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THE ENCYCLOPEDIA OF Mass Spectrometry

VOLUME 3

Biological Applications
Part B: Carbohydrates, Nucleic Acids and other Biological Compounds

RICHARD M. CAPRIOLI

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AMSTERDAM – BOSTON – HEIDELBERG – LONDON – NEW YORK – OXFORD
PARIS – SAN DIEGO – SAN FRANCISCO – SINGAPORE – SYDNEY – TOKYO

First edition 2006

Library of Congress Catalogue Number: 2004104294

A catalogue record for this book is available from the British Library.

ISBN (Vol. 3) 0-08-043803-2
ISBN (Set) 0-08-043850-4

Printed and bound in Spain

Foreword

Truly phenomenal changes have taken place in the field of mass spectrometry over the past several decades. By 1990, mass spectrometry had evolved as a discipline and an important tool for solving problems in organic and inorganic chemistry. It stood alongside nuclear magnetic resonance and optical spectroscopy as a means for the identification and structure proof of organic and organometallic compounds. Indeed, many journals required a mass spectrum or a measurement of an accurate mass before a description of a new compound could be published. Gas chromatography/mass spectrometry had become the premier analytical method for complex mixture analysis such as in environmental chemistry, flavors, aromas, petroleum and other energy materials, and in small molecule metabolism, as in drug research. Despite these advances, mass spectrometry did not yet play a major role in polar and large-molecule analysis. Nevertheless, the seeds for a revolution had been sown ten years or more earlier. Field desorption (FD), fast atom bombardment (FAB), and Cf-252 plasma desorption (PDMS) began to push mass spectrometry into applications such as peptide sequencing and molecular weight determination of large biological and synthetic polymers. Before long, FAB became routine and was an integral part of nearly all mass spectrometry laboratories, and Cf-252 PDMS and FDMS were utilized in more specialized applications. Tandem mass spectrometry was established as a vital tool for complex mixture analysis. The subject of mass spectrometry, both as a discipline and as a measurement method, began to flourish thereafter. The development of new instrumentation and methods positioned mass spectrometry as an invaluable tool in bioanalytical and biophysical chemistry that would extend its reach into the research laboratories of a vast array of disciplines.

The invention of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) in the late 1980s opened the door for mass spectrometry to assume a primary role in virtually all of the chemical sciences, including biological, medical, organic, organometallic, and elemental analysis. The discipline of mass spectrometry experienced a leap in activity with the chemical–physical studies of proteins, oligodeoxynucleotides, and other biological compounds in the gas phase.

The broad applicability of mass spectrometry to a multitude of chemical, physical, and biological problems has created the need for an encyclopedia on the subject. To permit a full and fruitful expansion in other disciplines, this encyclopedia is intended as a learning tool to the many newcomers who do not have the theoretical and practical background needed to take full advantage of the technology. Further, the field is now so broad that even the specialists are in need of a resource to allow them to explore the vast reaches of mass spectrometry and help them teach this discipline to scientists and students new to the field.

The Editors began discussion with staff at Elsevier aimed at establishing a comprehensive work in the field of mass spectrometry, a work that covers the theory of gas-phase ion chemistry, principles and designs of ionization and mass analysis, instrument developments and techniques, and a wide variety of applications. The Encyclopedia consists of nine volumes. To permit searching this literature, an index is provided in Volume 10. We estimate that the work will consist of c. 6500 pages in 600 articles, by 1000 authors. Included will be over 15,000 figures and 20,000 references. There is extensive cross-referencing and a subject index in every volume. The articles are intended to cover a specific subject field in three areas; (i) original or pioneering work in the area (ii) seminal contributions to the area, including appropriate examples illustrating a given application, and (iii) citations to useful review articles and additional reading. Articles are intended to be tutorial in nature, citing both advantages and disadvantages of a technique or method. Where possible, articles are divided into primary (basic) considerations, and advanced topics, the first aimed at the novice and the second to the experienced practitioner who is not necessarily an expert in that specific subject.

Volume 1 is devoted to fundamentals and to chemical physics. Work in these areas is aimed at understanding ionization phenomena and the properties of gas-phase ions. The research interacts productively with theory because often the first outcome of theoretical inquiry is the intrinsic property of a substance; that is, the property in the absence of solvent. Insight into the properties of gas-phase ions, the central subject of mass spectrometry, is illuminated by collision theory, molecular orbital theory, potential energy surfaces, ion spectroscopy, thermochemistry, equilibrium constants, and rate constants. Other ideas, some born in the past such as ion mobility, have been renewed because they can be applied to biomolecules in the gas phase.

The discipline of mass spectrometry has opened the door to new substances, some of which were unknown or only subjects of speculation in condensed-phase chemistry. The early research to produce CH_5^+, a species that violates the rule that carbon binds four times, is but one example. Distonic ions, ion-neutral complexes, clusters, isolated free radicals, and even highly reactive neutral species have become the subjects of mass spectrometry. The discovery of a new allotrope of carbon, the fullerenes, was made possible by workers in the discipline of mass spectrometry who were interested in the intrinsic properties of gas-phase clusters.

