m/z is used to denote mass-to-charge ratio and conventionally has been defined as a dimensionless quantity³¹ (see also <http://goldbook.iupac.org/M03752.html>).

However, a “dimensionless” mass-to-charge ratio is not consistent with equations of ion motion in the presence of electric and magnetic fields, which require units of mass divided by charge. Furthermore, the m/z scale sometimes is loosely discussed in terms of daltons (also known as unified atomic mass units [u]), although strictly speaking, Da is a unit of mass, not mass-to-charge ratio. Despite these somewhat confusing nomenclature issues, the present chapter generally follows convention by discussing m/z in terms of mass and Da.

To help avoid some of the confusion surrounding the use of m/z , it has been proposed that it should be defined

explicitly as quantity having units of mass-to-charge ratio, with mass specified in daltons and charge specified in elementary charges; this proposed unit would be called the *Thomson* (Th) in honor of one of the pioneers of MS.³²⁻³⁴ This terminology is sometimes seen in the literature but has not been widely adopted.

General Classes of Mass Spectrometers

Mass spectrometers are broadly classified into two groups: beam-type instruments and trapping-type instruments. In a beam-type instrument, the ions make one pass through the instrument and then strike the detector, where they are destructively detected. The entire process, from the time an ion enters the analyzer until the time it is detected, generally takes microseconds to milliseconds.

In a trapping-type analyzer, ions are held in a spatially confined region of space through a combination of magnetic and/or electrostatic and/or RF electrical fields. The trapping fields or supplemental fields are applied and manipulated in ways that allow m/z measurements to be performed. Trapping times may range from a fraction of a second to minutes, although most clinical applications are at the low end of this range.

Examples of trapping-type instruments include QITs, linear ion traps (which, along with QITs, also depend on RF electric fields), ICR mass spectrometers, electrostatic ion traps, and orbitraps (which are a type of electrostatic ion traps).

Detection of the ions in a trapping-type instrument may be destructive or nondestructive, depending on the specific type of mass spectrometer used. In this context, *destructive* means that ions are destroyed in the detection process. Additional discussions of mass analyzers, tandem mass spectrometers, and ion detectors can be found in the literature,^{33,35} and the CLSI documents C-50A and C-62A contain recommendations for matching the capabilities of different m/z analyzers to various application classes.^{1,2}

Beam-Type Designs. The main beam-type mass spectrometer designs are (1) quadrupole, (2) magnetic sector, and (3) TOF. It is convenient to categorize beam-type instruments into two broad categories, those that produce a mass spectrum by scanning the m/z range over a period (quadrupole and magnetic sectors) and those that acquire instantaneous snapshots of the mass spectrum (TOF). This categorization is not hard and fast. Certain instrument designs have been adapted to scanning or nonscanning operations. Nevertheless, the categorization is a useful one because it covers the majority of instruments currently available and because scanning and nonscanning instruments are adapted for different optimal usages.

Quadrupole. Quadrupole mass spectrometers, sometimes known as quadrupole mass filters (QMFs), are currently the most widely used mass spectrometers, having displaced magnetic sector mass spectrometers as the standard instrument. Although these instruments lag behind magnetic sector instruments in terms of (1) sensitivity, (2) upper mass range, (3) resolution, and (4) mass accuracy, they offer an attractive and practical set of features that account for their popularity, including (1) ease of use, (2) flexibility, (3) adequate performance for most applications, (4) relatively

low cost, (5) small size, (6) noncritical site requirements, and (7) highly developed data collection software systems.

A quadrupole mass spectrometer consists of four parallel electrically conductive rods arranged in a square array (Fig. 2.11). The four rods form a long channel through which the ion beam passes. The beam enters near the axis at one end of the array, passes through the array in a direction generally parallel to the axis, and exits at the far end of the array. The ion beam entering the quadrupole array may contain a mixture of ions of various m/z values, and in different modes of operation, different mass ranges can be selected. If a very narrow m/z range is selected (eg, $\Delta m/z < 1$) only ions of the specified m/z will be transported through the device to reach the detector. Ions outside this narrow range are ejected radially. The $\Delta m/z$ range represents a pass band, analogous to the pass band of an interference filter in optics. This is why quadrupole mass spectrometers are often referred to as *mass filters* rather than *mass spectrometers*.

Separation of ions in QMS is based on a superposition of RF and constant direct current, or DC potentials applied to the quadrupole rods. DC voltages are applied to the electrodes in a quadrupolar pattern. For example, a positive DC potential is applied to electrodes 1 and 3, as indicated in Figure 2.12, and an equivalent negative DC potential is applied to electrodes 2 and 4. The DC potentials are relatively small, on the order of a few volts. Superimposed on the DC potentials are RF potentials, also applied in a quadrupolar fashion. RF potentials range up to the kilovolt range, and frequency is on the order of 1 MHz. In the most frequently used mode of operation the frequency is fixed and highly stable, derived from a crystal controlled oscillator.

The physical principles underlying the operation of a quadrupole mass spectrometer are rigorously described by solutions of a complicated differential equation, the Mathieu equation.³⁶ When an ion is subjected to a quadrupolar RF field, its trajectory is described qualitatively as a combination of fast and slow oscillatory motions. For descriptive purposes, the fast component will be ignored here. The slow

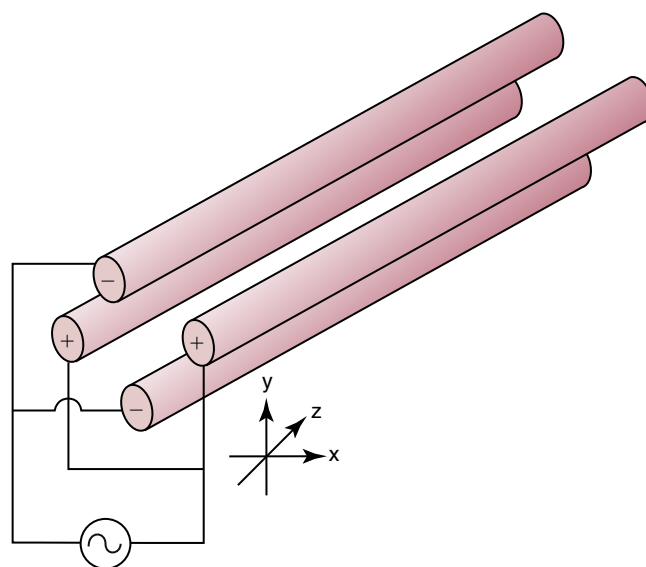


FIGURE 2.11 Diagram of quadrupole mass filter, including the radiofrequency part of voltages applied to the quadrupole rods.

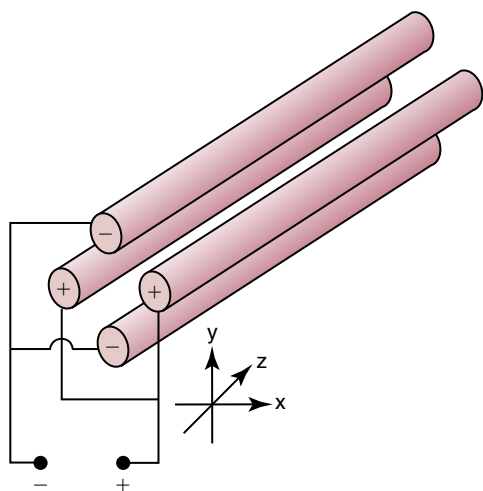


FIGURE 2.12 Direct current voltages applied to quadrupole rod assembly.

component oscillates about the quadrupolar axis; this resembles the motion of a particle in a fictitious harmonic *pseudopotential*. The frequency of this oscillation is sometimes called the *secular frequency*.

Effective force associated with the pseudopotential is directed inward toward the quadrupolar axis and is proportional to the distance from the axis. It therefore acts as a confining force, preventing ions from being ejected radially from the quadrupolar assembly. **Figure 2.13, A**, shows an example of an ion confined by an RF-only quadrupole. Below a certain m/z cutoff frequency (which depends on the frequency and amplitude of the RF field), ions are ejected rather than confined. **Figure 2.13, B**, shows an example of an ion ejected by an RF-only quadrupolar field. This establishes the low mass cutoff for the m/z pass band. The effective confining force is strongest just above the low m/z cutoff and then decreases asymptotically toward zero at high m/z .

The DC part of the quadrupolar potential is independent of m/z . Positive ions are attracted toward the negative poles. Negative ions are attracted toward the positive poles. Attraction increases as the distance from the quadrupolar axis increases. Because a quadrupolar DC potential always has both negative and positive poles, the quadrupolar DC potential always contributes to ejection in at least one direction. Whether ejection of an ion of a particular m/z actually occurs depends on whether the ejecting force caused by the quadrupolar DC potential overcomes the effective confining force caused by the pseudopotential generated by the RF field. Above a certain m/z value, the DC part dominates and ions are ejected radially from the device. This establishes an upper m/z limit for ion transmission. **Figures 2.13, C and D**, show examples of ion trajectories under the influence of combined RF-DC fields, one being confined and the other being ejected. Trajectories in **Figure 2.13** were calculated using the Simion ion optics computer program.³⁷

A rigorous description of low- and high-mass cutoffs is found in so-called stability diagrams, which graphically describe the lower and upper m/z cutoffs of a quadrupole mass spectrometer in terms of parameters related to voltages,

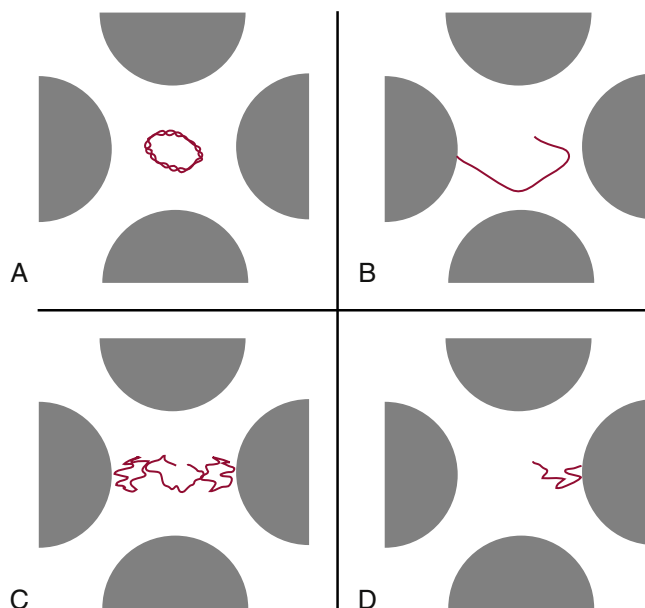


FIGURE 2.13 Ion trajectories showing confinement and ejection in quadrupole mass filters. A, Ion confinement by radiofrequency (RF)-only field. B, Ion ejection by RF-only field. C, Ion confinement with a combination of RF and direct current (DC) fields. D, Ion ejection with a combination of RF and DC fields. All trajectories were simulated using Simion software. (Courtesy Scientific Instrument Services, Ringoes, New Jersey.)

frequencies, and m/z . However, a full discussion of the stability diagram is outside of the scope of this chapter.

The combination of lower and upper m/z limits establishes a pass band ($\Delta m/z$) and ultimately a resolution $[(m/z)/(\Delta m/z)]$. With relatively few exceptions, quadrupole instruments are limited to a resolution of a few hundred to several thousand, which is sufficient to achieve isotopic resolution for singly charged ions of m/z as high as several thousand.

A quadrupole MS may be operated in SIM mode or scanning mode. In SIM mode, both DC and RF voltages are fixed. Consequently, both the center of the pass band and the width of the pass band are fixed. For example, the mass spectrometer may be set to pass ions of m/z 363 ± 0.5 . Both the center m/z and the $\Delta m/z$ are adjusted by the appropriate choice of DC and RF.

In the scanning mode of operation, the RF and/or DC voltages are continuously varied to scan a range of the specified m/z values. As with the SIM mode, the $\Delta m/z$ is determined by the RF and DC voltages. Usually the scan function is designed to maintain a constant $\Delta m/z$ across the full m/z range. Thus the resolution increases as m/z increases. The value of $\Delta m/z$ is frequently chosen in the range 0.5 to 0.7 to resolve isotopic peaks of singly charged species across the full m/z range.

