1. INTRODUCTION

At the beginning of the twentieth century, mass spectrometers were invented to help physicists and physical chemists to prove the existence of isotopes of the elements. As radioactivity and nuclear physics were explored, specialized mass spectrometers were used to characterize the fission products of radioactive elements as they were created or discovered. In addition, mass spectrometers were used for the measurement of isotopic enrichment of radioactive elements, their inorganic derivatives, and even the isotopic purification of radioactive elements as inorganic compounds. As this era of mass spectrometry reached maturity by the 1940s, some physicists announced that there would no longer be any need for mass spectrometry since virtually all of the elements had been discovered and characterized. Of course, these prognosticators were wrong because the entire field of organic mass spectrometry was about to begin.

1.1. Electron Impact (EI)

While mass spectrometers were being used for the purification of fissionable material for atomic weapons as part of the Manhattan Project of World War II, organic mass spectrometry was being invented for the analysis and quality control of petroleum distillates and petroleum-based fuels. By 1945, the application of mass spectrometry to organic chemistry had emerged as a productive new area of research and discovery. Commercial production of organic mass spectrometers began during the 1940s, and petroleum companies became the first customers for these new analytical instruments. Early commercial mass spectrometers used electron impact ionization (see Eqs 1 and 2) to generate ions from gas-phase molecules that were separated by acceleration through an electromagnetic field provided by either a fixed magnet or an electromagnet. After separation, the ions were detected using a simple impact detector such as a Faraday cup. This basic design is still in use today for the identification and quantitative analysis of volatile organic compounds.

$$M + e^-(70 \text{ eV}) \rightarrow M^+ + 2e^- \text{ formation of positive molecular ions using EI ionization}$$

$$M + e^-(2-10 \text{ eV}) \rightarrow M^+ \text{ electron capture EI ionization}$$

Toward the late 1950s, organic mass spectrometers began to be used for the analysis of a wider variety of organic molecules and eventually became a fundamental analytical tool for the characterization of synthetic organic compounds. Today, mass spectrometers are used routinely to confirm the molecular masses of organic compounds, to determine elemental compositions and to verify their structures based on fragmentation patterns. Fragmentation results from the cleavage of chemical bonds within an ion resulting in the formation of a product ion of lower mass and one or more neutral products. Of course, only the fragment ions and not the neutral species are detected in a mass spectrometer because this instrument measures the mass-to-charge ratio ($m/z$) of ions in the gas phase. The energy for fragmentation is either the result of excess energy imparted to the molecular ion during ionization or during a process known as collision-induced dissociation (CID) which will be discussed along with tandem mass spectrometry (MS–MS) below. Since the fragmentation pattern reflects the relative strengths of chemical bonds in a compound, mass spectra (a plot of ion relative abundance versus $m/z$) provide structurally significant fragment ions for compound identification. Rules for structure elucidation of chemical structures through the interpretation of mass spectra have been developed. (For a review of EI and
In many cases, EI imparts so much excess energy into a molecule that only fragment ions and no molecular ions are observed. Therefore, “softer” ionization techniques were developed to enhance molecular mass information. The first of these ionization methods was chemical ionization (CI). Developed by researchers in the petroleum industry [1], CI became another standard ionization technique for organic mass spectrometry. During CI, high-energy electrons (as in EI) are used to ionize a gas called a reagent gas at a constant pressure (usually ~1 mbar) in the mass spectrometer ionization source. The reagent gas in turn ionizes the sample molecules through ion-molecule reactions that usually involve the exchange of protons. Less frequently, sample molecule ionization might involve a charge exchange. Two of the most common ionization mechanisms in CI are summarized in Equations 3 and 4.

\[ M + RH^+ \rightarrow MH^+ + RCI \] through proton transfer, \( R = \) reagent gas \( \text{(3)} \)

\[ M + R^+ \rightarrow M^+ + R \]

CI through charge exchange \( \text{(4)} \)

1.2. Types of Mass Analyzers

During the 1960s, high-resolution double-focusing magnetic sector instruments became standard tools for the determination of elemental compositions using a type of analysis called accurate mass measurement. In mass spectrometry, resolving power is defined as \( M/\Delta M \) (resolution is the inverse of this term, \( \Delta M/M \)), where \( M \) is the \( m/z \) value of a singly charged ion, and \( \Delta M \) is the difference (measured in \( m/z \)) between \( M \) and the next highest ion. Alternatively, \( \Delta M \) may be defined in terms of the width of the peak. High resolution is typically regarded as a resolving power of at least 10,000 such that the molecular ions of most drug-like molecules (that is, compounds with molecular masses less than ~500) can be resolved from each other. After resolving a sample ion from others in a mass spectrum, an accurate mass measurement may be carried out by comparing the \( m/z \) value of the unknown to that of a calibration standard.

Since the 1960s, other types of mass spectrometers capable of high-resolution accurate mass measurements have become available as commercial products including Fourier transform ion cyclotron resonance (FTICR) mass spectrometers, reflectron TOF instruments, quadrupole time-of-flight hybrid (QqTOF) mass spectrometers, and recently, ion trap-TOF hybrid mass spectrometers (see Table 1 for a listing of types of organic mass spectrometers and a comparison of their performance characteristics). By the early 2000s, FTICR and QqTOF instruments became more popular than magnetic sector mass spectrometers for accurate mass measurements, high-resolution measurements, and drug discovery applications. As will be discussed below, accurate mass measurements are essential to many types of mass spectrometry-based screening and drug discovery today.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Resolving Power</th>
<th>( m/z ) Range</th>
<th>Tandem MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic sector</td>
<td>100,000</td>
<td>12,000</td>
<td>Low resolution</td>
</tr>
<tr>
<td>Quadrupole</td>
<td>&lt;4,000</td>
<td>4,000</td>
<td>None</td>
</tr>
<tr>
<td>Triple quadrupole</td>
<td>&lt;4,000</td>
<td>4,000</td>
<td>Low resolution</td>
</tr>
<tr>
<td>Time-of-flight (TOF)</td>
<td>15,000</td>
<td>&gt;200,000</td>
<td>None</td>
</tr>
<tr>
<td>FTICR</td>
<td>&gt;200,000</td>
<td>&lt;10,000</td>
<td>MS*, high resolution</td>
</tr>
<tr>
<td>Orbitrap</td>
<td>&lt;200,000</td>
<td>6,000</td>
<td>MS*, high resolution</td>
</tr>
<tr>
<td>Ion trap</td>
<td>&lt;4,000</td>
<td>&lt;10,000</td>
<td>MS*, low resolution</td>
</tr>
<tr>
<td>QqTOF</td>
<td>&gt;14,000</td>
<td>4,000</td>
<td>High resolution</td>
</tr>
<tr>
<td>Ion trap-TOF</td>
<td>&gt;10,000</td>
<td>4,000</td>
<td>High resolution</td>
</tr>
<tr>
<td>TOF-TOF</td>
<td>15,000</td>
<td>&gt;10,000</td>
<td>High resolution</td>
</tr>
</tbody>
</table>

Table 1. Types of Mass Spectrometers and Tandem Mass Spectrometers
1.3. Gas Chromatography-Mass Spectrometry

Biomedical applications of mass spectrometry began during the 1960s both at academic institutions and at pharmaceutical companies. These applications depended upon the volatilization (usually by heating) of pharmaceutical compounds and biochemicals prior to their gas-phase ionization using EI or CI. In order to increase the thermal stability and volatility of these compounds, a variety of derivatization methods were developed to mask polar functional groups and reduce hydrogen bonding between molecules. These methods were particularly useful for use with gas chromatography-mass spectrometry (GC-MS) which was introduced during the 1960s as a practical and powerful tool for qualitative and quantitative analysis of compounds in mixtures. Both EI and CI were immediately useful for GC-MS, since both of these ionization methods require that the analytes be in the gas phase. When capillary GC was incorporated into GC-MS, this technique reached maturity. GC-MS may be used to select, identify and quantify organic compounds in complex mixtures at the femtomole level. The speed of GC-MS is determined by the chromatography step, which typically requires several minutes up to one hour per analysis. By the 1970s, some organic chemists were announcing that organic mass spectrometry had reached maturity and that no new applications were possible. Like the physicists and physical chemists who had pronounced the end of mass spectrometry a generation earlier, this group would soon be proved wrong.

Although GC-MS remains important for the analysis of many organic compounds, this technique is limited to volatile and thermally stable compounds that comprise only a small fraction of all organic compounds and even fewer biomedically important molecules. Therefore, thermally unstable compounds including many pharmaceutical compounds such as nucleic acid analogs and biomolecules such as proteins, carbohydrates, and nucleic acids cannot be analyzed in their native forms using GC-MS. (For more details regarding GC-MS and its applications, see Watson, 2007 provided in Section 4) Although derivatization facilitates the GC-MS analysis of many of these compounds, alternative ionization techniques were needed for the analysis of the vast majority of polar and nonvolatile compounds of interest to drug discovery.