Perhaps the most exciting opportunity for mass spectrometry is in the area of biological, biochemical, and biomedical applications (**Volumes 2 and 3**). The end of the 20th century saw the technology of DNA sequencing

expand and be successfully applied to a variety of genomes including human. The genome is, however, relatively static, and it is clear that the next big challenge is to understand the proteome. Here, mass spectrometry has assumed the premier role for the identification of proteins, post-translational modifications, protein–protein and other molecular interactions in solution and ultimately in the cell. Ongoing research into the immune system (e.g., antigenic peptides), brain chemistry, and imaging of tissues and cells are part of exciting ongoing work. These opportunities will challenge mass spectrometrists to build new instruments, to develop new and powerful methods, and to understand the underlying ion chemistry.

Other areas of application of mass spectrometry to biology and biochemistry are also blossoming. Indeed mass spectrometry plays a role in DNA and oligonucleotide sequencing, in understanding DNA damage and the implications in cancer and other diseases. Structure elucidation of carbohydrates and glycoproteins, lipids, drugs, and drug metabolism is also greatly facilitated by mass spectrometry.

The applications of mass spectrometry to understand the properties of organic substances constitute subjects in **Volume 4**. Molecular mass spectrometry had its beginnings in the 1940s with applications in petroleum chemistry. These applications were quickly followed by efforts to understand fragmentation of organic molecules in the gas phase. Pioneering figures such as Fred McLafferty, John Beynon, Klaus Biemann, and Carl Djerassi saw the opportunity to use mass spectrometry to determine the structure of organic compounds. This capability helped develop areas such as organic and organometallic synthesis and natural product identification. Building on the early discoveries, mass spectrometry spread out to applied areas such as drug chemistry, environmental chemistry, food, flavor, and aroma chemistry, combinatorial chemistry, and geochemistry. Advances in macromolecular determination quickly impacted the area of synthetic polymers. These applications remain active today in the chemical and related industries, government laboratories, and academe.

Elemental analysis, one of the earliest areas touched by mass spectrometry, is the subject of **Volume 5**. The discovery of stable isotopes alone is one of the fundamental accomplishments of mass spectrometry. This important discovery by J.J. Thomson approximately 100 years ago set the stage for instrument development and measurement of the isotopic composition of the elements. The area of elemental analysis remains important today. Inductively coupled plasma, when interfaced to mass spectrometry, gives science the opportunity to measure nearly all of the elements of the periodic table. It is compatible with a number of ionization methods such as spark source, thermal ionization, electrospray, laser desorption, and secondary ionization mass spectrometry. Today, isotope ratio mass spectrometry is important in biomedicine, drug disposition, archeology, forensics, and materials dating.

Key to every area and application of mass spectrometry are developments in molecular ionization, mass analysis, and detection. **Volume 6** begins with ionization methods and reviews not only those that are used today but also those that were important in the past and that served as a foundation for new methods. Subjects include the venerable electron ionization, field ionization, and chemical ionization and the associated subjects of ion thermochemistry (e.g., proton affinities, electron affinities, gas-phase acidities, etc). The development of these subjects has impacted not only chemical analysis but also our understanding of physics and physical organic chemistry. Developments of the desorption methods (field, laser, Cf-252 plasma, and FAB) allowed mass spectrometrists to make in-depth studies in biochemistry, biophysics, and biomedicine. Further, their need helped to drive new technologies and helped the development of ESI and MALDI.

Mass analysis and detection are the subjects of **Volume 7**. These fields continue to occupy the attention of mass spectrometrists in all areas involving mass spectrometry. Although the early instruments were based mainly on magnetic sectors and double focusing spectrometers, the renaissance of the time-of-flight mass analyzer, made possible by modern electronics and computers, has significantly changed the face of mass spectrometry. The advent of the quadrupole, the quadrupole ion trap, and the Penning trap have had a major impact on contemporary mass spectrometry. The latter instrument is the basis for Fourier transform mass spectrometry (ion cyclotron resonance mass spectrometry). Many of today's inquiries into the basic properties of gas phase ions and applications in biochemistry and biomedicine also rely on these spectrometers, again demonstrating the close connection between the discipline and the technique.

The advent of soft (desorption) ionization was the stimulus for the development of the field of tandem mass spectrometry. Soft ionization often gives little or no structural information, and mass spectrometrists, starting in the 1980s, explored many combinations of analyzers to produce instrumental arrangements that are appropriate for structural chemistry and biochemistry. These instruments grew from earlier spectrometers in the 1970s that were used for ion chemistry and ion physics. A description of these tandem spectrometers appears in Volume 7 and also in Volume 1.

Combining mass spectrometry with separations methods or other types of fractionation techniques has provided major advances in performance with respect to targeted analyses and analysis of complex mixtures. This combination of techniques, such as liquid chromatography (LC-MS), capillary electrophoresis (CE-MS), supercritical fluid (SCF-MS), as well as others, is sometimes referred to as "hyphenated" techniques. **Volume 8** will be devoted to the instrumental setup, operational parameters, and applications of such combined

techniques. As a part of this technology, the volume will cover important aspects of the separation science as well in order to fully inform the reader of pertinent aspects involving its combination with mass spectrometry. Perhaps no other class of instruments has made a greater impact on critical issues of the past few decades than this instrumentation, particularly in the field of environmental and biological sciences. It is because of its extraordinary value that it is appropriate to dedicate a volume to this topic.

The final technical volume (**Volume 9**) will be Historical in nature. The history of mass spectrometry is rich, encompassing the early days where physics played an important role, through its important progression through many areas of chemistry and biochemistry, and today through nearly every area of science where molecular measurements are required. Indeed, it is still growing and is seeing new applications in areas of clinical significance.

