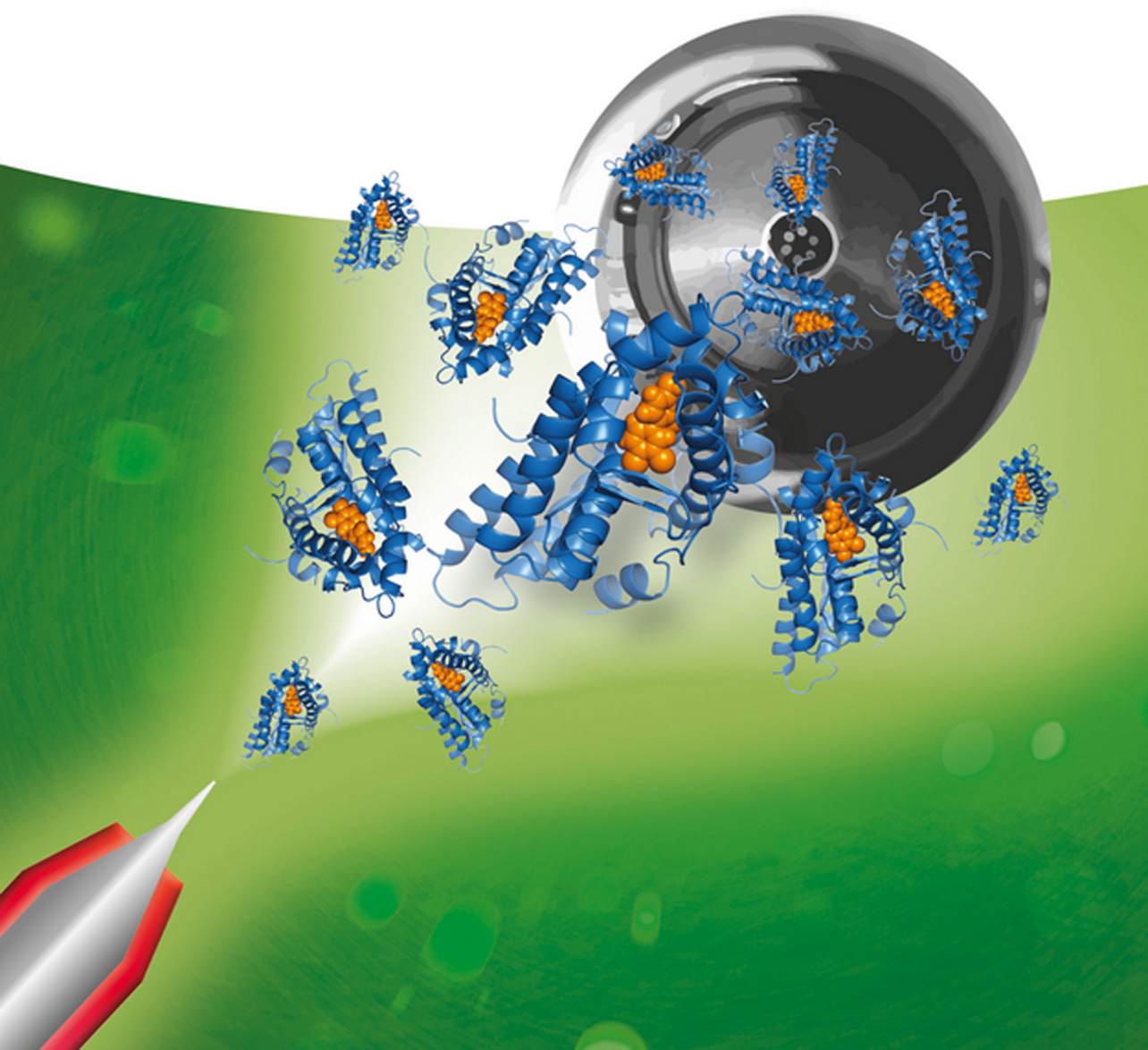


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Jeroen Kool and Wilfried M. A. Niessen