1.4. Desorption Ionization Techniques

During the 1970s and the early 1980s, desorption ionization techniques such as field desorption (FD), desorption EI, desorption CI (DCI), and laser desorption were developed to extend the utility of mass spectrometry toward the analysis of more polar and less volatile compounds (See Watson, 2007 provided in Section 4) for more information regarding desorption ionization techniques including DCI and FD). Although these techniques helped extend the mass range of mass spectrometry beyond a general limit of $m/z$ 1000 and toward ions of $m/z$ 5000, a breakthrough in the analysis of polar, nonvolatile compounds occurred in 1982 with the invention of fast atom bombardment (FAB) [2]. FAB and its counterpart liquid secondary ion mass spectrometry (LSIMS) facilitate the formation of abundant molecular ions, protonated molecules, and deprotonated molecules of nonvolatile and thermally labile compounds such as peptides, chlorophylls, and complex lipids up to approximately $m/z$ 12,000. FAB and LSIMS use energetic particle bombardment (fast atoms or ions from 3–30,000 V of energy) to ionize compounds dissolved in nonvolatile matrices such as glycerol or 3-nitrobenzyl alcohol and desorb them from this condensed phase into the gas phase for mass spectrometric analysis. Protonated or deprotonated molecules are usually abundant and fragmentation is minimal. Although still in use, FAB and LSIMS have been replaced by electrospray and matrix-assisted laser desorption ionization (MALDI) for most biomedical applications.

Introduced in the late 1980s, MALDI has helped solve the mass limit barriers of laser desorption mass spectrometry so that singly charged ions may be obtained up to $m/z$ 500,000 and sometimes higher [3]. For most commercially available MALDI mass spectrometers, ions up to $m/z$ 200,000 are readily obtained. Like FAB and LSIMS, MALDI samples are mixed with a matrix to form a solution
that is loaded onto the sample stage for analysis. Unlike the other matrix-mediated techniques, the solvent is evaporated prior to MALDI analysis leaving sample molecules trapped in crystals of solid-phase matrix. The MALDI matrix is selected to absorb the pulse of laser light directed at the sample. Most MALDI mass spectrometers are equipped with a pulsed UV laser, although IR lasers are available as an option on some commercial instruments. Therefore, matrices are often substituted benzenes or benzoic acids with strong UV absorption properties. During MALDI, the energy of the short but intense UV laser pulse obliterates the matrix and in the process desorbs and ionizes the sample (see Fig. 1). Like FAB and LSIMS, MALDI typically produces abundant protonated or deprotonated molecules with little fragmentation.

1.5. Liquid Chromatography-Mass Spectrometry (LC-MS)

By the time that GC-MS had become a standard technique in the late 1960s, LC-MS was still in the developmental stages. Producing gas-phase sample ions for analysis in a vacuum system while removing the HPLC mobile phase proved to be a challenging task. Early LC-MS techniques included a moving belt interface to desolvate and transport the HPLC eluate into a CI or EI ion source or a direct inlet system in which the eluate was pumped at a low flow rate (1–3 μL/min) into a CI source. However, neither of these systems was robust enough or suitable for a broad enough range of samples to gain widespread acceptance. Since FAB and LSIMS require that the analyte be dissolved in a liquid matrix, this ionization technique was easily adapted for use with HPLC in an approach known as continuous-flow FAB [4]. Because the matrix often interfered with HPLC and required postcolumn addition and because the LC-MS interface required frequent maintenance and cleaning, continuous-flow FAB is rarely used today.

Like continuous-flow FAB, the popularity of particle beam interfaces has diminished. During particle beam LC-MS, the HPLC eluate is sprayed into a heated chamber connected to a vacuum pump. As the droplets evaporate, aggregates of analyte (particles) form and pass through a momentum

![Figure 1. Scheme showing the process of MALDI from a solid matrix absorbing strongly at the wavelength of the incident laser pulse.](image-url)
separator that removes the lower molecular mass solvent molecules. Finally, the particle beam enters the mass spectrometer ion source where the aggregates strike a heated plate from which the analyte molecules evaporate and are ionized using conventional EI or CI. Particle beam LC-MS is limited to the analysis of volatile and thermally stable compounds that are amenable to flash evaporation and EI or CI mass spectrometry.

Thermospray became the first widely utilized LC-MS technique (during the late 1970s and the early 1980s). During thermospray, the HPLC eluate is sprayed through a heated capillary into a heated desolvation chamber at reduced pressure. Gas-phase ions remaining after desolvation of the droplets are extracted through a skimmer into the mass spectrometer for analysis. The sensitivity of thermospray is poor since there is no mechanism or driving force to enhance the number of sample ions entering the gas phase from the spray during desolvation. Also, thermally labile compounds tend to decompose in the heated source. These problems were solved when thermospray was replaced by electrospray.

Electrospray and atmospheric pressure chemical ionization (APCI) have become the most widely used ionization sources and HPLC-interfaces for drug discovery using mass spectrometry. Unlike thermospray, particle beam or continuous-flow FAB, electrospray and APCI interfaces operate at atmospheric pressure, do not depend upon vacuum pumps to remove solvent vapor, and are compatible with a wider range of HPLC flow rates. Also, no matrix is required. Both APCI and electrospray are compatible with a wide range of HPLC columns and solvent systems. Like all LC-MS systems, the solvent system should contain only volatile solvents, buffers or ion pair agents to reduce fouling of the mass spectrometer ion source. In general, APCI and electrospray form abundant molecular ion species. When fragment ions are formed, they are usually more abundant in APCI than electrospray mass spectra.

The APCI ion source and HPLC interface (see Fig. 2) uses a heated nebulizer to form a fine spray of the HPLC eluate, which is much finer than the particle beam system but similar to that formed during thermospray. Heated nitrogen gas is used to facilitate the

Figure 2. During APCI, eluate from a HPLC system is sprayed through a heated capillary and desolvated using heated nitrogen. Functioning as a CI reagent gas, solvent molecules are ionized by a corona discharge and then ionize sample molecules through proton transfer or charge exchange.
evaporation of solvent from the droplets. The resulting gas-phase sample molecules are ionized by collisions with solvents ions, which are formed by a corona discharge in the atmospheric pressure chamber. Molecular ions, \( M^+ \) or \( M^- \), and/or protonated or deprotonated molecules can be formed. The relative abundance of each type of ion depends upon the sample itself, the HPLC solvent, and the ion source parameters. Next, ions are drawn into the mass spectrometer analyzer for measurement through a narrow opening or skimmer that helps the vacuum pumps to maintain very low pressure inside the analyzer while the APCI source remains at atmospheric pressure. For example, the positive ion APCI mass spectrum of lycopene is shown in Fig. 3. The carotenoid lycopene is the red pigment of ripe tomatoes and is under clinical investigation for the prevention of prostate cancer [5].

During electrospray, the HPLC eluate is sprayed through a capillary electrode at high potential (usually 2000–7000 V) to form a fine mist of charged droplets at atmospheric pressure. As the charged droplets migrate toward the opening of the mass spectrometer due to electrostatic attraction, they encounter a cross-flow of heated nitrogen that increases solvent evaporation and prevents most of the solvent molecules from entering the mass spectrometer (see Fig. 4). Molecular ions, protonated or deprotonated molecules, and cationized species such as \([M+Na]^+\) and \([M+K]^+\) can be formed. (For additional information on electrospray ionization, see Cole, 1997 provided in Section 4). In addition to singly charged ions, electrospray is unique as an ionization technique in that multiply charged species are common and often constitute the majority of the sample ion abundance. The relative abundance of each of these species depends upon the chemistry of the analyte, the pH, the presence of proton donating or accepting species, and the levels of trace amounts of sodium or potassium salts in the mobile phase. In contrast, APCI, APPI, MALDI, EI, CI, and FAB/LSIMS usually produce singly charged species. A consequence of forming multiply charged ions is that they are detected at lower \( m/z \) values (i.e., \( z > 1 \)) than the corresponding singly charged species. This has the benefit of allowing mass spectrometers with modest \( m/z \) ranges to detect and measure ions of molecules with very high masses. For example, electrospray has been used to measure ions with molecular masses of hundreds of thousands or even millions of daltons on mass spectrometers with \( m/z \) ranges of only a few thousands. (For a review of LC-MS techniques, see Niessen, 2006 provided in Section 4.)