Magnetic Sector. Because magnetic sector mass spectrometers are rarely used in clinical laboratories, they will not be described in detail here. It should be noted, however, that these classic mass spectrometers are easy to understand (given a basic understanding of physics); are versatile, reliable, and highly sensitive; and in their “double focusing” design are capable of very high m/z resolution and mass accuracy. However, they are typically very large, expensive, and have the reputation of being difficult to use.

Consequently, other instruments have largely displaced magnetic sector mass spectrometers.

Time-of-Flight. TOF mass spectrometry (TOF-MS) is a nonscanning technique whereby a full mass spectrum is acquired as a snapshot rather than by sweeping through a sequential series of m/z values while acquiring the data. It is described here as a snapshot because, although ions of different m/z arrive at the detector sequentially (low m/z first), the samples are loaded into the ion source with little or no m/z discrimination with regard to time, and the duration of the acquisition of a single mass spectrum is measured in microseconds. One implication of this is that if the composition of the sample stream being presented to the mass spectrometer changes with time, there is essentially no distortion of the mass spectrum resulting from this time dependence, whereas with scanning-type mass spectrometers the mass spectrum may be distorted because of the interaction between scan time of the mass spectrometer and the changing concentration of the sample stream. This is particularly significant when dealing with fast chromatography coupled to MS.

TOF mass spectrometers have several advantages, including (1) a nearly unlimited m/z range, (2) high acquisition speed, (3) high mass accuracy, (4) moderate to high resolution, (5) moderate to high sensitivity, (6) absence of spectral distortions when used in conjunction with fast separations and narrow chromatographic peaks, and (7) reasonable cost. TOF-MS is also well adapted to pulsed ionization sources, which is an advantage in some applications, particularly with MALDI and related techniques.³⁸

A major advantage of modern TOF mass spectrometers is that they are capable of acquiring accurate mass measurements, sometimes loosely referred to as *exact mass*, which is typically accurate to a few parts per million (ppm). This allows TOF measurements to confirm the molecular formula of a compound and assist with identification of unknowns in the mass spectra. TOF mass spectrometers are conceptually simple to understand because they are based on the fact that in vacuum a lighter ion travels faster than a heavier ion, provided that both have the same kinetic energy.

Figure 2.14 presents a simplified conceptual diagram of a TOF mass spectrometer. It resembles a long pipe wherein ions are created or injected at the source end of the device and are then accelerated by the applied potential of several kilovolts. The ions travel down the flight tube and strike the detector at the far end. The time it takes to traverse the tube is known as the flight time; this is related to the mass-to-charge ratio of the ion.

The flight time for an ion of mass m and kinetic energy E to travel a distance L in a region free of electric fields is given by:

$$t = L \left(\frac{m}{2E} \right)^{1/2}$$

A sample calculation for an ion of molecular weight 200 Da (3.32×10^{-25} kg) with a kinetic energy of 10 keV (1.60×10^{-15} J), traveling through a distance of 1 m, yields a flight time of 10.18 μ s, and an ion of molecular weight 201 takes just 25 ns longer. To accurately capture such fleeting signals, the data recording system must operate on an

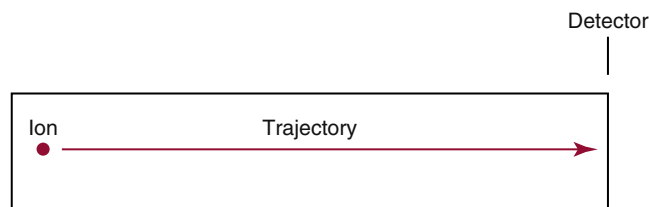


FIGURE 2.14 Diagram of simplified time-of-flight mass spectrometer.

approximately 1 ns or shorter time-scale. Advances in signal processing electronics have made these mass analyzers possible at a modest cost, and this has been a major factor in the rise in popularity of TOF-MS.

TOF is inherently a pulsed technique; it couples readily to pulsed ionization methods, with MALDI being the most common example, although TOF is also coupled with continuous ion sources such as EI, ESI, and APCI. However, the continuous nature of these sources causes a mismatch between continuous introduction of ions from the ion source and a pulsed detection with TOF-MS. This mismatch is overcome by using a technique known as orthogonal acceleration TOF-MS (OA-TOF-MS), in which the ion beam is injected orthogonal to the axis of the TOF-MS.³⁹⁻⁴¹

During the injection period, the acceleration voltage is turned off. Once the injection region is filled with the traversing beam, the acceleration voltage is quickly turned on and the TOF timing cycle starts. The process is cycled repeatedly. The overall duty cycle for this method can be more than 10%; this represents a vast improvement over the traditional method of gating the ion beam for TOF analysis. For full spectrum capability with continuous ion sources, orthogonal injection TOF mass spectrometers are generally considered to have the lowest detection limits of all mass spectrometers. However, for the monitoring of a single m/z rather than a full mass spectrum, the use of SIM mode with a quadrupole MS provides superior sensitivity.

Improved resolution is an additional benefit conferred by OA-TOF-MS. Although a complete explanation is beyond the scope of this chapter, in brief, orthogonal acceleration reduces the resolution-degrading effects that would normally accompany the kinetic energy variations of individual ions in the ion beam.

Use of an ion mirror is another technique often employed in TOF-MS design to improve resolution by compensating for kinetic energy variations.^{42,43} Such instruments are known as *reflectrons*. To date, TOF-MS has had a limited impact in clinical chemistry with only a few commercially offered TOF-MS assays, such as insulin-like growth factor I^{44,45} and drug screening,⁴⁶ but it could potentially play a greater role in the future. For example, full-spectrum capability, high resolution (up to 40,000 in some current instruments), high speed (10 to 100 stored spectra per second), and high mass accuracy of TOF-MS seem ideally suited to applications such as high-speed drug screens in toxicology when combined with fast chromatographic sample introduction.

Another area in which TOF-MS provides an advantage is high-mass analysis, where its mass range is nearly unlimited. In MALDI-TOF, for example, it is not unusual to detect proteins with molecular weights exceeding 100,000 (see

Chapter 4). The ability for high-mass analysis is expected to increase in importance as clinical laboratories embrace proteomic-based diagnostic methods.

Trapping Mass Spectrometers. In contrast to beam-type designs, these mass spectrometers are based on the trapping of ions to capture and hold ions for an extended length of time in a small region of space. Trapping times vary from a fraction of a second to minutes. Compared with beam-type instruments, the division between scanning and non-scanning instruments has less meaning for ion-trapping instruments. The main practical difference between scanning and non-scanning instruments is related to distortions in chromatographic peak shape (or peak skewing). These arise from the finite scan time of a mass spectrometer relative to the time-scale of the width of a chromatographic peak. The result is that the abundances of the peaks in mass spectra collected during the rising or falling portions of a chromatographic peak are distorted relative to the true mass spectrum. In other words, as an instrument collects a mass spectrum, scanning from low m/z to high m/z , the peak intensities observed for low m/z will reflect the concentration of analyte that elutes earlier than the concentration of analyte that elutes when detecting high m/z . As a result, mass spectra collected at the beginning of a chromatographic peak may have different relative peak intensities compared with those of spectra collected at the end of a chromatographic peak. In terms of producing skewed spectra, trapping devices are more similar to non-scanning instruments, such as TOF (does not cause skewing), than to scanning instruments. This is because the sample is captured in an instant and then analyzed at leisure. Because the sample is captured in an instant, no skewing of the spectra occurs, regardless of whether the mass analysis is performed by a scanning or a non-scanning technique.

Traditionally, ion traps have been classified as (1) a QIT, which relies on RF fields to provide ion trapping; (2) a linear ion trap, which is closely related to the QIT in its operating principles; (3) an ion cyclotron resonance (ICR) mass spectrometer, which relies on a combination of magnetic fields and electrostatic fields for trapping, and (4) an orbitrap, a more recent introduction into the field of ion trap MS.⁴⁷

Quadrupole Ion Trap. QITs are relatively compact, inexpensive, and versatile instruments that are excellent for (1) exploratory studies, (2) structural characterization, and (3) qualitative identification. They are also used for quantitative analysis, although precision of measurements is inferior when compared with quadrupole-based instruments.

Operation of the QIT is based on the same physical principle as the quadrupole mass spectrometer described earlier. Both devices make use of the ability of RF fields to confine ions. However, the RF field of an ion trap is designed to trap ions in three dimensions rather than to allow the ions to pass through as in a QMF, which confines ions in two dimensions. This difference has a large impact on the operation and limitations of the QIT.

The physical arrangement of a QIT is different from that of a QMF. If an imaginary axis is drawn through the y -axis of the quadrupole rods, and the rods are rotated around the axis, a solid ring with a hyperbolic inner surface results from the x -axis pair of rods. The two y -axis rods form two solid end caps. A diagram of an ion trap is given in

Figure 2.15. The description of the fields within the electrodes must now include a radial component and an axial (between the end caps) component. These design features have an effect on the conditions required for ion confinement when compared with a QMF, although the qualitative description of ion confinement discussed previously is valid.

A discussion of the several types of scanning experiments in QIT is beyond the scope of this chapter, except to mention that ions may be ejected from the trap in an m/z -dependent fashion for detection using an external electron multiplier.

Some advantages of QITs are an ability to perform multiple stages of tandem MS (MS^n), high sensitivity, and decoupling of the mass analysis from scanning, so no mass spectral peak skewing is seen in GC-MS and HPLC-MS. However, ion-ion repulsion effects (caused by large numbers of similarly charged species in a small space within the trap) limit the number of ions that can be trapped, simultaneously reducing dynamic range and producing mass misassignments at high signal levels. The previously mentioned features make QITs not well suited for quantitative analyses, which are typically required for majority of applications in clinical laboratories.

Linear Ion Trap. The linear trap is an RF ion trap that is based on a modified linear QMF. Rather than being a pass-through device, as in a traditional linear QMF, electrostatic fields are applied to the ends to prevent ions from exiting out the ends of the device. When trapped in this manner, ions can be manipulated in many of the same ways as in a QIT. An advantage of the linear quadrupole ion trap is that the trapping field can be turned off at will and the device operated as a normal QMF. Furthermore the trapping volume available within the QMF is much greater than the traditional QIT, allowing greater capacity of the ions to be trapped before ion-ion repulsion becomes an issue. Thus a single device combines most of the features of a QIT and QMF and is extremely versatile. Commercial triple quadrupole mass spectrometers are being offered in

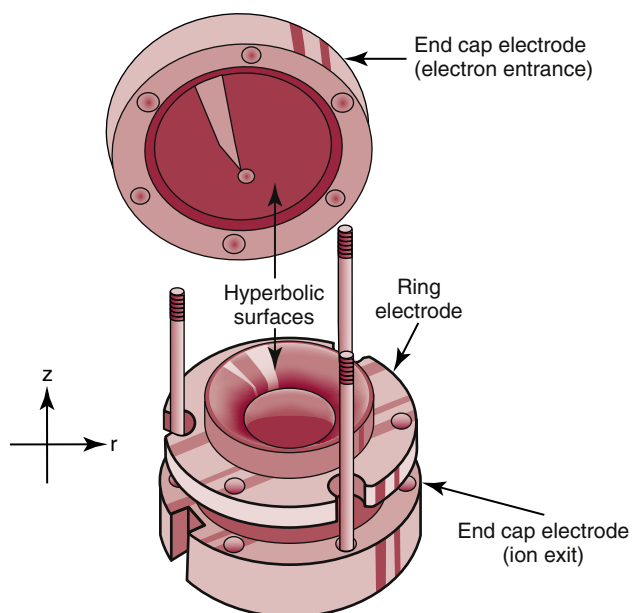


FIGURE 2.15 Diagram of quadrupole ion trap. r , Radial direction; z , axial direction.

which the third quadrupole is modified to function either as a linear trap or as a conventional third quadrupole mass spectrometer as selected by the user.

Ion Cyclotron Resonance. The ICR-MS excels in high-resolution and high mass accuracy measurements.⁴⁸ Measurements at resolution exceeding 1 million are not unusual. ICR is a trapping technique that shares many of the advantages of RF ion traps (QIT or linear ion traps). However, there are even more ways to manipulate ions in an ICR-MS than in RF ion traps, and MSⁿ (multiple stages of MS/MS) measurements are easily done with an ICR-MS. Sensitivity of an ICR-MS is generally high. Furthermore, sampling is decoupled from spectral acquisition, so no peak skewing is seen in chromatographic experiments—a feature that ICR shares with TOF and QIT, and the signal acquisition times are typically longer than for other types of mass analyzers.