Analyzing Biomolecular Interactions by Mass Spectrometry



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Preface

The introduction, in 1988, of two new ionization methods for mass spectrometry (MS) has greatly changed the application areas of MS, especially in the biochemical and biological fields. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) enabled the efficient analysis of highly polar biomolecules as well as complex biomacromolecules in an easy and user-friendly way and with excellent sensitivity. Multiple charging of proteins in ESI-MS enables the use of simple and relatively cheap mass analyzers in the analysis of peptides and proteins and even opened the way to study intact noncovalent complexes of proteins and drugs or other molecules, including protein–protein complexes. In addition, ESI provided an excellent means to perform online coupling of liquid chromatography (LC) to MS. MALDI-MS with its high level of user-friendliness and excellent sensitivity also boosted the applications of MS in studying biomacromolecules, being more recently even extended to the characterization of complete microorganisms. These developments encouraged further instrumental developments toward highly advanced (and more expensive) mass spectrometers, which provide additional possibilities in the study of biomolecules and their interactions. These new technologies opened a wide range of new application areas, of which perhaps proteomics and all derived strategies and applications belong to the most marked accomplishments. ESI-MS and MALDI-MS changed the way biochemists and biologists perform their research into molecular structures and (patho)physiological processes. Along similar lines, it also changed the ways drug discovery and development is being performed within the pharmaceutical industries. And in the slipstream of this, it changed analytical chemical research efforts in many other application areas.

The ability to study intact biomacromolecules and especially noncovalent complexes between biomolecules as well as other developments in the field, initiated by the introduction of ESI-MS and MALDI-MS, opened extensive research into the way MS can be used in the study of biomolecular interactions. Different distinct areas for analysis of bioaffinity interactions, and for analysis of biologically active molecules in general, can be recognized in this regard. These areas include precolumn-based ligand trapping followed by MS analysis, affinity chromatography following MS, and postcolumn online affinity profiling. Other methodologies are more indirect and relate to separately performed bioassays and (LC)-MS

analysis, such as effect-directed analysis, metabolic profiling, and antivenomics approaches. Besides these, direct approaches without the use of chromatography are nowadays also used in several research areas. These include direct MS-based bioassays and native MS studies in which the latter looks at intact protein complexes in the gas phase. Affinity techniques for trapping proteins and protein complexes toward bottom-up proteomics analysis could also be mentioned in this regard although these techniques are actually specific sample preparation strategies for proteomics research.

With so many new approaches and technologies being introduced in this area in the past 10–15 years, it seems appropriate to compile a thorough review of the current state of the art in the analysis of biomolecular interactions by MS. That is what this book provides in 12 chapters. Apart from a tutorial chapter on MS in the beginning and a conclusive overview at the end of the book, the various chapters are grouped into four themes:

- Native MS, that is, the study of liquid-phase and gas-phase protein–protein interactions by MS and ion-mobility MS
- The use of LC–MS to study biomolecular interactions via indirect assays, as, for instance, applied in effect-directed analysis and related approaches, MS-based binding and activity assays, and other ways to study and identify bioactive molecules, for example, via metabolic profiling or antivenomics.
- Precolumn and on-column technologies to assess bioaffinity, involving frontal and zone affinity chromatography, ultrafiltration and size exclusion chromatography, affinity capillary electrophoresis, and biosensor affinity analysis coupled to MS.
- Online postcolumn continuous-flow bioassays to study bioactivity or bioaffinity of compounds after chromatographic separation.

The contributors to this book did a great job in writing very good reviews and providing beautiful artwork to illustrate the principles and applications of their specific areas within the analysis of biomolecular interactions by MS. For us, it was a pleasure to work with them in this project. We would like to thank them all for their work and for their patience with us in finalizing the final versions of the various chapters.

We hope the readers will benefit from this book, value the overview provided in the various chapters, and perhaps even get stimuli for new research areas or new approaches to perform their research, for instance, by combining ideas and approaches from various chapters of the book into new advanced technologies.

Enjoy reading and get a high affinity with MS!

August 2014

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Abbreviations

μ	Electrophoretic mobility
2DE	Two-dimensional electrophoresis
5-HT	5-Hydroxytryptamine, serotonin
5-HT _{2A}	5-Hydroxytryptamine (serotonin) receptor subtype 2A
Ab	Antibody
ACE	Affinity capillary electrophoresis
ACE	Angiotensin converting enzyme
AChBP	Acetyl choline binding protein
Ag	Antigen
Ag–Ab	Antigen–antibody complex
AhR	Aryl hydrocarbon receptor
AMAC	Accelerated membrane assisted clean-up
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
AR-CALUX	Androgen chemically activated luciferase expression
BGE	Background electrolyte
BGF	Bioassay guided fractionation
BGT1	Betaine-GABA transporter
BLAST	Basic local alignment search tool
BS ² G	Bis(sulfosuccinimidyl)suberate
CCT	Chaperonin containing Tcp1
CDER	Center for drug evaluation and research
CE	Capillary electrophoresis
CECs	Chemicals of emerging concern
CHCA	α -Cyano-4-hydroxy cinnamic acid
CI	Chemical ionization
CID	Collision-induced dissociation
CID-MS/MS	Collision-induced dissociation tandem mass spectrometry
CRISPR	Clustered regularly interspaced short palindromic repeat
CZE	Capillary zone electrophoresis
D _{1–5}	Dopamine receptor subtypes D1 to D5
DAD	Diode array detector
DCC	Dynamic combinatorial chemistry