An example of the C18 reversed phase HPLC-negative ion electrospray mass spectrometric (LC-MS) analysis of an extract of the botanical *Trifolium pratense* L. (red clover) is shown in Fig. 5. Extracts of red clover are

![Figure 3](attachment:figure3.png)

**Figure 3.** Positive ion APCI mass spectrum of the red carotenoid lycopene in a solution of methanol and tert-butyl methyl ether (1 : 1; v/v). In this analysis, lycopene formed a protonated molecule detected at \( m/z \) 537 as the base peak of the mass spectrum instead of a molecular ion, \( M^+ \).
used as dietary supplements by menopausal and postmenopausal women and are under investigation as alternatives to estrogen replacement therapy [6]. The two-dimensional map illustrates the amount of information that may be acquired using hyphenated techniques such as LC-MS. In the time dimension, chromatograms are obtained, and a sample computer-reconstructed mass chromatogram is shown for the signal at $m/z$ 269. An intense chromatographic peak was detected eluting at 12.4 min. In the $m/z$ dimension, the negative ion electrospray mass spectrum recorded at 12.4 min shows a base peak at $m/z$ 269. Based on comparison to authentic standards (data not shown), the ion of $m/z$ 269 was found to correspond to the deprotonated molecule of genistein that is an estrogenic isoflavone [6]. Since almost no fragmentation of the genistein ion was observed, additional characterization would require CID and MS–MS as discussed in the next section.

When analyzing complex mixtures such as the botanical extract shown in Fig. 5, the use of chromatographic separation prior to mass spectrometric ionization and analysis is
essential in order to distinguish between isomeric compounds. Even simple mixtures of synthetic compounds might contain isomers that would require LC-MS for adequate characterization. Another problem overcome by utilizing a chromatography step prior to mass spectrometric analysis is ion suppression. No matter what ionization technique is used, the presence of multiple compounds in the ion source might enhance the ionization of one compound while suppressing the ionization of another. Usually, only some of the compounds in a complex mixture can be detected by mass spectrometry without chromatographic separation. The presence of salts and buffers in a sample can also suppress sample ionization. Therefore, LC-MS has become a powerful tool for analyzing natural products, synthetic organic compounds, and pharmaceutical agents and their metabolites.

In general, APCI facilitates the ionization of nonpolar and low molecular mass species and electrospray is more useful for the ionization of polar and high molecular mass compounds. In this sense, APCI and electrospray are often complementary ionization techniques. However, during the analysis of large or diverse combinatorial libraries, both polar

Figure 5. Two-dimensional map showing the LC-MS analysis of an extract of red clover under investigation for the management of menopause. Reversed phase separation was carried out using a C18 HPLC column in the time dimension and negative ion electrospray mass spectrometry was used for compound detection and molecular mass determination in the second dimension.
and nonpolar compounds are usually present. As a result, no one set of ionization conditions using APCI or electrospray is adequate to detect all the compounds contained in the library of compounds. Therefore, a UV ionization technique called atmospheric pressure photoionization (APPI) has been developed for use with combinatorial libraries and LC-MS [7]. During APPI, a liquid solution or HPLC eluate is sprayed at atmospheric pressure as in APCI. Instead of using a corona discharge as in APCI, ionization occurs during APPI due to irradiation of the analyte molecules by an intense UV light source. Obviously, the carrier solvent must not absorb UV light at the same wavelengths or interference would prevent sample ionization and detection.

1.6. Tandem Mass Spectrometry (MS–MS)

Desorption ionization techniques such as FAB, MALDI, and electrospray facilitate the molecular mass determination of a wide range of polar, nonpolar, low, and high molecular mass compounds including drugs and drug targets such as proteins. However, the “soft” ionization character of these techniques means that most of the ion current is concentrated in molecular ions and few structurally significant fragment ions are formed. In order to enhance the amount of structural information in these mass spectra, CID may be used to produce more abundant fragment ions from molecular ion precursors formed and isolated during the first stage of mass spectrometry. Then, a second mass spectrometric analysis may be used to characterize the resulting product ions. This process is called tandem mass spectrometry or MS–MS and is illustrated in Fig. 6.

Another advantage of the use of tandem mass spectrometry is the ability to isolate a particular ion such as the molecular ion of the analyte of interest during the first mass spectrometry stage. This precursor ion is essentially purified in the gas phase and free of impurities such as solvent ions, matrix ions, or other analytes. Finally, the selected ion is fragmented using CID and analyzed using a second mass spectrometry stage. In this manner, the resulting tandem spectrum contains exclusively analyte ions without impurities that might interfere with the interpretation of the fragmentation patterns. In summary, CID may be used with LC-MS-MS or desorption ionization and MS-MS to obtain structural information such as amino acid sequences of peptides, sites of alkylation of nucleic acids, or to distinguish structural isomers such as β-carotene and lycopene.

The types of tandem mass spectrometers used for biomedical research are shown in

Figure 6. Negative ion electrospray tandem mass spectrum of lycopene. CID was used to induce fragmentation of the molecular ion of m/z 536. As a result, the fragment ion of m/z 467 was formed by the loss of a terminal isoprene unit. This fragment ion may be used to distinguish lycopene from isomeric α-carotene and β-carotene that lack terminal isoprene groups.
Table 1 and include triple quadrupole instruments (ideal for quantitative analysis), ion trap and FTICR (high resolution) mass spectrometers which are capable of multiple stages of tandem mass spectrometry, and hybrid instruments such as QqTOF and ion trap-TOF mass spectrometers (capable of high-resolution tandem mass spectrometry). Except for the TOF–TOF mass spectrometers, all the instruments listed in Table 1 are compatible with electrospray, APPI, and APCI and may be interfaced with HPLC systems. The TOF–TOF mass spectrometer is ideally suited for use with a pulsed ionization technique such as MALDI.

Over the course of the last century, mass spectrometry has become an essential analytical tool for a wide variety of biomedical applications including drug discovery and development. By combining mass spectrometry with chromatography as in LC-MS or by adding another stage of mass spectrometry as in MS–MS, the selectivity of the technique increases considerably. As a result, mass spectrometry offers all of the analytical elements that are essential to modern drug discovery namely speed, sensitivity, and selectivity.

2. CURRENT TRENDS AND RECENT DEVELOPMENTS

Since the early 1990s, drug discovery research has focused on combinatorial chemistry [8,9] and high-throughput screening [10] in an effort to accelerate the pace of drug discovery. The goal has been to produce in a short time large numbers of synthetic organic compounds representing a great diversity of chemical structures through a process called combinatorial chemistry and then quickly screen them in vitro against pharmacologically significant targets such as enzymes or receptors. The “hits” identified through these high-throughput screens may then be optimized by quickly and efficiently synthesizing and then screening large numbers of analogs called targeted or directed libraries. As a result, lead compounds might emerge from such combinatorial chemistry drug discovery programs in a few weeks instead of several years. Furthermore, a single organic chemist using combinatorial synthetic methods might synthesize thousands of compounds or more in a single week instead of less than five in the same time using conventional techniques, and a single medicinal chemist might identify hundreds of lead compounds per month instead of just one or two in the same period of time.

Accompanying this new drug discovery paradigm, new scientific journals have been established such as Combinatorial Chemistry & High Throughput Screening, Journal of Combinatorial Chemistry, Journal of Biomolecular Screening, and Molecular Diversity (see the list of journal Web sites provided in Section 4). The variety of topics published in these journals reflects the multidisciplinary nature of the current drug discovery process and ranges from organic chemistry, medicinal chemistry, molecular modeling, molecular biology, and pharmacology to analytical chemistry. As described below, the most significant analytical component of drug discovery has become mass spectrometry. Only mass spectrometry has become an essential element at all stages of the drug discovery and development process.

Although a variety of spectroscopic and chromatographic techniques including infrared spectroscopy, nuclear magnetic resonance spectroscopy, fluorescence spectroscopy, gas chromatography, high-performance liquid chromatography (HPLC) and mass spectrometry are being used to support drug discovery in various capacities, some of them such as gas chromatography and fluorescence spectroscopy are not applicable to most new chemical entities, some are not specific enough for chemical identification (e.g., infrared spectroscopy), and other techniques suffer from low throughput (e.g., nuclear magnetic resonance spectroscopy). Unlike gas chromatography, HPLC is compatible with virtually all drug-like molecules without the need for chemical derivatization to increase thermal stability or volatility. In addition, mass spectrometry provides a universal means to characterize and distinguish drugs based on both molecular mass and structural features while at the same time providing high throughput. With the development of routine LC-MS interfaces and ionization techniques such as electrospray and APCI, mass spectrometry has also
become an ideal HPLC detector for the analysis of combinatorial libraries [11], and LC-MS, MS-MS, and LC-MS-MS have become fundamental tools in the analysis of combinatorial libraries and subsequent drug development studies [12–14].

The application of combinatorial chemistry and high-throughput screening to drug discovery has altered the traditional serial process of lead identification and optimization that previously required years of human effort. Consequently, neither the synthesis of new chemical entities nor their screening is limiting the pace of drug discovery. Instead, a new bottleneck is the verification of the structure and purity of each compound in a combinatorial library or of each lead compound obtained from an uncharacterized library using high-throughput screening. Since the number of lead compounds entering the drug development process has increased in part because compounds are entering development at earlier stages than in the past, the traditional drug development investigations concerning absorption, distribution, metabolism, and excretion (ADME) and even toxicology evaluations of new drug entities have become additional bottlenecks. As a solution to the drug development bottlenecks, high-throughput assays to assess the metabolism, bioavailability, and toxicity of lead compounds are being developed and applied earlier than ever during the drug discovery process so that only those compounds most likely to become successful drugs enter the more expensive and slower preclinical pharmacology and toxicology studies. In support of these new combinatorial chemistry synthetic programs and new high-throughput assays, mass spectrometry has emerged as the only analytical technique with sufficient throughput, sensitivity, selectivity, and robustness to address all of these bottlenecks.