Fourier transform ion cyclotron resonance—mass spectrometry (ICR-MS) is based on the principle that ions immersed in a magnetic field undergo circular motion (cyclotron motion). A typical ICR-MS uses a high-field (3 to 12 tesla) superconducting magnet. Within this field and within a high vacuum is mounted a cell typically composed of six metal electrodes, arranged as the faces of a cube. Ions are suspended inside the cell and undergo cyclotron motion, which keeps ions from being lost radially (the radial direction being defined as perpendicular to the magnetic field lines). A low (~ 1 V) potential is applied to the end caps to keep ions from leaving the trap axially. Thus the combination of electric and magnetic fields keeps ions confined within the cell.

Ions circulating in the ICR cell induce an electrical current in two parallel detection electrodes. The detection electrodes are on opposite sides of the ICR cell and are arranged parallel to the magnetic field. After certain mathematical operations are performed on the signal (principally a Fourier transform [FT]), a mass spectrum is recovered. Each m/z is associated with a specific cyclotron frequency, and each m/z value that is present in the sample produces a peak in the transformed signal. Because of the frequent use of FT in ICR, the technique is often referred to as FT-ICR or FTMS.

Although this technique has many advantages, including (1) high mass accuracy, (2) ultra-high resolution, and (3) the ability to perform MSⁿ, ICR-MS has several disadvantages, including (1) high instrument costs; (2) very demanding site requirements, in terms of both space and access restrictions; (3) requirement for a high-field superconducting magnet; (4) relatively long signal acquisition time, which limits the number of scans that can be acquired during the elution of a chromatographic peak; (5) safety concerns related to high magnetic fields; (6) demagnetization of credit cards and other magnetically encoded strips; (7) high costs of operation and maintenance because the instruments consume liquid helium and must never be allowed to run out of helium; and (8) necessity of a highly skilled individual to operate the instrument.

Orbitrap. The suitability of a new type of mass analyzer, the orbitrap, for clinical analysis has yet to be proved. However, the high resolution and mass accuracy of the orbitrap suggest that it has potential for use in clinical laboratories. The orbitrap mass analyzer has resolution and mass accuracy approaching that of an ICR mass spectrometer but does not require a magnetic field. This innovation minimizes many of the ICR disadvantages listed previously.

The principles of mass analysis in an orbitrap are based on an early ion storage device—the Kingdon trap.⁴⁹ After many variations over the years, Makarov and associates^{47,50-52} developed a modified version that was commercialized in 2006. The commercial instrument can easily achieve resolutions up to 100,000 and parts per million or even sub-parts per million mass accuracy, has four orders of magnitude dynamic range, and has sampling decoupled from spectral acquisition (as in the ICR). The resolution and mass accuracy are typically approximately 2 orders of magnitude greater than with a quadrupole mass spectrometer.

Orbitrap-MS is based on trapping within electrostatic fields.⁵³ The actual device is a spindle-like central electrode surrounded by a barrel-like outer electrode.⁵⁴ When ions are introduced perpendicular to the central electrode and a radial potential is applied between electrodes, the ions spiral (orbit) around the central electrode and are effectively trapped in a radial direction. Trapping in the axial direction is assisted by the shape of the electrodes, together with the potentials that are applied to the electrodes. Ion trapping therefore involves both orbital motion around the central electrode and axial oscillations.

The trapping potential in the axial direction is of the form of a harmonic oscillator, and because the frequency of a harmonic oscillator is independent of oscillation amplitude, this frequency is very stable and well behaved. The m/z can be calculated from the frequency of axial oscillation:

$$\omega = 2\pi f = (km/z)^{-1/2}$$

where ω is angular velocity, f is frequency, m/z is the mass-to-charge ratio, and k is a constant determined by the trap geometry, dimensions, and applied potential. (To be dimensionally correct, m/z in this equation must have units of mass divided by charge, which differs from the currently accepted definition of m/z as a unitless number³¹ (see also <http://goldbook.iupac.org/M03752.html>).

The image current (current induced by a motion of ions passing near a conductor) made in the outer electrode induced by the ion motion is acquired in the time domain and can be Fourier-transformed to produce a frequency spectrum that is then converted to m/z using the previous equation.

With the ability to perform accurate mass measurements, especially when combined with a linear ion trap or quadrupole to form a hybrid tandem mass spectrometer, orbitrap mass analyzers have excellent capabilities for proteomics research. One recent publication noted anomalous isotope ratios observed under high-resolution operating conditions.⁵⁵ It is a curious point that the anomalies are compound-dependent, and generally increase with increasing resolution. A theoretical explanation for these anomalies has been given.⁵⁶

Tandem Mass Spectrometers

Tandem MS, or MS/MS, has become the dominant MS-based technique used in clinical laboratories, where it has found extensive application in the quantitative analysis of routine samples.⁵⁷ However, it is also a useful technique for structural characterization and compound identification and therefore

often used for exploratory work. The most important features of this technique are its very high selectivity, ability to measure very low concentrations of analyte(s), and ability to multiplex the measurement of multiple analytes in a single method. Susceptibility of MS/MS to interferences is typically very low, especially if MS/MS is combined with chromatographic separation. The reason is that a detected compound is separated and characterized by three physical properties: chromatographic retention time, precursor ion mass, and product ion mass. Because of its high specificity, low consumable cost, and a potentially high sample throughput, increasingly more clinical laboratories are using tandem mass spectrometers for the routine analysis of samples.

The physical principle of MS/MS is based on the use of two mass spectrometers (or mass filters) arranged sequentially in tandem, with a collision cell placed between the two mass filters. The first filter is used to select a *precursor ion* of a particular m/z . The precursor ion is directed into the collision cell, where ions collide with background gas molecules and are broken into smaller product ions. The second mass filter acquires the mass spectrum of the product ions.

A variety of scan functions are possible with MS/MS. A product ion scan involves setting the first mass spectrometer (also called mass filter 1, MF1, MS1, or Q1) to select a given m/z , and scanning through the full mass spectrum of product ions using the second mass spectrometer or mass filter, MF2. This scan function is often used for structural characterization.

A precursor ion scan reverses this relationship, with the second mass filter, MF2, set to select a specific product ion, and MF1 is scanned through the spectrum of precursor ions. The scan tells which precursor ions produce a specific product ion—a capability that is often used to analyze for specific classes of compounds. For example, acylcarnitines are often analyzed using precursor ion scan mode by acquiring signal from all the precursors of the m/z 85.

In a constant neutral loss scan, the two mass filters are scanned synchronously, with a constant m/z offset between precursor and product ion. This scan indicates which ions lose a particular neutral fragment. For example, an offset of 176 m/z units would select for ions losing a glucuronide moiety in the dissociation process.

The most commonly used scan function in MS/MS is MRM (also referred as SRM). In this type of acquisition a series of precursor/product ion pairs are monitored, with the mass spectrometer set to step through the table of parent/product ion pairs in a cyclic fashion. MRM acquisition is primarily used for quantitative analysis of target compounds and is an analog to the SIM type of acquisition used in GC-MS.

As with single-stage mass spectrometers, MS/MS are roughly categorized as beam-type instruments and trapping instruments. The most popular beam-type instrument is the triple quadrupole. In this instrument, the first quadrupole (Q1) functions as MF1 and the third quadrupole (Q3) functions as MF2. Between these two quadrupoles is another quadrupole, Q2, which functions as the collision cell. The pressure is raised in Q2 (eg, $>10^{-3}$ torr) by the addition of a nonreactive gas (nitrogen, helium, argon, etc.) to the point that ions traversing Q2 undergo multiple collisions, leading to deposition of energy onto the analyte and subsequent

fragmentation of the precursor ion(s) into smaller fragments, followed by separation and detection of the product ions in a subsequent stage of mass analysis. The Q2 is operated as an RF-only quadrupole, ideally passing all ions regardless of m/z . The technique is typically used to fragment molecular or pseudomolecular ions in order to obtain analyte-specific fragments, which can be used for elucidating structure of the molecules of interest or in selective analysis of targeted molecules. In cases in which the collision-induced dissociation spectrum contains a large number of ions, the experienced investigator often can deduce the structure of a molecule from the mass spectrum of the product ion.

Two magnetic sector instruments also have been operated in tandem, with a collision cell placed between the two mass analyzers. These instruments permit high-resolution selection of both precursor and product ions. However, they are now rarely used because of the high cost and cumbersome operation. A single magnetic sector mass spectrometer (in the form of a double focusing mass spectrometer) has also been used as an MS/MS by a technique known as *linked scanning*. A product ion scan by linked scanning involves low resolution for the first m/z selection and high resolution for the second m/z selection.

Hybrid mass spectrometers include a combination of two different types of mass spectrometers in a tandem arrangement. The combination of a magnetic sector mass spectrometer with a quadrupole mass spectrometer was an early instrument of this type. More popular today is the combination of a quadrupole for the first stage of m/z selection and a TOF for the second m/z analyzer. Subsequently, linear ion trap and quadrupole mass analyzers have been combined with an orbitrap. Hybrid instruments are presently used mainly for proteomics research. These instruments cannot perform the true precursor ion scans or constant neutral loss scans, though it is possible to mimic these functions by postprocessing data, provided the full precursor-product ion map was generated in the experiment.

QIT, linear ion trap, and ICR mass spectrometers also can be used as MS/MS. Unlike beam-type instruments, which are referred to as “tandem in space,” trapping mass spectrometers are “tandem in time,” meaning that ions are held in one region of space while the parent ion is selected and dissociated and the daughter ion is analyzed sequentially in time in the same region of space. The ability to perform MS/MS is inherent in the design of most trapping mass spectrometers. Generally, little or no additional hardware is required, and tandem capability is supplied via software. An exception is the orbitrap, which is not amenable to MS/MS when used alone. However, when incorporated into a hybrid instrument, with a different type of mass spectrometer supplying the first stage of MS (such as a linear ion trap or a quadrupole), and with the orbitrap providing the final stage of MS, MS/MS is possible in an orbitrap-based instrument.

Most trap-based instruments are capable of multiple stages of MS. Thus, product ions may be further dissociated to produce another generation of product ions (MS/MS/MS, or MS³). In principle, any number of dissociation stages may be performed (MSⁿ). This capability finds its greatest use in structural characterization, such as in the sequencing of peptides, and is less useful for quantitative analysis.