In conclusion, this encyclopedia represents a grand tutorial of the subject of mass spectrometry. It will be a strong beginning for those who are new to the field and wish a centralized work and additional reading in order to learn the various subjects in more detail. By the same token, it is also intended to refresh and bring up to date the existing practioner in areas apart or adjacent to his immediate area of interest. Finally, through some of the articles that detail today's cutting edge advances in mass spectrometry, the encyclopedia will present a glimpse of tomorrow's technology and applications. We wish readers good fortune in their travels through this work and the hope that they experience some of the inspiration and fascination that mass spectrometry has brought to the world of science and industry.

Richard Caprioli and Michael Gross (Series Editors)

Preface to Volume 3

Mass spectrometry shares a rich history with biological research dating back many decades. Some of the technologies are very much in use today, such as electron impact and chemical ionization. Stable isotope ratio measurements by mass spectrometry were the major approaches in the discovery of fundamental processes in biology and continue even today to make significant inroads. The pharmaceutical industry has been a major leader in the use of quantitative mass spectrometry for the analysis of drugs and drug metabolites, and also in the use of the technology for the structural elucidation of natural compounds for potential drug candidates. Clinical applications have existed for several decades, albeit at a modest level. Analysis of blood and urine for metabolites for the diagnosis of a disease or those at risk has been a long-standing interest, for example, urinary organic acids for inborn errors of metabolism screening. Despite these successes, large, chemically complex structures such as oligosaccharides and nucleic acids were considered "intractable" and much of the work focused on subunit structure and the separation techniques that were amenable to the smaller subunits from digested macromolecules. In addition, some highly polar and ionic species were difficult to analyze by standard methods.

Over the past 10–15 years, enormous gains have been made by mass spectrometry toward the analysis of all the biomolecules present in cells. Although much early attention was focused on peptides and proteins, there has been a wealth of information and methodology with regard to other major biomolecule classes such as nucleic acids, lipids, and carbohydrates. In fact, it is in these areas that impressive gains have been made and in some measure, in a startling manner. In no small way, modern ionization methods, especially electrospray and matrix-assisted laser desorption, have provided a quantum leap in the capabilities of the tools that we can now deploy for answering biological questions involving structure and molecular weight of virtually every type of molecule in the cell.

The *Encyclopedia of Mass Spectrometry* has devoted two volumes to applications of mass spectrometry to biological molecules. The current volume, Volume 3, covers the major classes of biomolecules including carbohydrates, nucleic acids, and lipids. In addition, special areas of application such as pharmaceuticals, natural products, isotope ratio methods for biomolecule analysis, and clinical applications, are also included. The articles are arranged under general headings for continuity and ease of access, although several of these are of interest across various disciplines. The articles are intended to teach and therefore strive to cover basics and sufficient additional detail to bring the reader up-to-date on a given subject. Some advanced topics are also covered, either in a special section of an article or in additional reading citations.

Finally, I wish to thank the authors, and also the many unknown individuals who have made this work possible and whose major reward is to leave a detailed description and written legacy of this truly amazing technology. My thanks to all those who have helped in the conception, writing, and production of this volume.

Richard Caprioli

Contents

Chapter 4: Natural Products

Chapter 5: Isotope Ratio

Chapter 6: Lipids

CHAPTER 1

Nucleic Acids

DNA and RNA Sequence Analysis

1. Introduction

The recent developments in electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) now permit the gas-phase structural analysis of oligonucleotides and intact nucleic acids. The introduction of these new ionization methods has made the use of mass spectrometry for obtaining sequence information from oligonucleotides and nucleic acids feasible on a routine basis. Sequencing methodologies using mass spectrometry are broadly divided into two categories: gas-phase and solution-phase sequencing approaches. Gas-phase sequencing typically utilizes ESI-MS in combination with collisionally induced dissociation (CID) to generate fragment ions from a mass-selected oligonucleotide molecular ion. Sequence information is derived by interpretation of the CID mass spectrum. Solution-phase sequencing typically utilizes MALDI-MS for the analysis of a mixture of smaller oligonucleotides generated by the enzymatic or chemical digestion of the oligonucleotide of interest. The resulting mass ladder of peaks differs by a single nucleotide residue, and by measuring the mass difference between the ions present in the mass spectrum, the oligonucleotide sequence can be determined. Finally, large-scale DNA sequencing utilizing mass spectrometry as the read-out platform for Sanger dideoxy chain extension products may provide an alternative method to capillary electrophoresis in the future.

2. Nomenclature

Nucleic acids are high molecular weight biopolymers composed of repeating units of nucleotide (nt) residues (Fig. 1). The three major substituents of a nucleotide residue are the heterocyclic base, sugar, and phosphate group. The five most common heterocyclic bases are adenine (Ade), cytosine (Cyt), guanine (Gua), thymine (Thy), and uracil (Ura). Cytosine, thymine, and uracil are classified as pyrimidine bases, and adenine and guanine are classified as purine bases. Adenine, cytosine, guanine, and thymine are the major bases found in deoxyribonucleic acids (DNA), and adenine, cytosine, guanine, and uracil are the major bases found in ribonucleic acids (RNA). Nucleic acids are identified as either RNA or DNA, depending on the identity of the sugar. The sugar is a 2′-deoxy-D-ribose in DNA and a D-ribose in RNA. The phosphate group is usually attached through the 5′ or 3′ hydroxyl groups of the sugar. Oligonucleotides are most commonly joined together through the 3′ and 5′ sites of each nucleoside by a phosphodiester linkage. The primary sequence of an oligonucleotide is, by definition, determined from the 5′ to the 3′ end. Usually, the sequence is written in shorthand notation, using the single letter abbreviations for the nucleobases; for example, 5′-d(pACTG)-3′ and 5′-pACUG-3′.