DCL	Dynamic combinatorial library
DDA	Data dependent acquisition
DVB/CAR/PDMS	Divinyl-benzene/carboxen/polydimethylsiloxane
EC	Electrochemical conversion
ECD	Electron-capture dissociation
EDA	Effect-directed analysis
EI	Electron ionization
EIC	Extracted ion chromatograms
EICs	Extracted ion currents
ELSD	Evaporative light scattering detection
EOF	Electroosmotic flow
ER	Estrogen receptor
EROD	Ethoxyresorufin- <i>O</i> -deethylase
ESI	Electron spray ionization
ESI	Electrospray ionization
ESI-MS	Electrospray-ionization mass spectrometry
ETD	Electron-transfer dissociation
FA	Formic acid
FA	Frontal analysis
Fab	Fragment antigen-binding
FACCE	Frontal analysis continuous capillary electrophoresis
FDA	US Food and Drug Administration
FIA	Flow-injection analysis
FLD	Fluorescence detection
FRAP	Ferric reducing antioxidant power
FRET	Fluorescence resonance energy transfer
FWHM	Full width at half maximum
GABA	γ -Aminobutyric acid
GAT1–3	GABA transporter subtypes 1–3 (according to HUGO)
GC–MS	Gas chromatography mass spectrometry
GC-O	Gas chromatography olfactometry
GCxGC	Comprehensive two dimensional gas chromatography
GPC	Gel permeation chromatography
GPCR	G protein-coupled receptor
GSI	Global snakebite initiative
GST	Glutathione- <i>S</i> -transferase
HBH	Histidine–biotin–histidine
HDX	Hydrogen–deuterium exchange
HEK	Human embryonic kidney cells
HPLC	High performance liquid chromatography
HRS	High-resolution screening
HTLC	High-temperature liquid chromatography
HTS	High throughput screening
I.D.	Inner diameter
IA-CE	Immunoaffinity capillary electrophoresis

IC ₅₀	Half maximal inhibitory concentration
ICP	Inductively coupled plasma
ICP-MS	Inductively coupled plasma MS
ID	Inner diameter
IMS	Ion mobility spectrometry
ISD	In-source decay
IT	Ion-trap MS
IT-TOF	Tandem ion-trap – time-of-flight MS
K_a	Association constant
K_d	Dissociation constant
K_d	Equilibrium dissociation constant
kDa	kilodalton (10 ³ Da)
K_i	Affinity constant
k_{off}	Rate constant of complex dissociation
k_{on}	Rate constant of complex formation
L	Ligand
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LC-MS ^E	Liquid chromatography mass spectrometry in an alternating energy mode
LIF	Laser induced fluorescence
LLE	Liquid liquid extraction
LLOQ	Lower limit of quantification
MALDI	Matrix assisted laser desorption ionization
MS	Mass spectrometry/mass spectrometer
MS/MS	Tandem mass spectrometry
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromid
nAChR	Nicotinic acetylcholine receptor
NECEEM	Non-equilibrium capillary electrophoresis of equilibrium mixtures
NHS	<i>N</i> -Hydroxysuccinimide
NMR	Nuclear magnetic resonance
NMR	Nuclear magnetic resonance spectrometry
np-HPLC	Normal phase high performance liquid chromatography
p38	p38 mitogen-activated protein kinase
PAHs	Poly aromatic hydrocarbons
PDE	Phosphodiesterase
PEEK	Polyether ether ketone
PEG	Polyethylene glycol
PLE	Pressurized liquid extraction
POCIS	Polar organic chemical integrative sampler
PTFE	Polytetrafluoroethylene