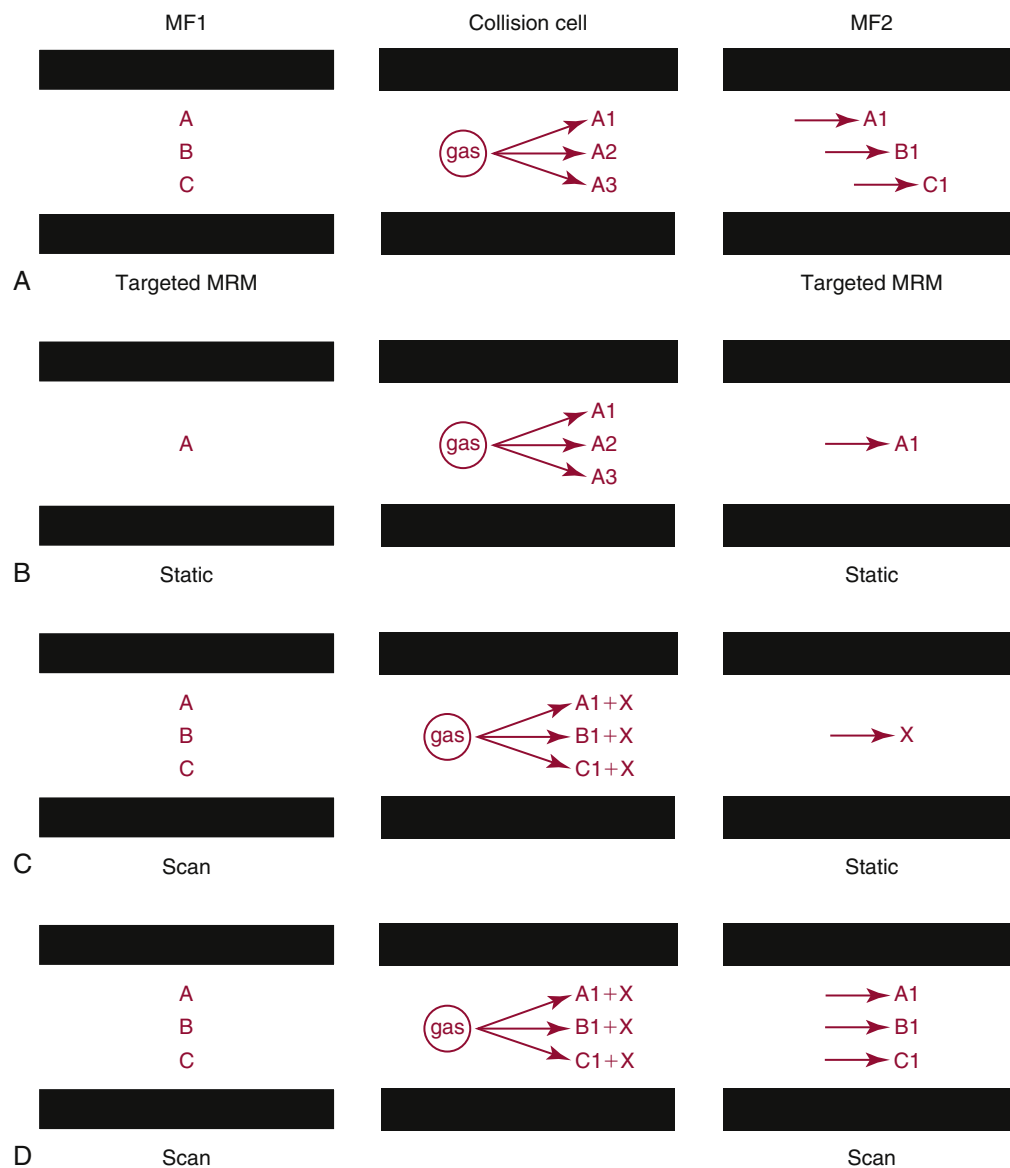


FIGURE 2.16 Scan modes in mass spectrometry/mass spectrometry (MS/MS). A, Multiple reaction monitoring (MRM), in which A , B , C , $A1$, $B1$, and $C1$ are ions. Monitoring of MS/MS transitions $A \rightarrow A1$, $B \rightarrow B1$, and $B \rightarrow C1$ is multiplexed. For simplicity, only the dissociation of A is shown in a collision cell in the figure. B, MRM of a single compound, where only one MS/MS transition is monitored. C, Precursor ion scan, in which A , B , C , and X are all ions. The second mass filter (MF2) is fixed to monitor the mass-to-charge ratio (m/z) corresponding to ionic species X , and the first mass filter (MF1) is scanned through a range of m/z values. D, Constant neutral loss scan, in which X is uncharged and A , B , C , $A1$, $B1$, and $C1$ are ions. The two mass filters are scanned with a constant m/z offset between the two corresponding to the mass of X .

Although trapping designs are extremely versatile (such as allowing multiple stages of fragmentation), these instruments are unable to perform true precursor ion scans or constant neutral loss scans, as illustrated in Figure 2.16, C and D, respectively. However, it is possible to simulate the effect of precursor ion scans or constant neutral loss scans by taking a series of product ion scans, one for each possible parent ion m/z . This will generate a complete MS/MS map. From this complete map data can be selected to simulate these two scan modes because a precursor ion scan is just a subset of the complete MS/MS map, as is a constant neutral loss scan. However, this procedure can be quite time-consuming, which would make it impractical in some applications.

Ion Mobility

Although strictly speaking, ion mobility spectrometers (IMS) are not mass analyzers, they are nevertheless often included as part of the field of MS, either as part of a hyphenated technique (eg, IMS-MS) or as a substitute for an MS analyzer.⁵⁸⁻⁶⁰ Ion mobility spectrometers are like mass spectrometers in the sense that they require the analyte to be ionized, but the separation mechanism is different. Rather than separating ions by their mass-to-charge ratio, ions are separated according to their mobility in an electric field. Thus ion mobility can be regarded in some respects as a form of gas phase electrophoresis.

The simplified schematic of a conventional ion mobility spectrometer strongly resembles a TOF-MS, but rather than

following a collisionless trajectory, ions undergo many collisions as they drift under the influence of an electric field. Other configurations for measuring gas phase mobility are also possible,⁵⁸⁻⁶⁰ but these will not be reviewed in detail here.

An IMS may operate at atmospheric pressure or at reduced pressure but not under a high vacuum because collisions are necessary for its operation. When used in conjunction with a mass spectrometer it is possible to place the mobility device before the first mass analyzer or following one or more stage of mass analysis.

A technique known as field asymmetric ion mobility spectrometry (FAIMS) is also based on ion mobility, but in this case ions are not separated strictly according to their mobility. FAIMS, sometimes known as differential mobility spectrometry (DMS), is based on the fact that the mobility of a gas phase ion is not strictly constant; that is, the drift velocity is not simply proportional to the electric field, but rather at high field there is a deviation from the proportional relationship. FAIMS uses a combination of an asymmetrical high-voltage RF field and a smaller DC field to separate ions according to a combination of low-field mobility and high-field mobility. FAIMS is beginning to find applications in clinical MS when used as a filtering device positioned between the ion source and the mass analyzer.⁶¹

IMS also has been used alone for clinical applications, without being combined with MS. Notably, it has been used to separate unmodified lipoproteins on the basis of size using a differential mobility analyzer (DMA, not to be confused with DMS). The instrument configuration differs from a conventional drift tube IMS, but like a drift tube IMS (and unlike FAIMS), the physical property being measured is gas phase ion mobility. After the separation, each lipoprotein particle is directly detected and counted as it exits the separation chamber, and the lipoprotein subfraction categorization is made based on the mobility of the particles.⁶²⁻⁶⁴

Detectors

With the exception of ICR-MS, orbitrap, and some ICP-MS instruments, most modern mass spectrometers use electron multipliers for ion detection. The main classes of electron multipliers used as MS detectors include the (1) discrete dynode multipliers; (2) continuous dynode electron multipliers (CDEMs), also known as channel electron multipliers; and (3) microchannel plate electron multipliers, also known as multichannel plate electron multipliers. Although different in design, all three work on the same physical principle. Additional types of detectors used in mass spectrometers are the Faraday cup, image current detection, and photomultipliers.

Figure 2.17 presents a conceptual diagram of the operation of a discrete dynode electron multiplier. When an ion strikes the first dynode, it causes the ejection of one or more electrons (secondary electrons) from the dynode surface. The electron is accelerated toward the second dynode by a voltage difference of approximately 100 V. On striking the second dynode, this electron causes the ejection of additional electrons, typically 2 or 3. The second group of electrons is then accelerated toward the third dynode and, on striking the third dynode, causes the ejection of several more electrons. This process is repeated through a chain of dynodes, numbering between 12 and 24 for most designs. The cascade process typically produces a gain of 10^4 to 10^8 ,

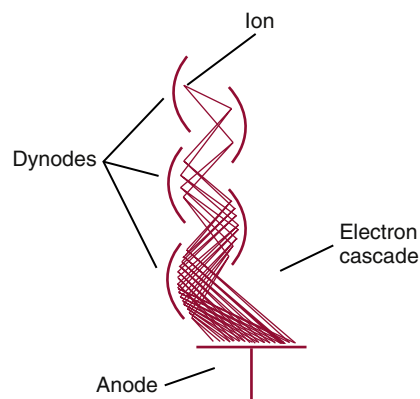


FIGURE 2.17 Discrete dynode electron multiplier showing dynode structure and generation of electron cascade.

meaning that one ion striking the first electrode produces a pulse of 10^4 to 10^8 electrons at the end of the cascade. The duration of the pulse is very short, typically less than 10 ns.

A CDEM works on the same principle as a discrete dynode electron multiplier but differs in design. The set of dynodes of a discrete dynode electron multiplier is replaced by a single continuous resistive surface that acts both as a (continuous) voltage divider to establish the potential gradient and as the secondary electron-generating surface. A microchannel plate electron multiplier is essentially a monolithic array of miniaturized CDEMs fabricated in a single wafer or disk of glass. Sometimes these are stacked into a chevron configuration for added gain.

The Faraday cup is not an electron-multiplying device, but rather a simple electrode that intercepts the ion beam directly. This current is amplified using electronic amplifiers. Because the Faraday cup measures signal intensity directly, rather than indirectly (as in saturation-prone electron multipliers), it provides an absolute measure of ion current and is useful when the magnitude of the signal is too high for electron multiplier-based detection. Some instruments use both electron multiplier and Faraday cup-based detection to provide extended dynamic range—a capability that is especially useful for elemental analysis of trace and toxic elements by ICP-MS.

Detection in ICR occurs via image current detection. This is closely related to the Faraday detection cup in the sense that the ion current is detected directly. However, ions are not destroyed in the process of image current detection and are available for remeasurement. This feature is one of the keys to the versatility of ICR mass spectrometers. Image current detection is also used in the orbitrap.

Closely linked to the detection system is the electronic and signal processing system. In instruments that use electron multiplier detection (the vast majority of mass spectrometers), the raw signal from the detector is processed in one of two ways: (1) individual pulses (corresponding to individual ions) may be counted, as in ion counting systems, or (2) the signal may be converted to a digital representation of the analog signal using an analog-to-digital converter, as in analog detection.

Computer and Software

Because of their (1) mass resolution capabilities, (2) scanning functions, (3) ability to automatically switch between positive

to negative ionization modes, and (4) speed with which multiple m/z signals are acquired, modern MS instruments generate enormous quantities of raw data. In addition, the use of MS in such areas as (1) proteomics, (2) biomarker discovery, (3) synthetic combinatorial chemistry, (4) high-throughput drug discovery, (5) pharmacogenomics, (6) toxicology, and (7) therapeutic drug monitoring requires that MS manufacturers provide powerful computers and software.

In toxicology laboratories, one important function of the data system is library searching to assist in compound identification. Several commercial libraries, including the Wiley Registry of Mass Spectral Data; the NIST Mass Spectral Database; and the Pflieger, Maurer, and Weber drug libraries, are available. In addition, many laboratories generate their own libraries. The quality and number of available spectra, the search algorithm, and whether condensed or full spectra are searched are all important factors in spectral matching.

In proteomics and biomarker discovery, complex mass spectra from single proteins, protein mixtures, or protein digests corresponding to complex samples are obtained. Data systems aid in characterization of spectral data to identify such properties as intact protein mass, amino acid subsequences, and posttranslational modifications. Fragmentation information also can be compared with peptide databases to identify structural mutations that may be present.

The most important function of software in MS systems is data collection and processing. Chromatographic peaks are integrated using data analysis software, and integrated peak intensities or peak areas serve as the basis for quantitative analysis. Calibration curves are generated during data processing, and quantitative results from individual samples are generated using the calibration curves; the data systems also contain report generation capabilities.

Deconvolution protocols have been developed that identify and characterize the mass spectra corresponding to the coeluting peaks. In addition to proprietary deconvolution protocols embedded in the data systems of mass spectrometers supplied by some vendors, there is a freely available deconvolution software program known as AMDIS.⁶⁵

CLINICAL APPLICATIONS

Mass spectrometers coupled with gas or liquid chromatographs (GC-MS or LC-MS) serve as versatile analytical instruments that combine the resolving power of a chromatograph with the specificity of a mass spectrometer.⁶⁶ Such instruments are powerful analytical tools that are used by clinical laboratories to identify and quantify biomolecules. The instruments are capable of providing structural and quantitative information in real time on individual analytes as they elute from a chromatographic column. Specific applications of these coupled instruments can be found in Chapters 5 and 6.

Gas Chromatography—Mass Spectrometry

GC-MS has been used for the analysis of biological samples for several decades. This technique is used by the US National Institute of Standards and Technology and other agencies for the development of definitive methods to qualify standard reference materials and assign accurate concentration to reference materials of many clinically relevant analytes,

including cholesterol, glucose, steroid hormones, creatinine, and urea nitrogen.