3. Instrumentation

Sequence analysis of oligonucleotides by mass spectrometry is performed using either ESI-MS or MALDI-MS. Smaller oligonucleotides ($n \leqslant 20$-mer) can be analyzed with either ESI- or MALDI-MS and larger oligonucleotides are typically analyzed with MALDI-MS. MALDI-MS is more amenable to mixture analysis than is ESI-MS, which argues for its use in the sequence analysis of larger oligonucleotides.

3.1 Electrospray Ionization

ESI-MS allows for the analysis of high molecular weight compounds through the generation of multiply charged ions in the gas phase. Because the basis of the mass spectrometric measurement is the m/z value of the molecule, the presence of multiple charges on the molecule will result in a decrease in the m/z values and allow characterization using mass analyzers with limited m/z ranges. A necessary requirement

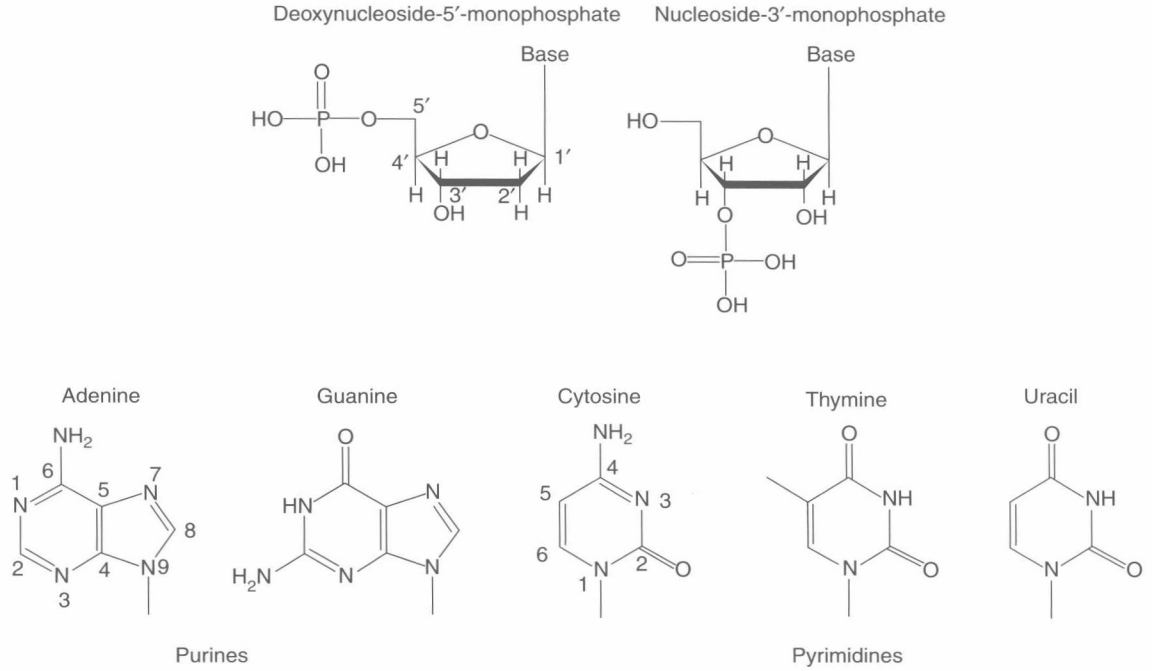
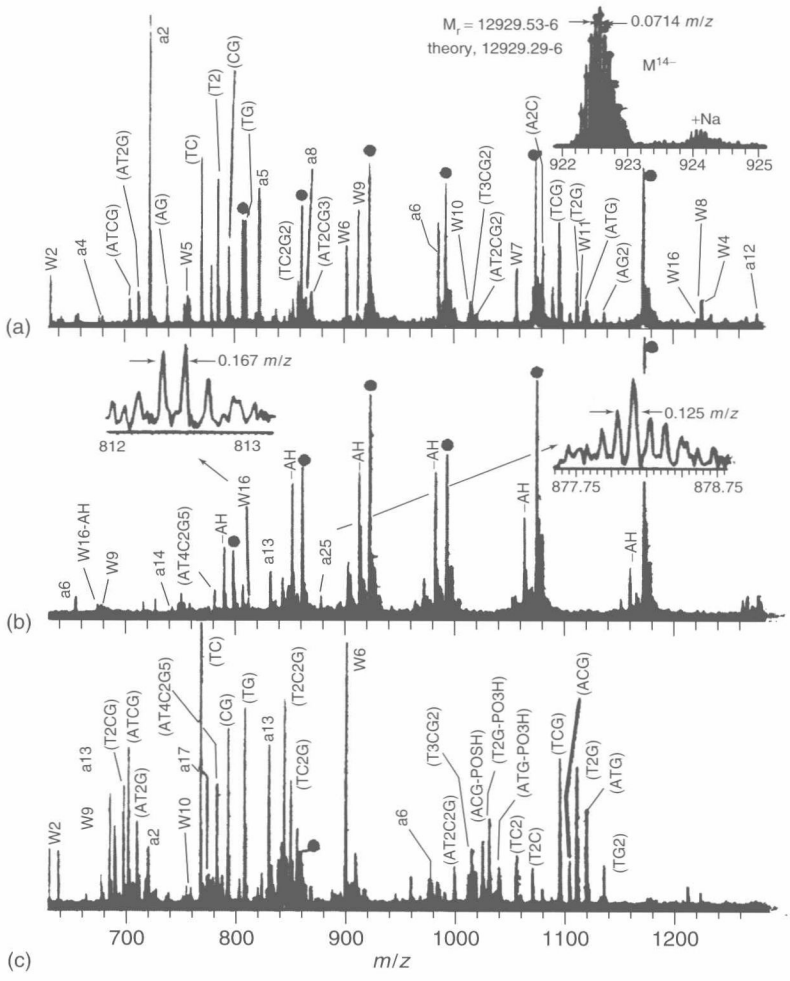