QSAR	Quantitative structure–activity relationships
QTAX	Quantitative analysis of tandem affinity purified <i>in vivo</i> cross-linked protein complexes
Q-TOF	Quadrupole time-of-flight
q-TOF	Tandem quadropole – time-of-flight MS
R	Receptor
rhSHBG	Recombinant human sex hormone binding globulin
RL	Receptor–ligand complex
RP	Reverse-phase
RP-HPLC	Reverse-phase high-performance liquid chromatography
RP-LC	Reversed phase LC
rTTR	Recombinant transthyretin
SAFE	Solvent assisted flavor extraction
SAXS	Small-angle X-ray scattering
SBSE	Stir bar sorptive extraction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
sEH	Soluble epoxide hydrolase
SERT	Serotonin transporter
SID	Surface-induced dissociation
SILAC	Stable isotope labeling of amino acids in cell culture
SLC6	Solute carrier family 6
SPE	Solid phase extraction
SPMD	Semi permeable membrane device
SPME	Solid phase microrxtraction
SRM	Selected reaction monitoring mode
T ₄	Thyroxine
T ₄ [*]	Radiolabeled thyroxine
TAP	Tandem affinity purification
TCA	Tricyclic antidepressants
TFA	Trifluoroacetic acid
TIC	Total ion chromatograms
TIE	Toxicity identity evaluation
TLC	Thin layer chromatography
TOF	Time-of-flight
TP	Transformation product
TTR	Transthyretin
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
UV/vis	Ultra violet/visible spectroscopy
WHO	World Health Organization
YAS	Yeast androgen screen
YES	Yeast estrogen screen

1

Introduction to Mass Spectrometry, a Tutorial

Wilfried M.A. Niessen and David Falck

1.1

Introduction

In the past 30 years, mass spectrometry (MS) has undergone a spectacular development, in terms of both its technological innovation and its extent of application. On-line liquid chromatography–mass spectrometry (LC–MS) has become a routine analytical tool, important in many application areas. The introduction of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) has enabled the MS analysis of highly polar and large molecules, including biomacromolecules. MS is based on the generation of gas-phase analyte ions, the separation of these ions according to their mass-to-charge ratio (m/z), and the detection of these ions. A wide variety of ionization techniques are available to generate analyte ions (Section 1.3). Mass analysis can be performed by six types of mass analyzers (Section 1.4), although quite frequently tandem mass spectrometers, featuring the combination of two mass analyzers, are used (Section 1.5). The data acquired by MS allow quantitative analysis of target analytes, determination of the molecular mass/weight, and/or structure elucidation or sequence determination of (unknown) analytes (Section 1.6).

This chapter provides a general introduction to MS, mainly from a functional point of view. Next to basic understanding of operating principles of ionization techniques and mass analyzers, the focus is on data interpretation and analytical strategies required in the study of biomolecular interactions using MS.

1.2

Figures of Merit

1.2.1

Introduction

An MS experiment typically consists of five steps: (i) sample introduction, (ii) analyte ionization, (iii) mass analysis, (iv) ion detection, and (v) data processing and

interpretation of the results. Sample introduction may involve individual samples or may follow (on-line) chromatographic separation. Mass analysis and ion detection require a high vacuum (pressure $\leq 10^{-5}$ mbar). Analyte ionization may take place either in high vacuum or at atmospheric pressure. In the latter case, a vacuum interface is required to transfer ions from the atmospheric-pressure ionization (API) source into the high-vacuum mass analyzer region.

In its basic operation with on-line chromatography or other forms of continuous sample introduction, the mass spectrometer continuously acquires mass spectra, that is, the instrument is operated in the full-spectrum (or full-scan) mode. This means that a three-dimensional data array is acquired, defined by three axes: time, m/z , and ion intensity (counts). This data array can be visualized in different ways (Figure 1.1). In the *total-ion chromatogram* (TIC), the sum of the ion counts in the individual mass spectra are plotted as a function of time. A *mass spectrum* represents a slice of the data array of the ion counts as a function of m/z at a particular time point. Summed, averaged, and/or background subtracted mass spectra can be generated. Mass spectra may be searched against libraries, when available, to assist in compound identification. In an *extracted-ion chromatogram* (XIC), the counts for the ion with a selected m/z are plotted as a function of time. The m/z selection window may be adapted to the resolution of the mass spectrometer. In instruments providing unit-mass resolution, the selection window in most cases is ± 0.5 m/z units (u), whereas with high-resolution mass spectrometry (HRMS, see below) selection windows as small as ± 10 mu can be used (narrow-window XIC). In a *base-peak chromatogram* (BPC), the ion count recorded for the most abundant ion in each spectrum is plotted as a function of time. BPCs are especially useful for peak searching in chromatograms with relatively high chemical background. More advanced tools of data processing are discussed in Section 1.6.1.