2.1. LC-MS Purification of Combinatorial Libraries

Although combinatorial libraries were originally synthesized as mixtures, today most libraries are prepared in parallel as discrete compounds and then screened individually in microtiter plates of 96-well, 384-well, or 1536-well formats. In order to facilitate subsequent structure–activity analyses and to assure the validity of the screening results, many laboratories verify the structure and purity of each compound prior to high-throughput screening. Semipreparative HPLC has become the most popular technique for the purification of combinatorial libraries on the milligram scale because of high throughput and the ease of automation. Typically during semipreparative HPLC, fraction collection is initiated whenever a UV signal is observed above a predetermined threshold. This procedure usually results in the collection of several fractions per analysis and hence creates additional issues such as the need for large fraction collector beds and the need for secondary analysis using flow injection mass spectrometry, LC-MS, or LC-MS-MS to identify the appropriate fractions. When purification of large numbers of combinatorial libraries is required, this approach can become prohibitively time consuming and expensive.

To enhance the efficiency of this purification procedure, the steps of HPLC purification and mass spectrometric analysis may be combined into automated mass-directed fractionation [15–17]. Any size HPLC column may be used, and only a small fraction of the eluant (≈μL/min) is diverted to the mass spectrometer equipped for APCI or electrospray ionization. Since all of the components including autosampler, injector, HPLC, switching valve, mass spectrometer, and fraction collector are controlled by computer, the procedure may be fully automated. For greatest efficiency, the system may be programmed to collect only those peaks displaying the desired molecular ions or alternatively, all peaks displaying abundant ions within a specified mass range. An example of the MS-guided purification of a compound synthesized during the parallel synthesis of a combinatorial library of discrete compounds is shown in Fig. 7. Although the crude yield of the reaction product was only 30% (Fig. 7a), the desired product was detected based on its molecular ion (Fig. 7b). After MS-guided fractionation, reanalysis using LC-MS showed that the desired product was >90% pure (Fig. 7c).

The use of MS-guided purification of combinatorial libraries provides a means for
reducing the number of HPLC fractions collected per sample and eliminates the need for postpurification analysis to further characterize and identify each compound as would be necessary when using UV-based fractionation. The ionization technique (i.e., electrospray, APCI, or APPI), and ionization mode (positive or negative) must be suitable for the combinatorial compound, so that molecular ion species are formed. Also, a suitable mobile phase and HPLC column must be selected. As an alternative to HPLC, supercritical fluid chromatography-mass spectrometry (SFC-MS) has been used for the high-throughput analysis of combinatorial libraries [18,19]. The advantages of SFC-MS relative to conventional LC-MS for the purification of combinatorial libraries of compounds are the lower viscosities and higher diffusivities of condensed CO₂ compared to HPLC mobile phases, and the ease of solvent removal and disposal after analysis. However, SFC instrumentation remains more expensive and less widely available than conventional HPLC systems. Furthermore, many drug-like molecules are poorly soluble in condensed CO₂.

### 2.2. Confirmation of Structure and Purity of Combinatorial Compounds

The determination of molecular masses, elemental compositions, and structures of compounds used for high-throughput screening, whether discrete compounds or combinatorial library mixtures, is typically carried out using mass spectrometry, since traditional spectroscopic and gravimetric techniques are too slow to keep pace with combinatorial chemical synthesis. In addition, mass spectrometry may be used to assess the purity of compounds being used for high-throughput screening. The highest throughput technique for confirming molecular masses and structures of drug candidates is flow injection analysis of sample solutions using electrospray, APCI, or APPI mass spectrometry. Typically, no sample preparation is necessary.
Although any organic mass spectrometer may be used to confirm the molecular mass of a compound, tandem mass spectrometers provide additional structural information through the use of CID to produce fragment ions. As discussed above (see also Table 1), tandem mass spectrometers include triple quadrupole instruments, QqTOF mass spectrometers, ion trap-TOF mass spectrometers, ion trap mass spectrometers, multiple sector magnetic sector instruments, FTICR instruments, and TOF–TOF mass spectrometers, and the new orbitrap mass spectrometers. For most medicinal chemistry applications, APCI or electrospray ionization is used.

In addition to molecular mass and fragmentation patterns, high-precision and high-resolution mass spectrometers such as QqTOF instruments, ion trap-TOF mass spectrometers, reflectron TOF mass spectrometers, double focusing magnetic sector mass spectrometers, and FTICR instruments are necessary for the measurement of exact masses of drugs and drug candidates for the determination of elemental compositions. The combination of high-resolution and high-precision is especially useful for determining the elemental compositions of compounds in combinatorial library mixtures without having to isolate each compound using chromatography or some other separation technique. Since FTICR instruments, orbitrap mass spectrometers, and hybrid ion trap-TOF and QqTOF mass spectrometers are capable of accurate mass measurements at high resolving power of both molecular ions and fragment ions generated during MS–MS, these instruments are becoming extremely popular within drug discovery programs.

Although accurate mass measurements of compounds in combinatorial libraries can usually be measured using electrospray or APCI mass spectrometry with infusion, online HPLC separation is sometimes required to overcome ion suppression problems due to the presence of buffer, contaminants or reaction by-products. However, LC-MS is a relatively slow process due to the slow chromatographic separation step. Several approaches have emerged to increase the throughput of this technique; parallel LC-MS, fast LC-MS and ultrahigh pressure liquid chromatography (UHPLC)-MS. One approach to increasing throughput of the rate-limiting chromatographic separation has been to simultaneously interface multiple HPLC columns to a single mass spectrometer. This approach is called parallel LC-MS. Commercial parallel electrospray interfaces and HPLC systems are now available that can accommodate up to 8 HPLC columns simultaneously [20–22]. Although the multiple sprays are introduced to the ion source simultaneously, these streams may be sampled in a time-dependent manner to minimize cross-contamination between channels.

Another solution to increasing the throughput of LC-MS has been to minimize the time required for HPLC separation through an approach called fast HPLC. HPLC separations are accelerated by using shorter columns and higher mobile phase flow rates. Since coelution of some species is likely to occur during fast chromatographic separations, the selectivity of the mass spectrometer is essential for the characterization and/or quantitative analysis of the target compound. However, samples prepared using combinatorial chemistry are usually simple mixtures of reagents, by-products, and product that require only partial chromatographic purification to prevent ion suppression effects during mass spectrometric analysis. A variety of HPLC columns are used for fast LC-MS that include narrow bore (2 mm) and analytical bore (4.6 mm) columns with length typically from 0.5–5 cm. The mobile phase flow rate for these fast LC-MS analyses is usually from 1.5–5 mL/min.

Similar stationary phase chemistries are used for UHPLC, but the particle size of the packing material is reduced to <2 μm for greater column efficiency. If the UHPLC columns are shortened compared to conventional columns, similar separation efficiencies may be obtained while the analysis times and solvent consumption can be reduced to two- to threefold. Alternatively, UHPLC may be operated at higher flow rates to obtain good separations in just seconds instead of minutes. However, the small particle size of UHPLC columns produce high back pressures of up to 800 bar, which is twice the maximum pressure of conventional HPLC systems. Therefore,
UHPLC systems are engineered to operate at pressures up to at least 800 bar. In addition to molecular mass determination using conventional MS or high-resolution accurate mass measurement and structural confirmation using MS–MS, fast LC-MS, and UHPLC-MS are also used to assess the purity and yield of combinatorial products [15,23]. Prior to high-throughput screening, many researchers analyze combinatorial libraries for both purity and structural identity using mass spectrometry so as to assure the validity of structure–activity relationships that might be derived from the screening data. Fast LC-MS, UHPLC-MS, and LC-MS–MS may be carried out to satisfy this requirement using gradients (usually a step gradient with a reverse phase HPLC or UHPLC column) with a total cycle time of <3 min [24] for fast HPLC or <1 min per analysis using UHPLC or isocratic systems.

2.3. Encoding and Identification of Compounds in Combinatorial Libraries and Natural Product Extracts

The utility of mass spectrometric identification in combinatorial chemistry is limited not only to the analysis of synthetic products as a means of quality control but also for the identification of active compounds or “hits” during high-throughput screening. Although the synthesis and screening of discrete compounds [25] enables them to be followed through the entire process by using partial encoding or bar coding, it is sometimes advantageous to screen libraries prepared as mixtures [26] and use a technique such as mass spectrometry to rapidly identify the hit(s) in the mixture. One approach to the rapid deconvolution of combinatorial library mixtures is to prepare libraries containing compounds of unique molecular mass, and then identify them using mass spectrometry. However, such libraries are necessarily small since the molecular mass of most drug-like molecules is between 150–400 Da. Because of the molecular mass degeneracy of larger combinatorial libraries, several encoding strategies have been devised to rapidly identify active compounds in these mixtures [27–29].