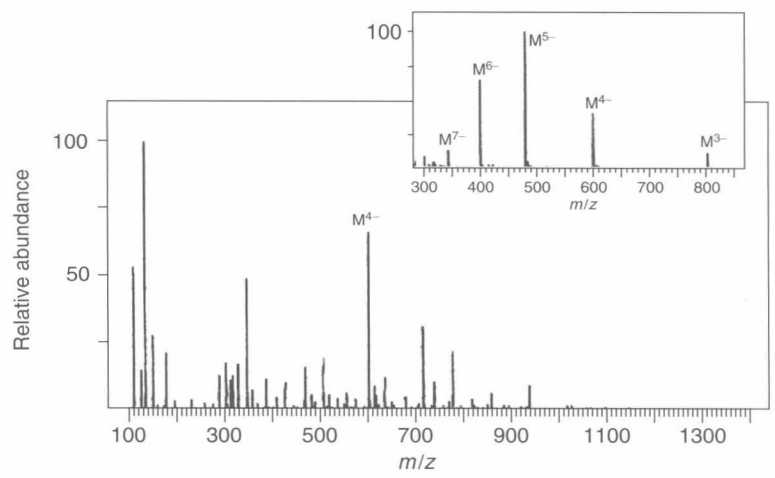
Figure 1
Subunit structures of the major nucleotides from deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

for ESI-MS is that the analyte molecules be charged in solution. The negatively charged phosphate backbone of oligonucleotides allows for negative-ion mode analysis of ESI-generated ions. Transfer of ions from solution phase to the gas phase is accomplished by generating an electric field between a spraying needle, which is held at a high negative potential, and a counter-electrode held at ground or a positive potential some distance from the needle. The solution being sprayed exits the needle as a conical distribution of droplets ("Taylor cone") each containing excess negative charge. A heated drying gas, such as nitrogen, is typically used to assist evaporation of the solvent sheath from the ion. The desolvated, multiply charged ion is then introduced into the mass spectrometer for analysis (*1*).

ESI is a gentler ionization technique compared to MALDI and generally produces multiply charged ions of intact molecules. Nonvolatile cation adduction (e.g., Na^+ and K^+) is a major problem with ESI-MS analysis of oligonucleotides and nucleic acid samples. The negative charge on the phosphate backbone of nucleic acids results in a large degree of Coulombic strain. In solution, solvent molecules help reduce these Coulombic interactions. In the gas phase, where solvent molecules are absent, relief of this strain is achieved by neutralization or cation adduction. These adduct peaks reduce the sensitivity

Figure 2
(Top) ESI/FTMS spectra of a 42-mer oligodeoxynucleotide: (a) nozzle skimmer dissociation; (b) infrared multiphoton dissociation; (c) collision-induced dissociation. A combination of these three dissociation approaches allows for the complete sequence of the 42-mer to be determined. Reproduced with permission of the American Chemical Society from Little, D. P.; Aaserud, D. J.; Valaskovic, G. A.; McLafferty, F. W. Sequence Information from 42–108-mer DNAs (Complete for a 50-mer) by Tandem Mass Spectrometry. *J. Am. Chem. Soc.* **1996**, *118*, 9352–9359. (Bottom) ESI triple quadrupole mass spectrum of d(CGAGCTCG) (inset), and CID mass spectrum resulting from the selection and dissociation of m/z 601.5 ion. Ions from this spectrum were utilized for sequence determination by the automated procedure of Ref. (*5*). Reproduced with permission of the American Chemical Society from Ni, J.; Pomerantz, S. C.; Rozenski, J.; Zhang, Y.; McCloskey, J. A. Interpretation of Oligonucleotide Mass Spectra for Determination of Sequence Using Electrospray Ionization and Tandem Mass Spectrometry. *Anal. Chem.* **1996**, *68*, 1989–1999.