Three figures of merit are relevant: mass spectrometric resolution, mass accuracy, and the acquisition speed, that is, the time needed to acquire one spectrum (or one data point in a chromatogram).

1.2.2

Resolution

Despite the fact that mass spectrometrists readily discuss (and boast) on the resolution of their instruments, it seems that there is no unambiguous definition available. The IUPAC (International Union of Pure and Applied Chemistry) recommendations [1] and ASMS (American Society for Mass Spectrometry) guidelines [2] are different in that respect [3, 4]. Most people in the MS community define *resolution* as $m/\Delta m$, where m is the mass of the ion (and obviously should be read as m/z) and Δm is either the peak width (mostly measured at full-width half-maximum, FWHM) or the spacing between two equal-intensity peaks with a valley of, for instance, 10% [1]. The FWHM definition is generally used with all instruments, except sector instruments where the valley definition is used. The *resolving power* is defined as the ability to distinguish two ions with a small difference in m/z . However, resolving power has also been defined as $m/\Delta m$ and the

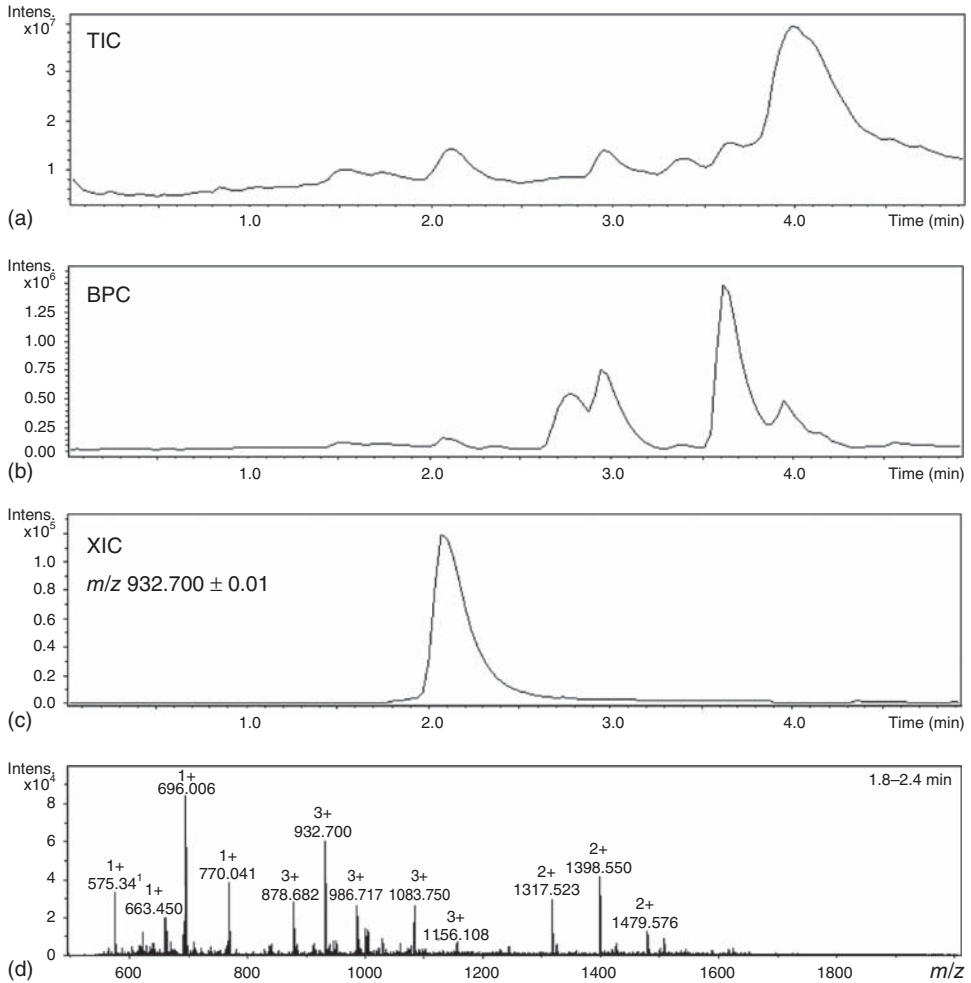


Figure 1.1 Visualization of the three-dimensional data array acquired in a full-spectrum MS experiment. (a) Total-ion chromatogram (TIC), (b) base-peak chromatogram (BPC), (c) extracted-ion chromatogram (XIC), and (d) mass spectrum. Data for an

N-glycopeptide from the LC-MS analysis of a tryptic digest of a commercial immunoglobulin G (IgG) standard, analyzed using a Dionex Ultimate 3000 nano-LC coupled via ESI to a Bruker Maxis Impact Q-TOF MS in the laboratory of one of the authors (D. Falck).

resolution as the inverse of resolving power [3]. The IUPAC definition is used throughout this text.