One of the most common applications of GC-MS is drug testing for clinical or forensic purposes. Many drugs have relatively low molecular weight and nonpolar and/or volatile properties, making these compounds particularly suitable for analysis by GC. Electron impact ionization with full scan mass detection is the most widely used approach for comprehensive drug screening. Unknown compounds can be identified by matching full mass spectrum of unknown peaks with a mass spectral library or a database. In addition, vendors have recently introduced GC tandem quadrupole (GC-MS/MS) mass spectrometers, which should expand the capability of GC-MS to perform improved targeted and untargeted analysis, thus enhancing existing screening and mass spectral identification capabilities of GC-MS.

GC-MS has many applications beyond drug testing. Numerous xenobiotic compounds are readily analyzed by GC-MS. Applications for anabolic steroids, pesticides, pollutants, and inborn errors of metabolism have been described.⁶⁷⁻⁶⁹

One important limitation to GC-MS is the requirement that compounds be sufficiently volatile to allow transfer from the liquid phase to the mobile carrier gas and thus to elute from the analytical column to the detector. Although many biologic compounds are amenable to chromatographic separation with GC, numerous other compounds are too polar or too large to be analyzed with this technique. In many cases, chemical derivatization is necessary to create sufficiently volatile forms of compounds. Knapp's classic work on derivatization⁷⁰ may be consulted for more information.

Despite its limitations, GC-MS has several positive attributes. High-efficiency separations have been achieved with numerous commercial capillary columns. This technique allows achieving high-efficiency chromatographic separation and excellent limits of quantification, and it allows use of commercial mass spectral libraries for identification of sample constituents. For some of the analytes, such as organic acids, GC-MS has advantages of higher specificity compared to soft ionization techniques used in LC-MS.

Liquid Chromatography—Mass Spectrometry

As discussed earlier, several interface techniques have been developed for coupling a liquid chromatograph to a mass spectrometer, notably ESI and APCI, which have allowed LC-MS and LC-MS/MS to be successfully applied to analysis of a wide range of compounds. In theory, as long as a compound can be dissolved in a liquid, it can be introduced into an LC-MS system. Thus, in addition to low molecular weight polar and nonpolar analytes, large molecular weight compounds, such as proteins, can be analyzed using this technique (see Chapter 6).

LC-MS/MS has gained momentum in the arena of toxicology screening and confirmation.⁷¹⁻⁷³ A majority of the currently used methods for targeted analysis use MRM acquisition using mass transitions corresponding to drugs of interest (see Fig. 2.16, A). For example, within the chromatographic time window of 1.0 to 2.0 minutes, the MRM transitions for selected sympathomimetic amines might be monitored. During the next defined time window,

a new set of MRM transitions are monitored and so on for the rest of the chromatographic run. A related approach is the use of targeted MRM, in which recognition of a chromatographic peak containing a preselected MRM transition triggers a product ion scan in a process called *information-dependent acquisition*, also known as *data-dependent acquisition*. One benefit of this approach is the ability to provide confirmation of the identity of the peaks identified during the analysis.

Coupling of TOF-MS to GC or LC provides a new approach to the identification of unknowns.^{46,74} Because TOF is capable of achieving high mass resolution and high sensitivity, the need for compound fragmentation may be minimized, allowing compound identification based on retention time and accurate mass.⁷⁵

The number of quantitative LC-MS/MS assays introduced for the measurement of clinically important compounds has markedly increased. For example, a few compounds that have been of special interest include (1) immunosuppressant drugs,⁷⁶ (2) biogenic amines,⁷⁷ (3) 25(OH)-vitamin D,⁷⁸ (4) antiretroviral drugs,^{79,80} (5) psychoactive drugs,⁸¹⁻⁸³ (6) methylmalonic acid,^{84,85} (7) thyroid hormones,⁸⁶ and (8) steroids.^{87,88} When quantification of a specific compound is desired, the most effective approach is MRM analysis (see Fig. 2.16, B). With MRM acquisition, both mass filters MF1 and MF2 are set in a static mode, whereby only precursor ions specific for the compound and the internal standard being measured are passed through MF1. This preselected precursor ion is then fragmented in the collision cell, and molecule-specific fragment ions derived from the compound of interest are passed by MF2 to the detector. Because only one ion is monitored in MF1 and typically two molecule-specific fragment ions monitored in MF2, as opposed to scanning for multiple ions, the MRM approach allows much greater specificity as well as lower limits of quantification.

Another area in which MS/MS is used clinically is screening and confirmation of genetic disorders and inborn errors of metabolism.^{89,90} The ability to analyze multiple compounds in a single analytical run makes this technique an efficient tool for screening purposes. In this application, in some cases MS/MS is of sufficient selectivity to eliminate the need to incorporate LC separation, a simplification that allows high-throughput analysis.

Electrospray-MS/MS is also used for carnitine and acylcarnitine analysis to detect organic acidemias and fatty acid oxidation defects.^{91,92} In the methods for acylcarnitine and amino acid analysis, these compounds vary widely in their polarity, which creates problems with consistency of response factors. To address this issue, most methods use a butyl ester derivatization of the carboxyl group to force cationic character on the amino acids and thus enhance the ionization efficiency.⁹³

Acylcarnitines can be analyzed without derivatization,⁹⁴ but most often are analyzed as butyl esters using a *precursor ion scan* mode of acquisition (see Fig. 2.16, C).^{95,96} This type of acquisition makes use of the fact that acylcarnitines have a common collision-induced m/z 85 product ion (represented by X in Fig. 2.16, C) that is selectively monitored in MF2. MF1 is set to scan for precursors with m/z 85, thus detecting and identifying acylcarnitines present in the sample (see Fig. 2.16, C). By incorporating in the method stable isotope-labeled analogs of the targeted acylcarnitines,

it is possible in addition to identification, to establish concentration of the acylcarnitines of interest.

Analysis of amino acids by LC-MS/MS is typically performed using traditional MRM monitoring but also can be performed using a data acquisition mode known as *constant neutral loss* (see Fig. 2.16, D).⁸⁸ Butyl derivatives of α -amino acids share a common neutral product, butylformate, which has a mass of 102 Da (represented by X in Fig. 2.16, D). By scanning for both product (MF2) and precursor (MF1) ions, and by keeping a constant offset between the two mass m/z analyzers (eg, a difference of 102 m/z units), any m/z differences that equal 102 Da can be used to detect and identify amino acids present in the samples.

One advantage of LC-MS/MS relative to GC-MS is that in many cases it allows avoidance of derivatization of the target compounds, but in some cases derivatization is useful for LC-MS/MS as well. An example of butyl ester derivatization was discussed previously. In this example, the derivative has more favorable fragmentation properties than the underivatized compounds. Similarly, the dibutyl ester of methylmalonic acid (MMA), when run in positive ion mode ESI, has more favorable MS/MS spectra than the underivatized compound run in negative ion mode ESI; in addition, the dibutyl esters of dicarboxylic acids are selectively ionized in positive ion mode ESI, whereas monocarboxylic organic acids are not efficiently ionized and are therefore not detected by the mass spectrometer.⁸⁴ By using MMA extraction at conditions specific for acidic compounds and detection specific for polycarboxylic acids, it is possible to perform the LC-MS/MS analysis using isocratic chromatographic separation without the need for reconditioning and reequilibration of the chromatographic column between injections.⁸⁴

The most frequent reason for using derivatization in LC-MS and LC-MS/MS is to achieve improved ionization efficiency. Gao and colleagues⁹⁷ have emphasized this issue in an extensive discussion of derivatization in ESI and APCI MS.

Product ion scan is another mode of acquisition using MS/MS; in this scan mode, the first mass filter is fixed to pass a specific m/z and the second mass filter is scanned over a specified range of m/z values. Product ion scan mode is very useful for structural elucidation, such as in peptide sequencing, but is less useful for routine quantitative analysis.

Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

MALDI (typically coupled with a TOF analyzer) has been used to analyze many different classes of compounds. Notably, it has been widely applied in discovery applications for the detection and identification of proteins and peptides (see Chapter 6). Primary limitations include high background noise and a higher coefficient of variation that seems inherent in the MALDI ionization process. In addition, MALDI is essentially a batch-type process that does not interface naturally with online separation processes using chromatographic techniques (eg, HPLC, capillary electrophoresis).

MALDI-TOF is often used to determine the identity of proteins through peptide mass fingerprinting. This technique has been used to identify a large number of two-dimensional (2D) gel spots for the bacterial pathogen *Pseudomonas*

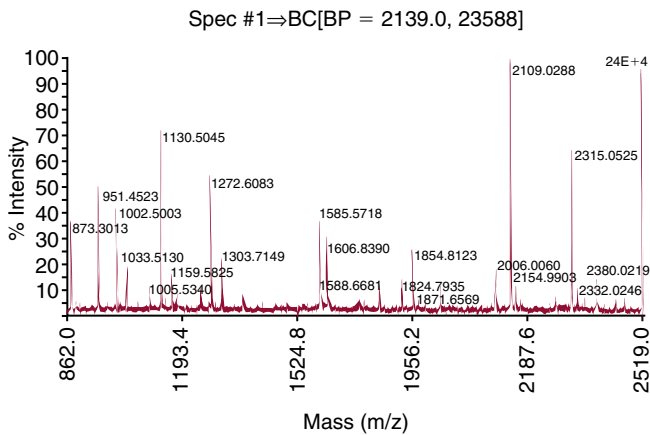


FIGURE 2.18 Example of a matrix-assisted laser desorption ionization–time-of-flight spectrum showing peptides generated in a tryptic digest of a spot cored from a two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. The 16 most abundant m/z values were submitted to the MS-Fit database for searching against the nonredundant database. The results for this search are shown in Table 2.1.

aeruginosa.⁹⁸ The procedure generally involves in-gel tryptic digestion followed by accurate mass measurement of the peptides produced during the digestion. The generated mass list is then compared with theoretical tryptic masses for proteins in a database (Fig. 2.18 and Table 2.1). This procedure, which works best for organisms with complete

and annotated genomes, is very rapid because 100 or more samples may be deposited on a single MALDI target plate and automatically processed. In the previous example,⁹⁸ the group rapidly identified a large number of proteins that were expressed differently among the studied bacteria. In addition, it was found that some proteins were listed as “hypothetical,” meaning they were previously undescribed or confirmed to be expressed, and that the theoretical molecular weight and/or isoelectric point (pI) in some cases were different from those measured in the gel, indicating possible loss of terminal amino acids and/or posttranslational modifications.

One clinical application of MALDI-TOF that has proved its clinical utility is identification of microorganisms (discussed in more depth in Chapter 4). Identification of the bacteria is performed by fingerprinting proteins and peptides extracted from cultures using gentle conditions.⁹⁹ The basis of this technique is that different bacteria express unique mixtures of proteins and peptides; when samples are analyzed using MALDI-TOF, the bacteria-specific mass spectra are observed in the 2- to 20-kDa mass range, allowing database searching and classification based on the protein mass fingerprint. One of the disadvantages⁹⁹ of the technique is the lack of actual protein information and the relative lack of specificity to different strains of the same bacteria. The protein mass fingerprints must be catalogued (entered in mass spectral library) for each bacterium and validated to be specific and reproducible for a given extraction method.