(a)
(b)
(c)
M_r = 12929.53-6
theory, 12929.29-6
0.0714 m/z
M14–
+Na
922
923
924
925
W2
a4
(ATCG)
(AT2G)
(AG)
W5
a2
(TC)
(T2)
(CG)
(TG)
a5
(TC2G2)
a8
(AT2CG3)
W6
W9
a6
W10
(T3CG2)
(AT2CG2)
W7
(A2C)
(TCG)
W11
(T2G)
(ATG)
(AG2)
W16
W8
W4
a12
0.167 m/z
812
813
0.125 m/z
877.75
878.75
AH
a6
W16-AH
W9
a14
(AT4C2G5)
W16
a13
a25
(T2C2G)
W6
(TC)
(CG)
(TG)
(T3CG2)
(ACG-POSH)
(T2G-PO3H)
(ATG-PO3H)
(ACG)
(TC2)
(T2C)
(TG2)
a17
W10
(AT2G)
(AT2C2G)
700
800
900
1000
1100
1200
m/z


100
M5–
M6–
M4–
M7–
M3–
300
400
500
600
700
800
m/z
Relative abundance
100
50
M4–
100
300
500
700
900
1100
1300
m/z

of mass measurement as the ion current is dispersed among multiple cationized ions and can also result in peak broadening in spectra if the mass analyzer has insufficient resolving power. Without question, sample preparation becomes the key to obtaining reliable and accurate sequence information when performing ESI-MS of nucleic acid samples. ESI sources can be interfaced to a variety of mass analyzers including quadrupole, quadrupole ion trap, Fourier transform ion cyclotron resonance (FTICR), sector, and quadrupole time-of-flight (Q-TOF) mass analyzers.

3.2 Matrix-Assisted Laser Desorption/Ionization

MALDI-MS has become a very powerful tool for the analytical characterization of a wide variety of samples. MALDI-MS owes its beginnings to early work performed on laser desorption/ionization mass spectrometry. However, it was not until the introduction of the strongly UV-absorbing matrix that this technique became a widely acclaimed and accepted instrumental method for chemical characterization. MALDI primarily generates singly charged intact molecular ions with multiply charged ions being observed for higher molecular samples. MALDI-MS is more adept at mixture analysis than is ESI-MS.

The vast majority of reported results for oligonucleotides have been obtained using ultraviolet (UV)-MALDI and solid matrices. However, infrared (IR)-MALDI with liquid matrices has recently been shown to yield a dramatic increase in the upper mass range amenable to this technique. As more research is performed on IR-MALDI of oligonucleotides, it may be likely in the future that this configuration will be the preferred approach for the analysis of high molecular weight nucleic acids. Both negative- and positive-ion modes can be used for oligonucleotide analysis by MALDI-MS, with the negative-ion mode preferred for lower molecular weight samples and positive-ion mode preferred for higher molecular weight samples. Positive polarity detection is used for higher molecular weight samples, not due to inherent increases in ionization efficiency in this polarity, but most likely due to the improved detection efficiency of high-mass ions in positive polarity with currently available commercial instruments.

Sample analysis is conceptually straightforward in MALDI-MS. The analyte is mixed with the matrix and spotted on a sample plate. The solvent is evaporated off the plate and the sample is analyzed. The pulsed nitrogen laser ($\lambda = 337$ nm) is the most common laser used in MALDI-MS and is standard on most commercial instruments. As mentioned above, IR lasers are recently receiving additional attention and many vendors offer IR lasers as an alternative radiation source. Operationally, laser fluences on the order of 10^6–10^7 W cm^{-2} with spot sizes around 10–100 μm are common in UV-MALDI-MS. The best results are achieved by working at or just above the threshold irradiance necessary to generate analyte signal.

The most common mass analyzer coupled to a MALDI source is a time-of-flight (TOF) mass analyzer because of its high sensitivity, high m/z range, and compatibility with pulsed lasers. Moreover, MALDI-TOF-MS can be used for high-throughput analysis because of the high duty cycle of the TOF analyzer and the possibility of a high degree of automation of the data acquisition process. Separation of ions of different m/z values in TOF mass spectrometry is accomplished by accelerating the ions through a short 20–30 kV electric field, allowing these ions to drift through a field-free region, and measuring the total flight time from ion formation to impact on the detector with an ion's flight time being proportional to the square root of its mass-to-charge ratio.