Since most combinatorial libraries contain compounds with degenerate molecular masses, various tagging strategies have been devised to uniquely identify library compounds bound to beads. Most of these tagging approaches are based on the synthesis of encoding molecules. For example, peptide [30] or oligonucleotide [31] labels have been synthesized on the beads in parallel to the target molecules and then sequenced for bead decoding. Alternatively, haloarene tags have been incorporated during synthesis and then identified with high sensitivity using electron-capture gas chromatography detection [32]. In addition to the increased time and cost for the synthesis of a library containing tagging moieties, the tagging groups themselves might interfere with screening giving false-positive or -negative results.

For peptide libraries, one solution to this problem uses MALDI mass spectrometry to directly desorb and identify peptides from beads that were screened and found to be hits [33]. This technique is called the termination synthesis approach. Since the peptide library compounds are analyzed directly, products with amino acid deletions or substitutions, side-reaction products, or incomplete deprotection are readily observed. Also, since there are no extra molecules used for chemical tagging, this source of interference is avoided. However, this approach is specific to peptide libraries and not necessarily applicable to other types of combinatorial libraries.

Another approach that eliminates possible interference from the chemical tags, “ratio encoding,” has been developed for the mass spectrometric identification of bioactive leads using stable isotopes incorporated into the library compounds [29,34]. Within the ligand itself, the code might be a single labeled atom that is conveniently inserted whenever a common reagent transfers at least one atom to the target compound or ligand. The code consists of an isotopic mixture having one of the many predetermined ratios of stable isotopes and can be incorporated in the linker or added through a reagent used during the synthesis. The mass spectrum of the compound shows a molecular ion with a unique isotope ratio that codes for a particular library compound. For example, Wagner et al. [29] used isotope ratio...
encoding during the synthesis of 1000 compound peptoid library and was able to identify uniquely all the components based on their isotopic patterns and molecular masses. Since isotope ratio codes are contained within each combinatorial compound, a chemical tag is not required. The speed of MS-based decoding outperforms most other decoding technologies, which are time consuming and decode a restricted set of active compounds.

Although combinatorial synthesis provides rapid access to large numbers of compounds for screening during drug discovery and lead optimization, these libraries are usually based on a small number of common structures or scaffolds. There is a constant need for increasing the molecular diversity of combinatorial libraries and finding new scaffolds, and natural products have always been a rich source of chemical diversity for drug discovery. The traditional approach to screening natural products for drug leads utilizes bioassays to test organic solvent extracts for activity. If strong activity is detected, then activity-guided fractionation of the crude extract is used to isolate the active compound(s), which are identified using mass spectrometry (including tandem mass spectrometry and accurate mass measurements), IR, UV/VIS spectrometry, and NMR. Recently, a variety of mass spectrometry-based affinity screening methods have been developed to streamline the tedious process of activity-guided fractionation. These approaches are discussed in Section 2.4.

Whether lead compounds in natural product extracts are isolated using bioassay-guided fractionation or mass spectrometry-based screening, there is a high probability that the structure of the active compound(s) has already been reported in the natural product literature. In such cases, the tedious process of complete structure elucidation using a battery of spectrometric tools should be unnecessary. Instead, mass spectrometry alone may be used to quickly “dereplicate” or identify the known compounds based on molecular mass, fragmentation patterns and elemental composition in combination with natural product database searching [35–39]. Commercially available natural products databases include NAPRALERT [40], Scientific & Technical information Network (STN) [41], and the Dictionary of Natural Products [42]. Since some of these databases also contain UV–vis absorbance data, it is also advantageous to use a photodiode array detector between the HPLC and mass spectrometer to obtain additional spectrometric data during LC-UV-MS dereplication [36,37].

2.4. Mass Spectrometry-Based Screening

The earliest approaches to combinatorial synthesis used portioning and mixing [26] and enabled the synthesis of combinatorial libraries containing hundreds of thousands to millions of compounds. However, efficient screening techniques did not exist to rapidly identify the “hits” within large combinatorial mixtures. Therefore, chemists were motivated to develop ways to prepare large numbers of discreet compounds using massively parallel synthesis, which could be assayed quickly for pharmacological activity using high-throughput screening one compound at a time. Although high-throughput screening of discreet compounds has become the standard approach to drug discovery in medicinal chemistry, there is a shortage of chemically diverse novel structures to serve as lead compounds for combinatorial synthesis and lead optimization. A traditional source of molecular diversity for drug discovery, natural products are receiving renewed interest in drug discovery programs. However, the complexity of natural products sources such as botanical or microbial extracts requires screening techniques that are suitable for mixtures. To address this requirement, mass spectrometry-based screening assays have been developed that are suitable for screening complex mixtures including natural product extracts. All of the mass spectrometry-based screening methods use receptor binding of ligands as the basis for identification of lead compounds.

2.4.1. Affinity Chromatography-Mass Spectrometry

Since the introduction of affinity chromatography approximately 40 years ago, this technique has become a standard biochemical tool for the isolation and identification of new binding partners to specific target molecules. Therefore, the coupling of affinity chromatography to mass spectrometry is a logical ex-
tension of this approach, and the application of affinity LC-MS to the screening of combinatorial libraries has been demonstrated by several groups [43,44]. During affinity LC-MS screening, a receptor molecule such as a binding protein or enzyme is immobilized on a solid support within a chromatography column. The library mixture is pumped through the affinity column in a suitable binding buffer so that any ligands in the mixture with affinity for the receptor would be able to bind. Then, unbound material is washed away. Finally, the specifically bound ligands are eluted using a destabilizing mobile phase and identified using mass spectrometry. This affinity-column LC-MS assay is summarized in Fig. 8.

In some applications [43], ligands are eluted from the affinity column and then trapped on a second column such as a reverse phase HPLC column. LC-MS or LC-MS–MS identification of the ligands (hits) is then carried out using the trapping column. In other systems, ligands are identified directly from the affinity column using mass spectrometry. For example, Kelly et al. [44] prepared an affinity column containing immobilized phosphatidylinositol-3-kinase and used it for direct LC-MS screening of a 361-component peptide library. Electrospray mass spectrometry and tandem mass spectrometry were used to identify the ligands released from the affinity column using pH gradient elution.

Advantages of affinity chromatography-mass spectrometry for screening during drug discovery include versatility and reuse of the column. Both combinatorial libraries and natural product extracts can be screened using this approach, and a wide range of binding buffers may be used. Mass spectrometry-compatible mobile phases are only required during the final LC-MS detection step. Furthermore, a single column may be used multiple times to screen different samples for ligands unless the destabilization solution irreversibly denatures, releases, or inhibits the receptor.

Despite these advantages, affinity chromatography has numerous drawbacks that have prompted the development of alternative mass spectrometer screening tools. For example, immobilization of the receptor might change its affinity characteristics causing false-negative or false-positive hits. This is particularly problematic for receptors that are solution-phase in their native state. Also, developing and then implementing an immobilization scheme is often a slow, tedious, and even expensive process, which is unique for each new receptor. Finally, false-positive hits are often obtained when screening large, molecularly diverse libraries, since there are usually compounds in such mixtures that have affinity for the stationary phase or linker molecule instead of the receptor.

**Figure 8.** Affinity chromatography combined with LC-MS–MS for screening combinatorial library mixtures.
2.4.2. Gel Permeation Chromatography-Mass Spectrometry

Another type of chromatography that has been combined with mass spectrometry as a screening system for drug discovery is gel permeation chromatography (GPC) [45,46]. Also called size exclusion chromatography, GPC separates molecules according to size as they pass through a stationary phase containing particles with a defined pore size. During GPC-based screening, a library mixture is preincubated with a macromolecular receptor to allow any ligands in the library to bind, and then GPC is used to separate the large receptor–ligand complexes from the unbound low molecular mass compounds in the mixture. Finally, ligands are released from the receptor during reversed phase HPLC and identified either on-line or off-line using tandem mass spectrometry. This screening method is illustrated in Fig. 9.

During the preincubation and GPC steps, any binding buffer may be used, since the binding buffer will be removed during reverse phase LC-MS analysis. However, the GPC separation step must be carried out quickly, since ligands begin to dissociate from the receptor immediately and can become lost into the size exclusion gel. Despite this disadvantage, this approach allows both receptor and ligand to be screened in solution, which avoids some of the problems associated with the use of affinity columns for screening. The GPC LC-MS-MS screening method is suitable for screening natural product extracts as well as combinatorial library mixtures.