TABLE 2.1 Example of Printout of Bacterial Identification Through Peptide Mass Fingerprinting Using Matrix-Assisted Laser Desorption Ionization MALDI-Time of Flight*

Rank	Mowse Score	# (%) Masses Matched	Protein Mw (Da)/pI	Species	NCBI nr.81602 Accession #	Protein Name
1	1.07e+008	14/16 (87%)	101754.9/9.15	<i>Saccharomyces cerevisiae</i>	6321275	(Z72685) ORF YGL163c
1. 14/16 matches (87%). 101754.9 Da, pI = 9.15. Acc. #6321275. <i>Saccharomyces cerevisiae</i> . (Z72685) ORF YGL163c.						
m/z Submitted	MH ⁺ Matched	Delta ppm	Start	End	Peptide Sequence (Click for Fragment Ions)	Modifications
870.4746	870.4797	-5.8732	598	606	(K) GVGGSQLR(A)	
873.3981	873.3929	5.9793	774	779	(K) DCFIYR(F)	C ² H ² O ²
951.4901	951.4900	0.1050	814	821	(R) LFSSDNLR(Q)	
1002.5385	1002.5373	1.2224	515	522	(K) NFENPILR(G)	
1033.5513	1033.5543	-2.8793	46	55	(K) NTHIPPAAGR(I)	
1130.6349	1130.6322	2.4037	120	128	(R) LSHIQYTLR(R)	
1130.6349	1130.6322	2.4037	514	522	(R) KNFENPILR(G)	
1159.6039	1159.6071	-2.7957	56	67	(R) IATGSDNIVGGR(S)	
1272.6508	1272.6483	1.9865	734	746	(K) AGGCGINLIGANR(L)	C ² H ² O ²
1303.7573	1303.7599	-1.9457	270	280	(K) ILRPHQVEGVR(F)	
1585.7190	1585.7215	-1.5602	446	459	(K) NCVNGLMLADEGHR(L)	C ² H ² O ²
1606.8861	1606.9029	-10.4650	22	35	(R) LVPRPINVQDSVNR(L)	
2138.0756	2138.0704	2.4250	747	765	(R) LILMDPDWNPAAADQQALAR(V)	
2315.1093	2315.0951	6.1321	401	423	(K) SSMGGNTTVSQAIHAWAQAQGR(N)	
2388.0671	2388.0731	-2.5004	293	313	(K) DYLAEEAFNTSSSEDPLKSDEK(A)	

MH⁺, Ion formed by attachment of a proton to molecule M; MOWSE, MOlecular Weight SEarch method; MW, molecular weight; m/z , mass-to-charge ratio

*A generated mass list is compared with theoretical tryptic masses for proteins in a database. Match quality is used for pathogen identification.

Some of these drawbacks were addressed in a MALDI technique that targets ribosomal proteins.¹⁰⁰ This technique was evaluated using 1116 isolates collected in a routine clinical microbiology laboratory and was described as being fast, reliable, and easy to use. More than 95% of clinical isolates were correctly identified, and most of the previously incorrectly identified isolates were assigned to the correct genus or a closely related genus. Bacterial identification by MALDI MS is rapidly becoming a routine method in microbiology laboratories.

MALDI MS has the reputation of being a nonquantitative technique. However, some progress has been made toward its use as a quantitative technique.¹⁰¹ If this application becomes routine, it could have major benefits for clinical MS because the time to acquire a mass spectrum by MALDI is only a few seconds. This could dramatically improve throughput. However, it seems likely that this application will require off-line separation (or sample fractionation) before loading on the MALDI target, to obtain sufficient selectivity necessary for clinical applications.

Inductively Coupled Plasma Mass Spectrometry

ICP-MS is used for the determination of trace and toxic elements in many types of samples (see references 10, 102, and 103). However, it is known that the toxicity of an element may depend on the organic or inorganic state in which the element is present. In these cases, it is more important to ascertain the concentrations of toxic species rather than the total concentration of the element. To extend the usefulness of this technique, GC and HPLC systems have been coupled to ICP-MS to separate different compounds containing the targeted element before ICP-MS analysis.¹⁰⁴

Proteomics, Genomics, and Metabolomics

The past 20+ years have seen tremendous progress in genomics, with hundreds of genomes completed or near completion and many now parsed and annotated. This information is highly complex, mainly because of the myriad changes that occur to proteins produced from the genome throughout the life cycle of a cell, but potentially will provide a better understanding of the cellular functions and allow discovery of novel disease biomarkers.¹⁰⁵ In the mid-1990s, MS came to the forefront of analytical techniques used to study proteins, and the term *proteomics* was coined. Although the definition of proteomics is still debated, for the present discussion it is taken to encompass knowledge of the structure, function, and expression of all proteins in the biochemical or biological contexts of all organism.¹⁰⁶ In a more basic and practical sense, proteomics refers to the identification and quantification of proteins and their posttranslational modifications in a given system or systems. Proteome analysis is a powerful tool for investigating (1) biomarkers of disease, (2) antigens of pathogens, (3) drug target proteins, and (4) posttranslational modifications, as well as for other investigations. This is a challenging task in that a given gene may have many distinct chemical protein isoforms. In addition, many other molecules (metals, lipids, etc.) interact with proteins in a noncovalent fashion. Therefore in a genome, such as the human genome, a repertoire of more than a million proteins may require identification and quantification. Two foundations are necessary to begin

this daunting challenge. The first is the basic sequence expected for each possible protein in a cell (ie, information from a completed genome). The second is instrumentation, which currently consists of advanced mass spectrometers that identify and quantify protein isoforms in an automated fashion at very low limits of detection. Both foundations are now essentially in place. However, the goals previously stated are far from being reached, and considerable advances will need to be made in the field of systems biology for better understanding of the biological systems.

Currently, MS is routinely used to accomplish many tasks in proteomics. The most basic task is protein identification. The typical approach is known as the *bottom-up* method, whereby proteins are separated—by gel electrophoresis or by solution-based methods—and then digested. The resulting enzymatic fragments are analyzed and used to identify the protein(s) present. This process is time-consuming and has many pitfalls. Increasingly, much research has been devoted to analysis of mixtures of proteins. These mixtures are derived from biological fluids, cellular compartments, tissue, or immunoprecipitation. Currently, both instrumentation and data analysis software are not sufficiently advanced to allow unambiguous identification of all the proteins in highly complex biological samples. As a result, much emphasis has been placed on separation methods and enrichment techniques for preparing samples for analysis of proteins and peptides.

Another approach that was shown to enable sequencing of intact proteins and posttranslational modifications is known as the *top-down* method. Top-down proteomics involves identification of proteins in complex mixtures without prior digestion of proteins into peptides. Approaches used for protein top-down characterization include extraction of the proteins from samples, fractionation and analysis of the samples using high-resolution accurate mass MS/MS with CID, higher energy collision dissociation, and electron-transfer dissociation fragmentation. Main benefits of the top-down analysis are in the ability to detect in the samples proteins containing posttranslational modifications and their sequence variants.

Many research groups have introduced methods allowing handling of highly complex biological samples. The most popular approaches include subcellular fractionation, multidimensional chromatography, affinity enrichment, and multiplexing. By combining these approaches, several thousand protein species can be identified routinely. Obviously these numbers are better than those obtained through bottom-up methods from gels, but they still fall far short of those necessary for complete understanding of biological systems.

The term *proteomics* is often used in the context of biomarker discovery. To date, very few markers have been discovered using proteomics methods that have migrated to the clinical laboratory. Some of the reasons for the dearth of new protein biomarkers have recently been discussed.¹⁰⁷ From a broader view, however, proteomics also may include the application of MS for the analysis of known protein and peptide biomarkers. For example, mass spectrometric methods for the analysis of carbohydrate-deficient transferrin have been developed, including a reference method¹⁰⁸ and a method for routine patient testing. Additional areas of

application of MS include analysis of the proteome of the pathogenic mold *Aspergillus fumigatus* with the aim of identifying vaccine candidates and new allergens.¹⁰⁹

Analysis of thyroglobulin, a widely used marker of the recurrence of thyroid cancer, has been described by several groups.¹¹⁰⁻¹¹² The initial digestion of the thyroglobulin by trypsin also digests and therefore removes autoantibodies which cause interference in immunoassays. Currently assays based on the above principle are offered in several commercial laboratories and represents substantial progress in the application of MS for routine analysis of proteins as well as providing important information to treating physicians.

Promising proof-of-principle research has been performed on the characterization of hemoglobinopathies by MS.^{113,114} Most methods for hemoglobin analysis use MS to detect separate hemoglobin chains or peptide products from enzymatic digests of hemoglobin. However, as shown by Rockwood and coworkers,¹¹⁵ and shortly thereafter by Ganem and associates,^{116,117} the retention of higher order structure, such as noncovalent complexes, is possible when ions are transferred from solution to gas phase, and hemoglobin tetramers have been observed by MS.¹¹⁸ Another hemoglobin application, a reference method for hemoglobin A_{1c} using MS, has been approved by the International Federation of Clinical Chemistry and Laboratory Medicine.¹¹⁹ LC-MS methods for quantitative analysis of hepcidin, a peptide hormone believed to be a master regulator of iron status, have been published.¹²⁰⁻¹²²

Genetic applications for clinical MS are beginning to emerge. For example, MALDI-TOF has been used for mutation detection in myeloproliferative disorders,¹²³ DNA methylation analysis,^{124,125} and gene expression analysis.¹²⁶

A promising genomic approach for pathogen identification uses polymerase chain reaction amplification of selected regions of a pathogen genome, followed by accurate mass measurement using ESI-TOF-MS. From the accurate mass information, a DNA base composition is computed, and the results are matched to pathogen DNA base compositions in a database.^{127,128}

A burgeoning area in which MS plays a role is the emerging field of *metabolomics*. This scientific area involves the investigation and characterization of small molecules, including intermediates and products of metabolism, present in biological fluids under different conditions that include (1) normal homeostasis, (2) disease states, (3) stress, (4) dietary modification, (5) treatment protocols, and (6) aging. In a fashion similar to a mass spectrum providing a fingerprint signature for a specific molecule, it has been speculated that compounds identified and evaluated in metabolomic studies may provide a fingerprint signature for different physiologic states.

In practice, metabolites are identified through comparison with (1) known reference materials, (2) commercial or in-house developed mass spectral libraries or metabolite databases, (3) interpretation of mass spectra, or (4) ancillary techniques such as nuclear magnetic resonance. As with other applications of MS, both GC-MS and LC-MS have a place in such studies. GC-MS has some potential advantages that were described earlier. To use GC-MS, however, the metabolites in the sample must be volatile, or derivatization

needs to be used to enhance detectability of a larger number of compounds.

LC-MS has its own usefulness in metabolomics because it has potentially wider applicability to polar and nonpolar compounds and allows the observation of the molecular or pseudomolecular ions. However, because reference materials or isotope-labeled internal reference materials do not exist for validating ionization efficiencies or recoveries for some of the biologically relevant compounds, the effects of ion suppression (discussed later) remain a potential confounding factor. Compared with proteomic research, metabolomics faces the added difficulties in that the MS/MS spectra are more difficult to interpret, scarce information is available in the MS/MS libraries, and DNA and protein sequence databases are of no use in interpreting the results.

Mass spectral imaging of tissue sections is another emerging technology that has potential for clinical applications and holds a great promise. The most common approach is to apply MALDI MS to image tissue sections.^{129,130} A mass spectrum is acquired at each spot on a regularly spaced array across the sample. From these data, an image is constructed for each *m/z*. The images provide a spatial map of chemical composition (peptides or small molecules) from the sample. Another approach uses laser ablation, followed by ICP-MS, to provide a spatial map of the inorganic elemental composition of the sample.¹³¹ This technique can be extended to immunohistochemical imaging by using metal-labeled antibodies.¹³² With this scheme, it is possible to use different labels on different antibodies to do multiplexed imaging of several different targets on the same sample. However, it should be noted that at the present time mass spectral imaging is an extremely time-consuming process because the beam has to be rastered across the tissue by the laser many thousands of times and very large amounts of data need to be processed and analyzed. At this time this is mainly a research technique that is not used in routine diagnostic laboratories.

Practical Aspects of Mass Spectrometry: Logistics, Operations, and Quality

In many respects, the logistics, operations, quality control, and quality assurance processes for clinical MS laboratories follow the well-established clinical laboratory standards and guidelines. However, mass spectrometers are complex instruments and most manufacturers of instrumentation are still learning how to best support their clients in clinical laboratories. Consequently, the adoption of MS, and especially the more complex technologies such as LC-MS/MS, places added demands on training, competency, and manufacturers' support beyond those of more familiar and well-established technologies used in clinical laboratories.

In contrast to techniques such as optical spectrophotometry, mass spectrometers tend to require more frequent troubleshooting, tuning, calibration, and optimization, and the laboratory inspection checklist of the College of American Pathologists specifies that mass spectrometer performance should be verified daily.¹³³ In addition, the frequency of calibrations and optimizations needed to maintain instruments in fit-for-purpose condition will vary, depending

on the requirements of the assays being performed, the instrumentation used, and other factors.

The term *calibration* in relation to MS is used in at least two distinct ways. The first is calibration of the m/z scale of the instrument, usually referred to as *mass calibration*. The other is calibration for quantitative analysis.