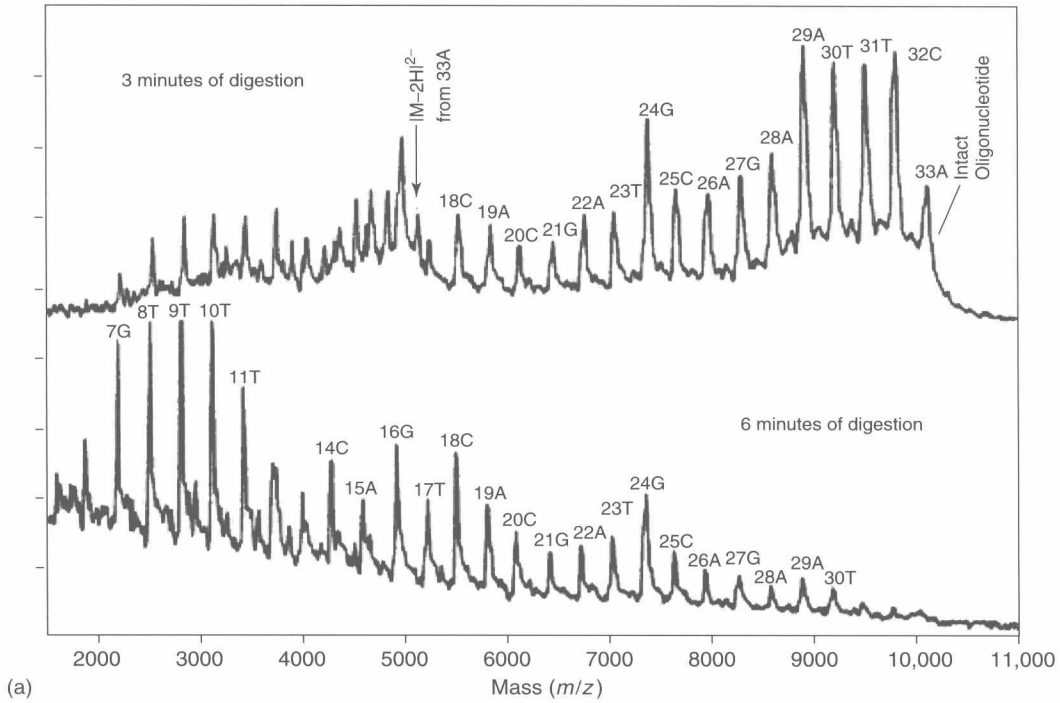
4. Sequence Determination

There are two general approaches to determining oligonucleotide sequence by mass spectrometry. This first approach is a gas-phase approach in which the oligonucleotide of interest is dissociated in the gas phase to generate fragment ions that contain sequence-specific information (*2*). The second approach is a solution-phase approach in which the oligonucleotide of interest is reacted with an enzyme or chemical to yield a series of products that are analyzed by mass spectrometry (*3*). The former approach typically is utilized with ESI-MS and the latter approach utilizes MALDI-MS.

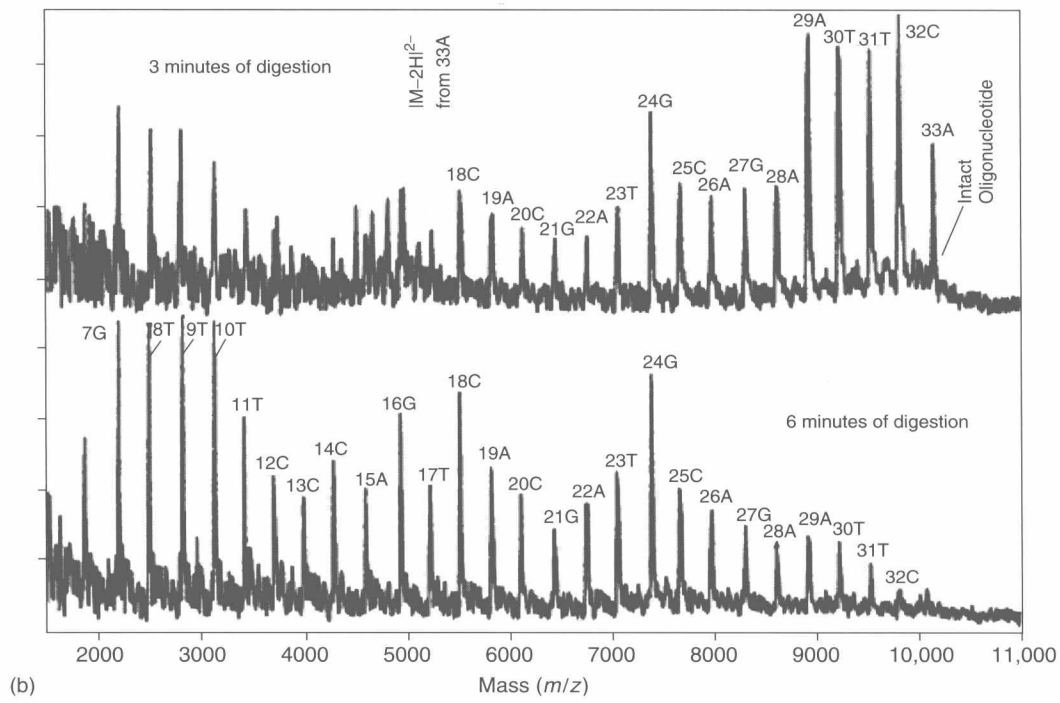
4.1 Gas-Phase Sequencing

Gas-phase dissociation of oligonucleotides can occur either through excess energy deposited into the analyte