2.4.3. Affinity Capillary Electrophoresis-Mass Spectrometry

Affinity capillary electrophoresis was originally used for the determination of the binding constants of small molecules to proteins [47–49]. This solution-based technique is rapid and requires only small amounts of ligands. Affinity constants are measured based on the mobility change of the ligand upon interaction with the receptor present in the electrophoretic buffer [50]. By combining affinity capillary electrophoresis with on-line mass spectrometric detection and identification, affinity constants for multiple compounds can be measured in a single analysis [51]. Recognizing that on-line mass spectrometric detection was helpful for the identification of each ligand, Chu et al. [52] extended this approach to include the screening of combinatorial libraries as a means of drug discovery. The data in Fig. 10 show the results of screening a 100-tetrapeptide library for affinity to vancomycin using affinity capillary electrophoresis-mass spectrometry. Without vancomycin in the electrophoresis buffer, all the peptides eluted within 3 min. When vancomycin was present, the peptides eluted in

Figure 9. GPC followed by LC-MS–MS for screening mixtures of combinatorial libraries. After incubation of a receptor with a library of compounds, the ligand–receptor complexes (L–R) are separated from the low molecular mass unbound library compounds using GPC. Next, the L–R complexes are denatured during reversed phase HPLC to release the ligands for MS–MS identification.
order of affinity with the highest affinity compounds being detected between 4.5 and 5 min. Positive ion electrospray tandem mass spectrometry was used to identify the highest affinity ligands (see Fig. 10B).

Note that some peptide ligands such as Fmoc-DDFA were detected as adducts with Tris that was used in the electrophoresis buffer. Although the identification of this peptide was not prevented by the formation of this adduct, some buffers used during electrophoresis might interfere with mass spectrometric ionization and detection. Also, the types of libraries that have been screened using this approach have contained modest numbers of synthetic analogs such as peptides. Libraries exceeding 400 members required preliminary purification using affinity chromatography to reduce the number of compounds [52]. As a result, this approach is probably not ideal for screening libraries containing molecularly diverse compounds or for screening natural product extracts. However, affinity capillary electrophoresis-mass spectrometry is fast with each analysis requiring less than 10 min. Also, it may be used to measure affinity constants for ligand–receptor interactions.

2.4.4. Frontal Affinity Chromatography-Mass Spectrometry

Like affinity chromatography-mass spectrometric screening (see Section 2.4.1), frontal affinity chromatography utilizes an affinity column containing immobilized receptor molecules [53]. The difference between the two screening methods is that the ligands are continuously infused into the column during frontal affinity chromatography and detected using mass spectrometry. Compounds with no affinity for the immobilized receptor elute immediately in the void volume,

Figure 10. Affinity capillary electrophoresis-UV-mass spectrometry of a 100-tetrapeptide library screened for binding to vancomycin (104 µM in the electrophoresis buffer). (a) The elution of peptides was monitored with UV absorbance during capillary electrophoresis, and the elution time increased with increasing affinity for vancomycin. (b) Positive ion electrospray mass spectrum with CID of the Tris adduct of the protonated peptide detected at ~5 min in the electropherogram shown in A. (Reproduced from Ref. [52] by permission of the American Chemical Society.)
but the elution of the ligands is delayed. As compounds compete for binding sites on the affinity column, these sites become saturated until ligands begin to elute from the column at their infusion concentration (see Fig. 11). In this manner, frontal affinity chromatography may be used to measure affinity constants for ligands, and by using a mass spectrometer for on-line identification of ligands, this technique becomes a screening method [54,55].

During frontal affinity chromatography–mass spectrometry, signals for compounds eluting from the affinity column are recorded on-line by a mass spectrometer, and the last compounds to elute at their infusion concentrations represent the highest affinity compounds or "hits." Since frontal affinity chromatography utilizes a conventional affinity column, this technique would be most accessible to investigators already using affinity-mass spectrometry (see Section 2.4.1). However, the same limitations and disadvantages of using immobilized receptors still apply such as nonspecific binding to the stationary phase, the development time and cost of preparing the affinity columns, and the possibility that immobilizing the receptor might alter its binding characteristics and specificity. In addition, mass spectrometric detection creates some additional limitations. Since all library compounds must be monitored simultaneously, the compounds must be selected so that they have unique molecular masses. Also, one compound in the mixture should not suppress the ionization of another. Therefore, this approach is probably restricted to the screening of small combinatorial libraries that are similar in chemical structure and ionization efficiencies. Finally, the binding buffer used for affinity chromatography must be compatible with on-line APCI or electrospray mass spectrometry. This means that the mobile phase must be volatile and usually of low ionic strength (i.e., typically <40 mM for electrospray ionization). However, the mobile phase limitations may be overcome by collecting fractions from the affinity column and analyzing them off-line using LC-MS as indicated in Fig. 11 [55].

2.4.5. Solid-Phase Mass Spectrometric Screening Since drugs are usually in a soluble form in order to be transported to the active sites in cells and tissues, it is logical that most mass spectrometry-based screening methods utilize solution-phase analysis of these compounds, and it is no surprise that most successful mass spectrometry screening assays use electrospray ionization or APCI. However, solid-phase ionization techniques such as MALDI
might be effective also provided that ligand–receptor interactions are allowed to take place in an environment similar to in vivo conditions and provided that a suitable separation step is carried out prior to the preparation of the MALDI sample.

In order to utilize MALDI mass spectrometry for screening, several research groups have developed immobilized receptors on MALDI targets or on solid supports that can be placed on a MALDI target for use in the affinity purification of potential drugs from test solutions. Following procedures originally developed for affinity chromatography, the preparation of affinity surfaces for MALDI mass spectrometry has been achieved quite easily. However, the utility of these affinity MALDI chips for screening mixtures of small molecules during drug discovery has been unproductive. One of the problems has been the high background noise at low m/z values caused by the matrix used for MALDI. This problem may be mitigated by eliminating the matrix or using alternative sample stages such as porous silicon chips [56,57]. However, noise persists due to the affinity support and immobilized receptor molecules. Another problem to be overcome is to eliminate the high background noise caused by nonspecific binding of test compounds to the affinity target. Although this problem is similar to the false-positive results and nonspecific binding that occurs during affinity chromatography–mass spectrometry (see Section 2.4.1), the signals for nonspecific binding is magnified by the fact that the actual affinity surface is being irradiated and sampled by the MALDI laser beam. As a result, affinity-based screening coupled with MALDI mass spectrometry has not been a successful drug discovery approach.

A recent method termed self-assembled monolayers for MALDI (SAMDI) offers a solution to the problems associated with MALDI screening [58]. In an anthrax lethal factor inhibition assay, self-assembled monolayers of an anthrax protein were constructed against a background of triethylene glycol groups on a glass plate. The self-assembled monolayers provided well-defined arrays of exposed receptor sites for ligand interaction, and the background noise and nonspecific binding problems pervasive in previous MALDI screening appear to have been largely subdued. Next, MALDI-TOF mass spectrometry was used successfully to screen a library of 10,000 molecules for inhibitors of the receptor. The SAMDI technique was even used to derive quantitative values (IC\textsubscript{50} and Z-factor) in good agreement with those obtained by conventional screening methods.

Progress is being made in the use of affinity probes for the capture of proteins and other macromolecules from biological solutions followed by MALDI mass spectrometric detection and identification [59,60]. One affinity MALDI mass spectrometry method has been paired with affinity probes using surface plasmon resonance systems [61]. These affinity-based MALDI mass spectrometry screening assays are promising approaches for testing blood or other biological fluids for the presence of specific proteins or other macromolecules. As a result, these have the potential to become clinical diagnostic tools or might even lead to the identification of new therapeutic targets. Recently, MALDI mass spectrometry has been used for molecular imaging of drug compounds, metabolites, biomarkers, proteins, and other chemicals in tissue sections [62].

2.4.6. Pulsed Ultrafiltration-Mass Spectrometry
A versatile approach to screening solution-phase combinatorial libraries and natural product extracts is pulsed ultrafiltration-mass spectrometry [63,64], which utilizes a standard LC-MS system with an ultrafiltration chamber substituted for the HPLC column. The principle of pulsed ultrafiltration screening of combinatorial libraries is shown in Fig. 12. During pulsed ultrafiltration, ligand–receptor complexes remain in solution in the ultrafiltration chamber while unbound library compounds and buffers are washed away. After unbound compounds are removed, the hits from the library are eluted from the chamber by destabilizing the ligand–receptor complex using an organic solvent, a pH change, or a combination of both. The released ligands are identified on-line using APCI or electrospray mass spectrometry [63] or collected and analyzed off-line using mass spectrometry, LC-MS, or LC-MS–MS [65].