Schedules for mass calibration vary among laboratories, types of instruments used, and types of assays being run. For example, if accurate mass measurements are an important part of a method, as with many assays that employ TOF-MS, then very frequent mass calibration is typically required. In some cases, internal mass reference materials are included within each run, or even within each sample. For applications that are less dependent on mass accuracy, such as most quantitative methods performed on quadrupole mass analyzers, mass calibration may be performed less frequently. For example, mass calibration may be performed every few weeks, with verification of mass calibration performed more frequently—as often as daily in some laboratories.

Similarly, based on validation results obtained in individual laboratories, schedules for calibration for quantitative analysis may vary. For example, some laboratories calibrate an assay daily, whereas others calibrate with every run.

One advantage of MS is that most methods in the MS laboratories avoid the use of highly specialized reagents such as commercial kits of reagents and antibodies. Consumables are mostly generic items, such as solvents, chromatographic columns, and sample vials or 96-well plates. This tends to buffer the laboratory from supply disruptions of specialized reagents, and in some cases can decrease consumable costs as well. However, consumables must be carefully selected and monitored for quality, because contaminated reagents and supplies may negatively affect performance of the methods; this problem is far too common. Solvent quality is of particular concern. For example, one study documented wide variations in methanol quality from different suppliers, which can lead to large differences in ionization efficiency and cause interferences in the analysis.¹³⁴

Whenever possible, a quantitative method should use isotopically labeled internal standards, which typically differ from the analyte of interest by substitution of monoisotopic ions with isotope labels (typically deuterium, ^{13}C , or ^{15}N). However, it is not always possible to obtain isotopically labeled versions of each target analyte, in which case a closely related chemical analog should be selected as an internal standard.

MS provides several opportunities for enhancing analytical quality and therefore improving patient care. The high degree of selectivity of MS, particularly when included as a part of hyphenated techniques (GC-MS, LC-MS, LC-MS/MS, etc.) reduces the likelihood of interference compared with immunoassays or separation-based techniques (GC or LC) using nonspecific detection, particularly for small molecule analysis, where cross-reactivity is a concern.¹³⁵ Perhaps as important as a high degree of selectivity, MS provides a means to detect the presence of interferences when they occur. With methods that produce fragmentation, in the ion source (as in EI) or in a collision cell (as in MS/MS), fragment ions are produced with reproducible relative intensity (compared to the base peak). By monitoring one or more ratios of the ion fragments, and by comparing these ratios to ratios

obtained from authentic reference materials measured in the same run, it is possible to detect the presence of interfering compounds on a sample-by-sample basis.¹³⁶

In addition, accurate mass measurements are useful for detecting interferences if one is using an instrument capable of such measurements, such as a TOF mass spectrometer. To illustrate, the accurate mass of protonated cortisol ($\text{C}_{21}\text{H}_{31}\text{O}_5^+$) is 363.2166 Da, whereas the accurate mass of one of the isotope peaks of protonated molecule of the drug fenofibrate ($\text{C}_{20}\text{H}_{22}\text{ClO}_4^+$) is 363.1180 Da—a difference of 271 ppm. Therefore an interference of even a few percent by fenofibrate (drug used for treating patients with high cholesterol and high triglycerides) in a cortisol analysis would be detectable as a shift in mass of the observed peak on an instrument capable of low single-digit parts per million mass accuracy.¹³⁷

Obviously, detection of interferences by accurate mass measurement alone becomes more difficult as the mass of the interfering compound approaches that of the target compound, but given the ability to detect interferences at a $\approx 20\%$ level or better, and assuming a mass spectrometer of ≈ 3 ppm mass accuracy, a reasonable estimate is that interferences could be detected for all compounds with $|\Delta m/z|$ greater than 30 ppm relative to the target compound. Given the cortisol example discussed earlier, 22 chemical formulas for ions are within 30 ppm of the mass of protonated cortisol, provided we limit our list to the composition constraints listed earlier. Interferences from these would be difficult or impossible to detect by mass measurement alone. Thus accurate mass would likely detect the majority of possible interferences, but some of the potentially interfering compounds would be difficult or impossible to detect.

OPTIMIZATION OF INSTRUMENT CONDITIONS

When developing an MS-based method there are many parameters to optimize. This applies to both the mass spectrometer and the separation method.

Selection of Mass Transitions and Operating Conditions

MRM is the most commonly used type of acquisition in LC-MS/MS methods for targeted analysis. MRM-based methods allow sensitive and specific quantitation of analytes in samples with complex matrices. Typical MRM chromatograms contain one peak or a few peaks, which are easy to integrate, particularly if the sample preparation has been well designed.

When developing MRM methods, it is useful to start with identifying all analyte-specific mass transitions. The typical approach for selection and optimization of mass transitions is through infusion of solution of pure standard of the targeted compound using syringe pump. During the infusion, the signal can be optimized by adjusting the ion source conditions, declustering potential, the ion transmission conditions, and collisional fragmentation. When ions are transported from atmospheric pressure to the vacuum region, they typically exist in the form of clusters. Application of the declustering potential during the ion focusing causes low-energy collisions, which lead to declustering of the ions.

The MRM experiments are set by specifying m/z of the precursor ion (typically the molecular ion) of the targeted molecule and the m/z of the molecule-specific fragments, produced by fragmentation in the collision cell. While developing a method, it is important to carefully assess which mass transitions to be used in the method. The best sensitivity and specificity are typically achieved using high-intensity unique fragment ions, which have minimal background noise and no interfering peaks coeluting with the analyte of interest. Use of fragment ions corresponding to the loss of water, ammonia, carbonyl (CO), and CO₂ groups generally should be avoided because they result in nonspecific mass transitions.

The optimal values of the voltages needed for declustering the molecular ion and the ion transmission are established by scanning the voltages and finding the apex values that correspond to the maximum signal intensity. Optimization of the collision energy (CE) is accomplished by scanning the CE used for fragmentation of the molecular ion, and plotting the abundances as a function of CE produces a profile, called a breakdown curve (Fig. 2.19).

In the majority of cases, the voltage corresponding to the apex is selected for use in the method. At this value of the CE there is maximum signal intensity. A second advantage of operating at the apex is that slight fluctuations in the instrument conditions do not result in large changes in the signal intensity, so the acquired signal is more stable. However, on some occasions, when unresolved chromatographic peaks could interfere with the analysis, it is beneficial to select CE on the leading slope or the trailing slope of the breakdown curve (more commonly on the leading slope) to improve specificity of analysis by avoiding fragmentation of the substance that potentially interferes with target analyte. This can be useful in cases in which the breakdown curve of an MS/MS transition of a potentially interfering compound partially overlaps that of the target compound. In such cases, by operating on the slope of the breakdown curve of the selected MS/MS transition of the target compound it may be possible to more strongly discriminate from the interfering substances.

In addition to evaluating breakdown curves, CE should be selected with the following principles in mind. If the CE is too low, ion fragmentation will be inefficient, and the signal

abundance will be low. If the CE is too high, fragmentation can become too extensive; the number of peaks in the product ion spectrum may increase intensity of the peaks that are selective to the product ion of interest may have insufficient intensity to be useful, and the possibility of interfering peaks from coeluting isobars may increase.

Depending on the type of ionization source used, ionizability of molecules is influenced by their volatility, pKa, proton affinity, electronegativity, hydrophilicity/hydrophobicity, surfactant properties, solution pH, ionization energy, electron collision cross-section, etc. Of the ionization methods most commonly used in the MS applications in clinical laboratories, ESI is typically used for analysis of polar and ionizable molecules and APCI and APPI are used for nonpolar molecules. When a new method is being developed, available ionization techniques and polarity modes should be evaluated to assess effect of the conditions on the signal response and specificity of the detection.

The electrospray voltage and ion source conditions have a great effect on the ionization efficiency; the optimal voltage depends on the molecular structure, mobile phase composition, and flow rate. At higher electrospray voltages, greater fluctuation in the ionization efficiency can be observed and a larger number of impurities present in the sample may get ionized, potentially causing the loss of specificity and poor reproducibility. Therefore the lowest voltage resulting in an adequate sensitivity for the analyte is typically preferred. In general terms, there tends to be a threshold voltage, below which ionization is very inefficient; if the ESI voltage is too high, corona discharge or other undesirable effects may occur.

Online Two-Dimensional Separations

Two-dimensional chromatographic separation is a technique in which separation is performed using two HPLC columns with phases having different selectivity. The chromatographic columns are connected to each other through a switching valve, the sample is injected in the first column, and effluent of the column is directed to waste. At the time when the targeted peak is eluted from the first column, the effluent is redirected into the second column, then during the column reconditioning and equilibration the switching valve is turned back to the original position. Using this approach, chromatographic columns with complementary (orthogonal)

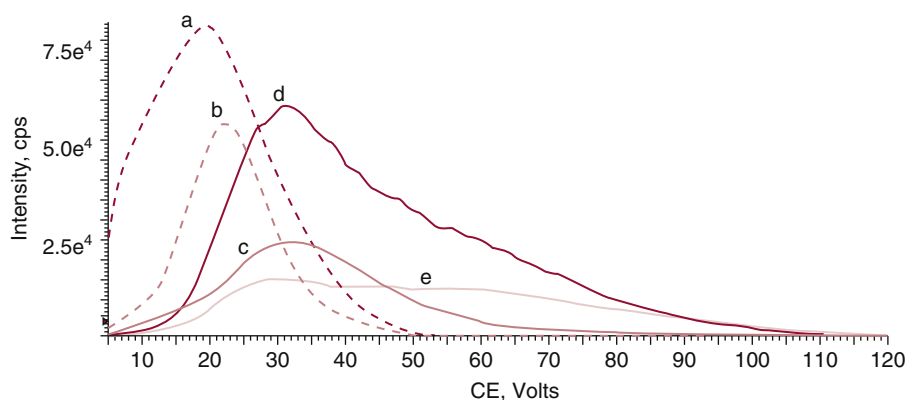


FIGURE 2.19 Breakdown curves for collision energy scans of cortisol. Curves correspond to mass transitions: a— m/z 363 → 345, b— m/z 363 → 327, c— m/z 363 → 171, d— m/z 363 → 121, e— m/z 363 → 97. cps, Counts per second; CE, collision energy.

or partially orthogonal) selectivity are typically used, so that peaks that are poorly resolved or unresolved by the first column would get separated on the second column.

In addition to the use of different stationary phases with complementary retention mechanisms, the selectivity may be modified through selection of the optimum for each separation mobile phase and temperature. Some advantages of well-designed 2D separations may include greater resolving power, faster analysis time (while the separation takes place on one column, the other column could be conditioned and reequilibrated), reduced contamination of the mass analyzer (major fraction of the effluent from the first column is directed to waste and not transferred into the second column), and ability to use for the second separation a mobile phase that is favorable for the optimal ionization efficiency. Various coupling strategies have been developed for the switching valve configuration and the peak transfer from the first to the second column; the choice of the specific strategy is method-dependent and would affect the robustness of the assay. Two-dimensional separations are more difficult to develop and troubleshoot, but in many cases the benefits in the methods' performance outweigh the drawbacks.