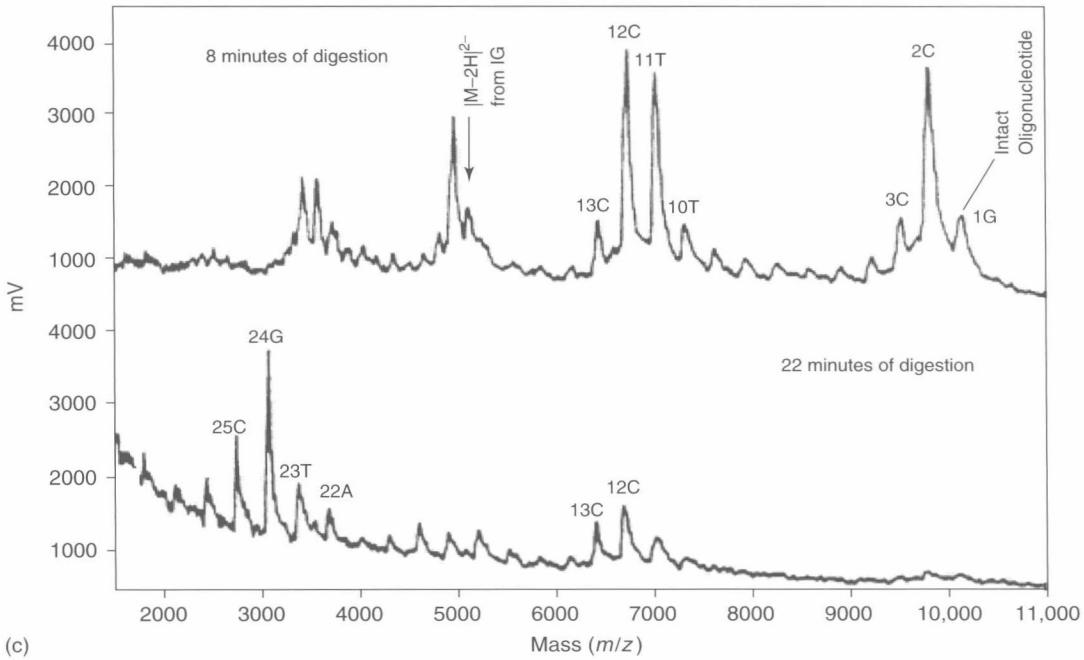
Figure 3
Negative-ion MALDI-TOF mass spectra of the exonuclease digestion of an oligodeoxynucleotide 33-mer, d(GCCAGGGTTTTCCCAGTCACGATGCAGAATTCA). (a) Digestion with snake venom phosphodiesterase, standard MALDI. (b) Digestion with snake venom phosphodiesterase, DE-MALDI. (c) Digestion with calf spleen phosphodiesterase, standard MALDI. (d) Digestion with calf spleen phosphodiesterase, DE-MALDI. Reproduced with permission of Academic Press from Smirnov, I. P.; Roskey, M. T.; Juhasz, P.; Takach, E. J.; Martin, S. A.; Haff, L. A. Sequencing Oligonucleotides by Exonuclease Digestion and Delayed Extraction Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry. *Anal. Biochem.* **1996**, *238*, 19–25.


3 minutes of digestion
IM–2HI2– from 33A
18C
19A
20C
21G
22A
23T
24G
25C
26A
27G
28A
29A
30T
31T
32C
33A
Intact Oligonucleotide
7G
8T
9T
10T
11T
6 minutes of digestion
14C
15A
16G
17T
18C
19A
20C
21G
22A
23T
24G
25C
26A
27G
28A
29A
30T
2000
3000
4000
5000
6000
7000
8000
9000
10,000
11,000
Mass (m/z)

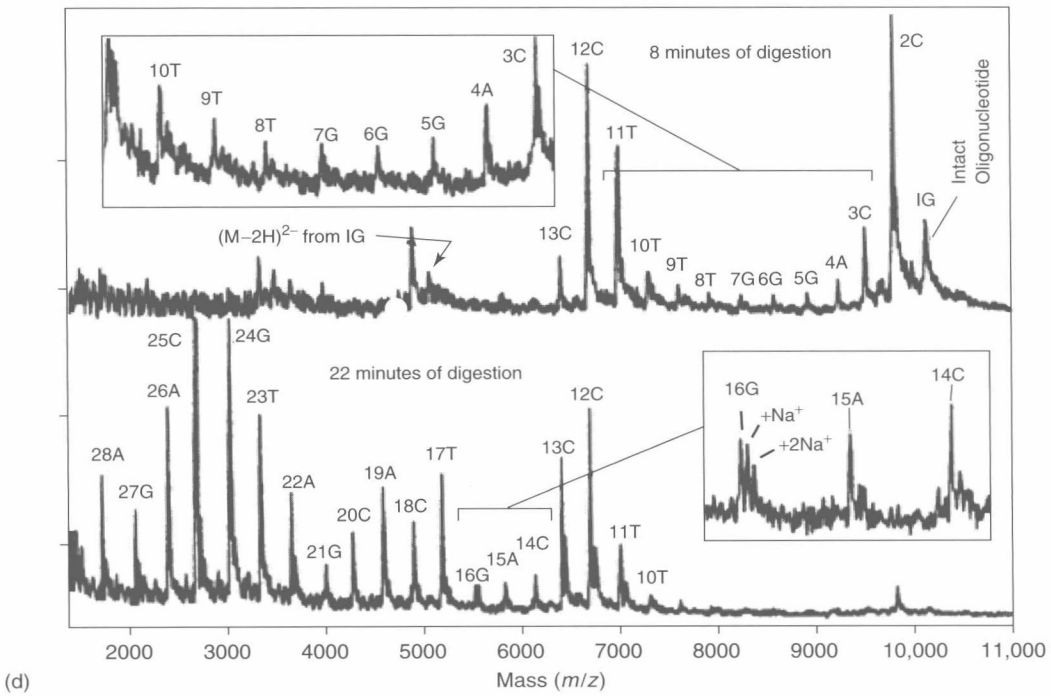
(a)


3 minutes of digestion
IM–2HI2– from 33A
18C
19A
20C
21G
22A
23T
24G
25C
26A
27G
28A
29A
30T
31T
32C
33A
Intact Oligonucleotide
7G
8T
9T
10T
11T
12C
13C
14C
15A
16G
17T
18C
19A
20C
21G
22A
23T
24G
25C
26A
27G
28A
29A
30T
31T
32C
6 minutes of digestion
2000
3000
4000
5000
6000
7000
8000
9000
10,000
11,000
Mass (m/z)

(b)



(c)



(d)

Figure 3
(Continued).