An example of pulsed ultrafiltration mass spectrometry for the screening of a library of
20 adenosine analogs for ligands to adenosine deaminase is shown in Fig. 13. After a 15 min preincubation of the library compounds (17.5 μM each except for EHNA, which was present at 1.75 μM) with 2.1 μM adenosine deaminase in 50 mM phosphate buffer, an aliquot containing 420 pmol of the receptor was injected into the ultrafiltration and washed for 8 min at 50 μL/min with water to remove the phosphate buffer and unbound or weakly binding library compounds. Methanol was introduced into the mobile phase to dis-
sociate the enzyme–ligand complex and release bound ligands for identification by electrospray mass spectrometry. During methanol elution, only EHNA (erythro-9-(2-hydroxy-3-nonyl) adenine) was detected as the [M + H]^+ ion of m/z 278 (Fig. 13). In control experiments using the library without enzyme, no library compounds were detected during methanol elution (Fig. 13 control). Despite being present at a 10-fold lower concentration than the natural substrate adenosine analogs, EHNA was easily identified since it had the highest affinity among the library compounds (K_d = 1.9 nM). This demonstrates the utility of ultrafiltration electrospray mass spectrometry for identifying a high-affinity ligand among a set of analogs that bind to a specific receptor. In a follow-up lead optimization study using pulsed ultrafiltration mass spectrometry, a synthetic combinatorial library of EHNA analogs was screened for binding to adenosine deaminase, and structure–activity relationships for EHNA binding were identified [67].

As an illustration of the versatility of pulsed ultrafiltration-mass spectrometry, binding assays for a variety of receptors have been reported including dihydrofolate reductase [65], cyclooxygenase-2 [64], serum albumin [68,69] and estrogen receptors [70]. Pulsed ultrafiltration is not only useful for identifying ligands to different receptors but also a wide range of combinatorial libraries and natural product extracts in any suitable binding buffer may be screened. In addition to combinatorial libraries, complex natural product extracts such as the number of binding sites, two pulsed ultrafiltration measurements are carried out. First, an aliquot or pulse of a liquid is injected through the chamber, and the elution profile is recorded. Then, the chamber is loaded with a receptor, and the ligand is re-injected. If binding occurs, the elution profile will be delayed in proportion to the affinity constant. The control injection is used to control for nonspecific binding to the apparatus. Since the concentration of receptor and total amount of liquid are known, and since the concentration of free ligand is measured as it elutes from the chamber over a wide range of concentrations, the affinity constant and other binding parameters may be calculated.

Metabolism and toxicity screening applications of pulsed ultrafiltration use hepatic microsomes in the ultrafiltration chamber. For metabolic screening, drugs and the cofactor NADPH are flow injected through the ultrafiltration chamber (oxygen is dissolved in the mobile phase), and the metabolites formed by microsomal cytochrome P450 and any unreacted compounds flow out of the chamber for mass spectrometric identification and/or quantitative analysis [72]. On-line applications require the use of volatile buffers, but LC-MS and LC-MS-MS may be used off-line to analyze the ultrafiltrate no matter what buffer had been used. Screening drugs for metabolic activation using pulsed ultrafiltration-mass spectrometry is carried out in a similar manner, except that glutathione is coinjected along with NADPH and the drug substrate [73]. MS-MS may be used on-line or LC-MS-MS used off-line to screen for glutathione adducts as an indication that the drug was metabolized to a reactive intermediate(s) that was trapped by reaction with glutathione. Finally, pulsed ultrafiltration may be used with UV or mass spectrometric detection to measure affinity constants of individual compounds [68].

In order to measure affinity constants and other physicochemical properties of binding such as the number of binding sites, two pulsed ultrafiltration measurements are carried out. First, an aliquot or pulse of a liquid is injected through the chamber, and the elution profile is recorded. Then, the chamber is loaded with a receptor, and the ligand is re-injected. If binding occurs, the elution profile will be delayed in proportion to the affinity constant. The control injection is used to control for nonspecific binding to the apparatus. Since the concentration of receptor and total amount of liquid are known, and since the concentration of free ligand is measured as it elutes from the chamber over a wide range of concentrations, the affinity constant and other binding parameters may be calculated.

In most of the applications of pulsed ultrafiltration to date, serial analyses were carried out with a throughput of approximately one or two assays per hour. Since the purpose of these assays was to screen complex mixtures or to obtain metabolism data for new drug entities, the throughput of these analyses was acceptable but not high throughput. The rate
limiting step in these analyses is the ultrafiltration separation and not the mass spectrometric detection. Several solutions have been reported to increase the throughput of pulsed ultrafiltration mass spectrometry. van Bremen et al. [72] used a multiplex ultrafiltration system with ultrafiltration chambers arranged in parallel and interfaced to a single mass spectrometer. An off-line variation of this approach is to use centrifuge tubes fitted with ultrafiltration membranes to obtain ultrafilters of multiple samples simultaneously [74]. Then, each ultrafiltrate can be analyzed using LC-MS. Another solution to increasing the throughput of pulsed ultrafiltration mass spectrometry has been to miniaturize the ultrafiltration chamber volume while maintaining the flow rate and chamber pressure. Since the ultrafiltration membrane cannot withstand high pressure without rupturing, the ultrafiltration process cannot be accelerated simply by increasing the flow rate through the chamber. For example, Beverly et al. [75] fabricated a 35 μL ultrafiltration chamber which was approximately threefold lower in volume than previously reported versions [63]. As a result, ultrafiltration mass spectrometric analyses could be carried out at the rate of at least three per hour, which corresponded to a threefold enhancement of throughput. This study suggests that chip-based ultrafiltration mass spectrometry would have the potential to result in a truly high-throughput system.

The advantages of pulsed ultrafiltration-mass spectrometry include the variety of different applications that may be carried out, the convenience of on-line screening, solution-phase screening, the ability to screen combinatorial libraries and natural product extracts, the diversity of receptors that may be screened, and the freedom to use either volatile or nonvolatile binding buffers. For metabolomic and toxicology screening, flow injection analyses have the additional advantages that product feed-back inhibition is prevented so that the metabolic profile more closely approximates the in vivo system [72]. Finally, the disadvantages of pulsed ultrafiltration screening for drug discovery include the washing step, during which dissociation and loss of weakly bound ligands might occur, and the slow speed of each experiment, which can take up to one hour.

2.4.7. Drug Development Assays Based on Mass Spectrometry

Often, lead compounds identified during high-throughput screening have undesirable properties that preclude their use as drugs, such as poor oral bioavailability, unacceptably rapid metabolism, or the formation of toxic metabolites [76]. Identification of these problems early in the drug development process can save time and reduce the costs of drug development by guiding medicinal chemists toward lead compounds more likely to become drugs. The application of mass spectrometry to drug development assays such as the high-throughput estimation of lipophilicity, metabolic stability, and the detection of reactive metabolites are just a few examples of how LC-MS and LC-MS-MS are being used to expedite drug development. Many drug development applications such as screening for metabolic stability, in vitro metabolism and the formation of reactive metabolites can be carried out using pulsed ultrafiltration mass spectrometry as described in Section 2.4.6. Some other drug development assays utilizing mass spectrometry are described below.

**Determination of log P Using LC-MS**

The lipophilicity of a drug candidate is usually estimated by its log P, where P is the partition coefficient between water and octanol. Because most compounds contain ionizable groups that significantly affect solubility, the partition coefficient is often measured at a specific pH in which case the value is known as the log D. Traditionally, log P and log D measurements have been carried out using the shake-flask method, in which the test compound is added to water and octanol, agitated and the phases allowed to separate. The concentration of the analyte is then measured in both solvents by a suitable technique such as LC-UV or LC-MS. The shake-flask method is the most accurate method for log P or log D estimation; however, it is the most costly and time consuming. Faster and less expensive alternatives to the shake-flask method include computer-based in silico prediction, LC-MS retention time-based estimation, and a recent 96-well plate format coupled to
LC-MS [77]. In the microtiter plate method, a deep 96-well plate is loaded with aqueous and organic phases at the appropriate pH along with the compound of interest, and then vortex mixed. The phases are separated by centrifugation, and the plate is loaded into an autosampler for quantitative analysis using LC-MS. The needle depth of the autosampler can be controlled to sample from either the aqueous or organic layers (see Fig. 14). A needle wash is performed to eliminate carryover. Quantitation of the analytes in each phase is carried out using LC-MS. This approach facilitates the rapid and accurate determination of log \( P \) or log \( D \) while eliminating tedious liquid handling operations.

**Drug Metabolism and Metabolic Activation**

Metabolism studies of lead compounds are usually initiated *in vitro* by incubating the lead compound with liver microsomes and enzymatic cofactors such as NADPH or hepatocytes. The products are analyzed by using LC-MS and compared to control reactions to find new peaks, which correspond to drug metabolites. To detect metabolites of lead compounds formed *in vivo*, laboratory animals are administered the investigational compound followed by LC-MS analysis of body fluids and tissues. To enhance the throughput of metabolite identification and quantification, several LC-MS and LC-MS–MS approaches have been developed [78].