Conventional Versus Microflow Separations

As more LC-MS methods are developed, greater sensitivity and reduced sample volume are often required. This is especially true for analysis of novel biomarkers. Other trends in modern analytical laboratories are aimed at reducing the volume of solvents used, the cost of the used mobile phase disposal, and the costs of labor. The benefits of microflow separations in LC-MS analysis have been widely reported¹³⁸; they include a higher sensitivity, greater efficiency of ion sampling, reduced solvent consumption and waste, and reduced contamination of the ion source. Despite the advantages, there are relatively few micro-flow based LC-MS methods currently used in routine laboratories. The main reasons are related to the fact that these separations historically were insufficiently rugged, required greater technical expertise of the staff, and caused frequent interruptions in the workflow of a laboratory. However, recent publications on comparison of the microflow and high-flow-rate traditional LC-MS/MS methods demonstrated the rugged method's performance with up to 10-fold gain in the signal-to-noise ratios and up to 20-fold reduction in the use of the solvents.¹³⁸

Ion Suppression

Ion suppression is another quality issue that should be evaluated during method development and validation.¹³⁹ First described in 1993,^{140,141} ion suppression is a matrix effect that results from the presence of coeluting nonvolatile or less volatile compounds (or compounds with greater proton affinity) that change the efficiency of spray droplet formation, ionic properties, and evaporation. These interfering substances, which include salts, ion-pairing agents, endogenous compounds, surfactants, drugs and/or metabolites, compete with analyte ions for access to the droplet surface or transfer to the gas phase, which, in turn, affects the number of charged ions in the gas phase that ultimately reach the detector.¹⁴² Anions, such as phosphate or borate

in buffers, also can neutralize the effective ionization of an analyte. Phospholipids present in biological samples and impurities introduced during the sample preparation and analysis have been demonstrated to be major contributors to ion suppression.^{134,143}

Ion suppression refers to the effect of the constituents of the sample that suppresses ionization of the analyte of interest. Factors contributing to ion suppression include greater ionizability of the substances coeluting with the peak of interest, and concentration of the coeluting substance causing ion suppression. Ion suppression can have an adverse effect on the accuracy, precision, and sensitivity of the assay, particularly if the internal standard does not perfectly coelute with the targeted analyte, in which case the internal standard and the analyte may undergo different extents of ion suppression and thus compromise quantitative measurements. This is most likely to happen if the internal standard is highly deuterated. Ion suppression also may reduce the signal of the target analyte to nearly undetectable levels, in which case it is essentially impossible to obtain an accurate quantitative result. Of the different types of ionization techniques used in LC-MS methods, ESI tends to be most susceptible to ion suppression; methods using APCI are typically less prone to the effects of ion suppression, but the possibility of ion suppression in APCI should not be dismissed without validation.

Considering that biological samples contain a large number of endogenous molecules with concentrations ranging over a very wide dynamic range, ion suppression should be expected and the effects of ion suppression should be evaluated for all new or modified methods. The presence of ion suppression or other deleterious matrix effects can be evaluated via several experimental protocols.¹ One involves comparison of (1) the instrument response for reference materials (including any internal standards) injected directly in the mobile phase, and (2) the same amount of compound spiked into preextracted samples.¹⁴⁴ Data for the standard in the mobile phase provide a relative 100% response value. Data for the same amount of compound spiked into preextracted samples show the effects of sample matrix on MS response (ion suppression).

A second, more commonly used and preferred protocol involves postcolumn continuous infusion of compound into the MS detector, while analyzing samples (type intended for the evaluated method, eg, serum, plasma, urine) prepared according to the protocol of the evaluated method.¹⁴⁵⁻¹⁴⁷ The instrumental setup includes a syringe pump connected via a tee to the column effluent (Fig. 2.20). Because the compound being tested is introduced into the ion source at a constant rate, a constant instrument response should be observed if no ionization suppression or enhancement occurs while analyzing biological specimens (Fig. 2.21, A). Typically there is suppression of the signal at the portion of the analysis that corresponds to the void volume of the HPLC column (see Fig. 2.21, B). The void volume is that portion of a chromatogram corresponding to no retention other than the time it takes for the mobile phase to flow through the column. Thus it represents a chromatographic time region, despite the word *volume* in the terminology.

The degree of ion suppression and the recovery time to full response can vary from assay to assay¹⁴⁵ and among the

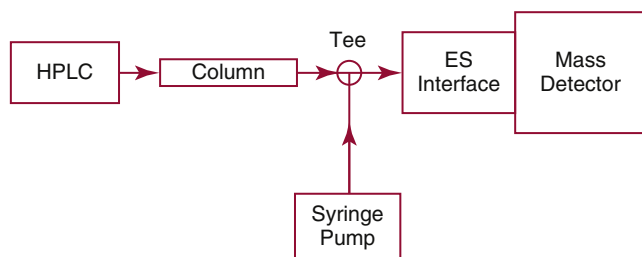


FIGURE 2.20 Postcolumn infusion system. Mobile phase or specimen extracts are injected into the high-performance liquid chromatography (HPLC) system. The analyte being evaluated is continuously infused, post column, and is mixed with the column effluent through a tee before entering the electrospray interface (ES).

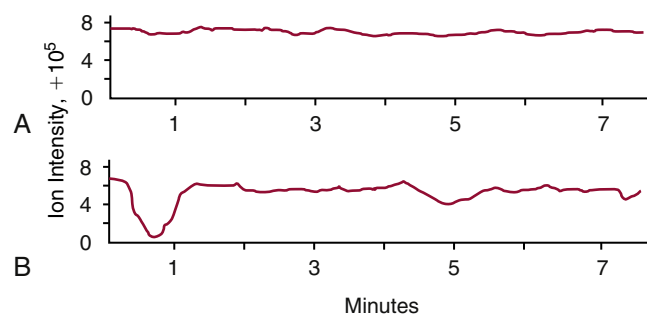


FIGURE 2.21 Infusion chromatograms for hypothetical analytes. A, Mobile-phase injection. B, Serum liquid-liquid extract injection. These profiles illustrate that ion suppression can be greater than 90%, that a recovery time may exist, and that suppression is not limited to the solvent front region. For a comprehensive presentation of these types of effects, the reader is referred to references 145 to 147.

samples and can be dependent on the sample preparation method, chromatographic column used, and LC separation conditions. Because endogenous compounds from the specimen matrix may elute at any time during the chromatographic run, ion suppression is not limited to the column void and not limited to the analysis time of the evaluated sample. In the case of strongly retained compounds, substances causing ion suppression may elute in subsequent injections. Considering this, the detector response should be monitored during analysis of multiple patient samples, to ensure that ion suppression will not affect subsequent injections. The observed degree of ion suppression also can be dependent on the sample volume aliquotted for the analysis, the injection volume, and the concentration of the analyte being monitored,¹⁴⁸ which is related to the matrix-to-analyte concentration ratio.¹⁴⁹ It should be noted that the degree of the matrix effect might differ among the samples of the same biological material, as has been ably shown by Matuszewski and colleagues.^{150,151}

To control for ion suppression, it is highly desirable to use matrix-matched calibration standards and controls. It is important to evaluate ion suppression for all types of sample matrices intended for analysis by the method; in addition, considering the complexity of biological samples and the between-subject differences, there may be substantial

fluctuations in the concentrations of the ion-suppressing species among samples. Because of this, a significant number of individual samples of all sample matrices intended for the method (serum, plasma, urine, etc.) should be used during the evaluation of the ion suppression.

In cases in which the isotope-labeled internal standards do not completely coelute with the analytes of interest, ion suppression cannot be completely compensated by the internal standard. This problem is particularly acute when using an internal standard that is highly deuterated, because these compounds are likely to not totally coelute with the native compound, and this can lead to significant quantitative errors in the analysis.¹⁵² The ¹³C- or ¹⁵N-labeled compounds are chromatographically retained identically to the nonlabeled analogs and are not susceptible to the previously described problem.

Ion suppression is not limited to HPLC-MS or ESI/APCI ion sources. For MALDI analysis, arginine-containing peptides have been reported to dominate over the signal from other peptides in protein digests,¹⁵³ with the extent depending on the matrix used. The presence of ionic detergents, such as Triton X-100 and Tween 20, has also been shown to cause signal suppression in MALDI experiments, which can be countered by modifications to the matrix.¹⁵⁴

Noise Reduction Techniques

In MS, background noise refers to the sum of electronic and chemical noise, which is independent of the data signal. Presence of the background noise interferes with the measurements and affects accuracy and specificity of analysis, especially at low concentrations. Reduction of chemical noise has been one of the aims for improvement since introduction of MS as an analytical technique. This is sometimes known as the “peak-at-every-mass” problem. The problem has long been known to mass spectrometrists, and it affects virtually all ionization methods to some degree.

Chemical noise is often dominant over electronic noise in MS. Background ions are inherent of atmospheric pressure ionization and related to the presence of impurities in the samples and in the mobile phases, residues accumulating on the surfaces of the ion source, and in part the high efficiency of atmospheric pressure ionization. Approaches used for noise reduction include optimizing the sample preparation, improving selectivity of ionization, optimization of the declustering conditions and ion transmission, maintaining cleanliness of the ion sources, and the flow path of the separation device.

One effective way for significant reduction of the effect of the background noise on the methods’ performance is the use of MS/MS acquisition (MRM, neutral loss scan, product ion scan, and precursor ion scan), which allows substantial improvement of the detection specificity and reduction of the effects of chemical noise. Other approaches for reduction of the background noise include the use of mass analyzers with high resolving power (see Fig. 2.3); the use of multidimensional separations along with MS, such as ion mobility separations (IMS); high FAIMS; multidimensional chromatographic separations; and the incorporation of additional stages of fragmentation (MS/MS/MS). Software-based approaches (eg, dynamic background subtraction, active background noise reduction) also have been applied

as noise reduction techniques. The previously mentioned techniques allow a reduction in the interference from the chemical background noise, but do not affect its cause. The best approach to the reduction of chemical background noise is the use of more extensive and efficient sample cleanup (as a way of minimizing introduction of contaminants into the ion source) and the use of high-purity solvents and additives for the mobile phases. This brings up the general issue of developing methods that are fit for purpose, but not so complex or expensive that their use will be impractical. Every laboratory needs to balance these factors in a way that is consistent with their goals, throughput, and constraints.

With regard to reducing background noise by using mass analyzers with high resolving power, it is important to understand the relationships among resolution, background noise (primarily chemical noise), electronic noise, and total signal level. A complete discussion is beyond the scope of the chapter, but a few general concepts can be useful to the clinical chemist without necessarily delving into all of the subtleties. For the sake of discussion, let us consider TOF detection. As mentioned earlier, this type of mass spectrometer acquires the full mass spectrum; that is, it is not possible to operate the instrument in SIM mode (or MRM mode in the case of a quadrupole TOF). However, it is possible to simulate SIM or MRM mode in postprocessing by integrating the mass spectrum over a limited m/z range and plotting the result as a function of spectrum number or chromatographic retention time.

If the m/z window is wide compared to the mass spectral peak width, the portion of the integrated signal arising from the targeted peak is independent of peak width. However, the signal arising from chemical noise generally increases with increasing width of the m/z window. This corresponds to the conditions in [Figure 2.3, A](#)—the FWHM of the TOF mass spectrometer was approximately 0.06 Da, whereas the window for generating the simulated SIM was 1 Da. Thus one would expect the chemical noise to decrease as the width of the integration window decreases.

The simulated SIM in [Figure 2.3, B](#) has the integration window set at 0.0072 Da, nearly an order of magnitude narrower than the peak width of the mass spectrometer. Under these conditions (ie, with the integration window much narrower than the peak width), narrowing the integration window still further affects chemical noise and target analyte signal nearly equally, so there is very little to be gained in terms of improving the signal-to-noise ratio by making the integration window narrower. Furthermore, in some cases there is a danger of increased statistical noise in the signal because ion numbers are quantized, which shows up as shot noise in the integrated signal. The signal-to-noise ratio in the shot noise-limit scales as 1 over the square root of the integration window width—that is, it gets worse as the window is made narrower. Discussion of the effect of the electronic noise on the noise budget is outside of the scope of this chapter, but it can be evaluated using methods analogous to those discussed previously.

Thus, in many cases, there is an optimum operating condition wherein the optimal integration window is often roughly equal to the peak width of the mass spectrometer. One additional comment is in order. Narrowing the integration window does nothing to reduce chemical noise if an

interfering species is strictly isobaric with the targeted species. This implies that it is not at all useful if the goal is to reduce interferences from isomers. Furthermore, it is of very little usefulness if the mass offset of the interfering compound from the targeted compound is less than the peak width of the mass spectrometer. Nevertheless, as [Figure 2.3](#) illustrates, a narrow window can be useful in improving the quality of chromatograms, and, as one can infer from the earlier discussion, this technique works best when using a high-resolution mass spectrometer.

POINTS TO REMEMBER

- MS is a highly sensitive and selective technique for analyzing a wide variety of clinically relevant analytes.
- MS relies on ionizing analytes in a sample, followed by separation of the ions according to their mass to charge ratios.
- When coupled with a separation technology such as gas chromatography or liquid chromatography, the hybrid technique is well suited for the quantitative analysis of clinically relevant molecules from bodily tissues and fluids.
- The use of MS for clinical applications has led to the development of highly specific assays that can overcome many of the issues faced when using immunoassays, such as cross-reactivity.
- Although highly selective, MS is not immune from interferences; molecules with same m/z (isomers and isobars) and similar fragmentation pattern may interfere with analysis

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