The use of LC-MS with electrospray or APCI facilitates the detection and characterization of metabolites resulting from common biotransformations, such as metabolic oxidation or dealkylation. Accurate mass measurements recorded using high-resolution mass spectrometers such as TOF, QqTOF, orbitrap, or IT-TOF mass spectrometers (see Table 1) facilitate the discrimination of drug metabolites from endogenous compounds by providing molecular formulas of metabolites. Data-dependent product ion tandem mass spectrometry combined with accurate mass measurements on QqTOF, orbitrap, or IT-TOF mass spectrometers further enhance metabolite identification by providing structural information as well as elemental composition data. Commercially available software is available from several vendors, which enables automatic data analysis and metabolite detection based on elemental composition, mass defect filtering and isotope pattern-matching functions [79]. Mass defect filtering relies on the unique difference between the nominal mass of a compound (an integer value) and its exact mass (value including the fractional mass), which is specific to its elemental composition. The mass defects and isotope patterns of drug metabolites are usually similar to those of the precursor drug. For example, Bateman et al. [79] investigated the metabolism of the antiretroviral agent indinavir by using
UHPLC-MS on a QqTOF mass spectrometer. By applying a mass defect filter, the background was dramatically reduced and several new metabolites were detected that were not otherwise apparent.

Electrophilic metabolites of lead compounds that might cause toxicity may be detected by trapping the metabolite with glutathione (GSH) or other water-soluble nucleophile and then detecting the GSH conjugate using LC-MS–MS. Since GSH is an endogenous nucleophile, LC-MS–MS screening may be used to detect GSH conjugates formed in vitro or in vivo. Glutathione conjugates formed in vivo are either excreted in bile or as mercapturic acids in the urine. The potential of LC-MS–MS to be used as a screening tool to detect GSH conjugates with selectivity was reported in the 1990s by Nikolic et al. [73]. Initially described as an application of pulsed ultrafiltration LC-MS–MS, screening for reactive drug metabolites trapped as GSH conjugates using tandem mass spectrometry has become widely accepted, although sample cleanup may involve protein precipitation or solid-phase extraction instead of ultrafiltration.

During LC-MS–MS with CID, GSH conjugates form characteristic GSH fragment ions that are useful for their selective detection. For example, during positive ion electrospray tandem mass spectrometry, GSH conjugates fragment to eliminate a neutral molecule of pyroglutamic acid (129 mass units) [80]. The tandem mass spectrometry technique of constant neutral loss scanning may be used on instruments such as a triple quadrupole mass spectrometer to detect GSH conjugates due to their characteristic loss of 129 units. Since some GSH conjugates do form this type of fragment ion in high abundance, negative ion electrospray may be used instead to detect another characteristic GSH ion of m/z 272 that is formed by cleavage at the sulfur linkage between the drug metabolite and the GSH moiety [81]. Whether used for the characterization of drug metabolites or the detection of reactive metabolites after trapping with nucleophiles such as GSH, LC-MS, and LC-MS–MS are unsurpassed for their sensitivity, speed and selectivity. By applying LC-MS, LC-MS–MS and drug metabolite analysis software, information about the types of metabolites, their structures, and rates of formation may be obtained and used to select the best lead compounds to be advanced in the drug discovery and development process.

**Other Drug Development Assays** High-throughput LC-MS assays to measure other important pharmacokinetic parameters such as serum protein binding have been reported. Serum protein binding can be measured by online ultrafiltration MS [68] or with a 96-well filrate assembly [82]. Intestinal permeability measurements are usually carried out using the Caco-2 cell model in a multi-well format that can be analyzed by LC-MS [83]. It should be noted that LC-MS or LC-MS–MS quantitative analysis to support drug discovery and drug development assays may be accelerated using UHPLC. These UHPLC-MS systems reduce the runtime per sample to below 5 min while maintaining or even improving analytical sensitivity and reproducibility [84].

### 3. THINGS TO COME

Mass spectrometry has become an essential analytical tool at every stage of the drug discovery and development process. In this chapter, the various applications of mass spectrometry to combinatorial chemistry, drug discovery, and lead optimization have been highlighted. In the past decade, advances in instrument technology have progressed rapidly. In particular, UHPLC, high-resolution mass spectrometers, and tandem mass spectrometers are becoming more widely available and are being used to make drug discovery and development more rapid than ever before.

Intense competition between instrument vendors and the demands of biomedical mass spectrometrists (the consumers) continue to drive technological innovation. For example, innovations in ion sources and ion optics improve detection limits by an order of magnitude with each generation instruments, although this trend may not be able to continue indefinitely. New combinations of ion traps with TOF and orbitrap mass spectrometers make it possible to explore MS^n fragmentation patterns with accurate mass measurements without the high cost of FT-ICR...
mass spectrometers. A full exploration of the applications of these hybrid instruments has only begun. Among the most promising of the new mass spectrometry technologies just becoming commercially available is ion mobility spectrometry (IMS). Specialized IMS instruments are already in use by security teams for the detection of drugs, explosives, and toxins in the field. Recently, IMS has been interfaced with MS to add a new dimension of separation in an instrument termed ion mobility mass spectrometry (IMMS). These instruments direct ions along a high-pressure path constrained by an electric field. The ion packet undergoes multiple collisions with a drift tube gas, which retards the motion of molecules with larger cross-sectional areas. Distinct from both gas chromatography and time-of-flight, ion mobility spectrometry separates ions based on their shape and charge within a few tens of milliseconds. After exiting the drift tube, the ions can be separated and detected by conventional mass spectrometer analyzers. IMMS is ideal for the separation and analysis of large biomolecules in the gas phase, such as studies of protein conformation and binding. Small molecule separations, particularly for complex natural products extracts, might also benefit from the ability of IMMS to separate and characterize isomers or other compounds with similar structures within just a few microseconds instead of the minutes required for HPLC or even the seconds needed for UHPLC. 

To match the increased analytical performance of LC-MS technology, data analysis software must be developed that is both user-friendly and highly functional. Tools such as automatic feature picking, mass defect or isotope pattern filtering, and metabolite identification are only just becoming available. These tools will become more sophisticated, and will become better integrated into the data analysis workflow. Even more powerful will be software that allows rapid profiling of large sample sets such as is needed for metabolomics. As the quality and quantity of mass spectrometry data increases, robust strategies to manage and archive the data will need to be implemented to allow efficient data mining and interchange of data between vendor platforms. 

In conclusion, mass spectrometry provides rapid, reliable, sensitive, and selective analysis of combinatorial libraries for structure confirmation, purity analysis, and library deconvolution. In addition, mass spectrometric screening methods have been developed and applied to drug discovery and development. High-throughput LC-MS methods have become routine for solubility and bioavailability testing and metabolite screening to ensure that only high-quality lead compounds are advanced to more expensive clinical testing. In the case of natural products, mass spectrometry facilitates the rapid and accurate activity screening, dereplication, and characterization of complex extracts. At different times during the last 100 years, first physicists and physical chemists and then organic chemists pronounced that mass spectrometry had run out of new applications and had no future. Gladly, they were wrong. Today, medicinal chemists recognize the potential of mass spectrometry to contribute to all facets of drug discovery and development. Mass spectrometry has become a fundamental analytical tool for drug discovery, and this role should continue to grow in the future.

4. WEB SITE ADDRESSES AND RECOMMENDED READING FOR FURTHER INFORMATION

http://www.asms.org Homepage of the American Society for Mass Spectrometry. This web site contains additional information about mass spectrometry and links to a variety of reference materials regarding biomedical mass spectrometry.
http://benthamscience.com\echts Combinatorial Chemistry & High Throughput Screening
http://pubs.acs.org/journals/jchff Journal of Combinatorial Chemistry
http://www.5z.com/moldiv Molecular Diversity
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Abstract

In the twenty-first century, medicinal chemists are producing large numbers of organic compounds through a process called combinatorial chemistry and then testing them against pharmacological targets such as enzymes or receptors using high-throughput screening. As a result, lead compounds can emerge from drug discovery programs in a few weeks instead of several years. Although a variety of spectroscopic and chromatographic techniques are used to support drug discovery, only HPLC or UHPLC combined with mass spectrometry (LC-MS) are compatible with virtually all drug-like molecules. In addition to providing a universal means to characterize drug molecules based on molecular mass and structural features, mass spectrometry provides high throughput. With the development of routine LC-MS interfaces and ionization techniques such as electrospray and atmospheric pressure chemical ionization, mass spectrometry has also become an ideal HPLC detector for the analysis of combinatorial libraries. Furthermore, a variety of mass spectrometry-based screening assays have been developed to screen complex mixtures of compounds including natural product extracts as a means of expanding the molecular diversity of drug leads. The use of mass spectrometry in support of combinatorial library synthesis and purification is addressed as well as screening applications such as frontal affinity chromatography-mass spectrometry, gel permeation chromatography-LC-MS, affinity chromatography-mass spectrometry, and pulsed ultrafiltration mass spectrometry. Since drug development issues such as metabolic stability and the formation of reactive metabolites are now being considered during the drug discovery process, some applications of mass spectrometry to early drug development are addressed.

Keywords: affinity chromatography; combinatorial chemistry; drug discovery; drug development; frontal affinity chromatography; gel permeation chromatography; high-performance liquid chromatography; high-throughput screening; liquid chromatography-mass spectrometry; liquid chromatography-tandem mass spectrometry; ultrafiltration; ultrahigh pressure liquid chromatography; pulsed ultrafiltration.