In a simple and straightforward way, mass analyzers can be classified as either unit-mass-resolution or high-resolution instruments (see Table 1.1). For unit-mass-resolution instruments such as quadrupoles and ion traps, calculation of the resolution as $m/\Delta m$ is not very useful, as the FWHM is virtually constant over the entire mass range.

Table 1.1 Characteristics and features of different mass analyzers.

Analyzer	Resolution ^{a)}	Mass accuracy	Full-spectrum performance ^{b)}	Selected-ion performance ^{b)}	Pressure (mbar)
Quadrupole	Unit-mass	±0.1	+	++	<10 ⁻⁵
Ion-trap	Unit-mass	±0.1	++	+	10 ⁻⁵
Time-of-flight	≤70 000	<3 ppm	++	–	<10 ⁻⁷
Orbitrap	≤140 000	<1 ppm	++	–	<10 ⁻⁹
FT-ICR	≤400 000	<1 ppm	++	–	<10 ⁻⁹
Sector	≤60 000	<3 ppm	+	++	<10 ⁻⁷

a) Resolution based on FWHM definition, except for sector (5% valley definition).

b) ++, instrument highly suitable for this operation; +, instrument less suitable for this operation; and –, instrument not suitable for this operation (post-acquisition XIC possible).

1.2.3

Mass Accuracy

In MS, the mass of a molecule or the m/z of an ion is generally expressed as a monoisotopic mass (molecular mass) or m/z , referring to the masses of the most abundant natural isotopes of the elements present in the ion or molecule. In chemistry, the average mass or molecular weight is used, based on the average atomic masses of the elements present in the molecule. The *exact mass* (or better m/z) of an ion is its calculated mass, that is, its theoretical mass. In this respect, the charge state of the ion is relevant, because the electron mass (0.55 mDa) may not be negligible. The *accurate mass* (or better m/z) of an ion is its experimentally determined mass, measured with an appropriate degree of accuracy and precision. The accurate mass is the experimental approximation of the exact mass. The *nominal mass* (or better m/z) is the mass of a molecule or an ion calculated using integer values for the masses of the most abundant isotopes of the elements present in the molecule or ion. The *mass defect* is the difference between the exact mass and the nominal mass of ion or molecule [1, 5].

The achievable mass accuracy in practice depends on the resolution of the mass analyzer and the quality and stability of the calibration of the m/z axis. An instrument providing unit-mass resolution generally allows m/z determination for single-charge ions with an accuracy of ±0.1 u (nominal mass determination). In HRMS, the *mass accuracy* is generally expressed either as an absolute mass error (accurate mass – exact mass, in mu) or as a relative error (in ppm), calculated from

$$\frac{(\text{accurate mass} - \text{exact mass})}{(\text{exact mass})} \times 10^6$$

In HRMS of small molecules, the error in m/z determination will typically be in the third decimal place (accurate mass determination).

From the accurate m/z of an ion, one can use software tools to calculate its possible elemental compositions. The number of hits from such a calculation

obviously depends on the m/z value, the number of elements considered, and the mass accuracy achieved [6]. The number of hits may also be reduced by taking an accurately measured isotope pattern of the ion into consideration [7, 8]. For a given ion with m/z M , the relative abundances of the ions with m/z $M+1$, $M+2$, and $M+3$ reveal the presence (or absence) and even the number of specific elements, for example, Cl, Br, and S from the $M+2$ ion. For small molecules (<1 kDa), the maximum number of carbon atoms in the molecule can be estimated by dividing the relative abundance (in percent) of the $M+1$ peak by 1.1. Ultra-HRMS instruments have additional possibilities to derive elemental composition, as they can even separate the contributions of different atoms to the $M+2$ isotope peak. This is illustrated for an unknown compound with $C_{13}H_{24}N_3O_6S_2$ in an onion bulb in Figure 1.2 (see also [9]).

As discussed in Section 1.6.6, mass accuracy also has a distinct influence on the ease and quality of protein identification from peptide-mass fingerprints or peptide-sequence analysis approaches.

1.2.4

General Data Acquisition in MS

The general mode of data acquisition of a mass spectrometer is the full-spectrum (or full-scan) mode. In this mode, mass spectra are continuously acquired between

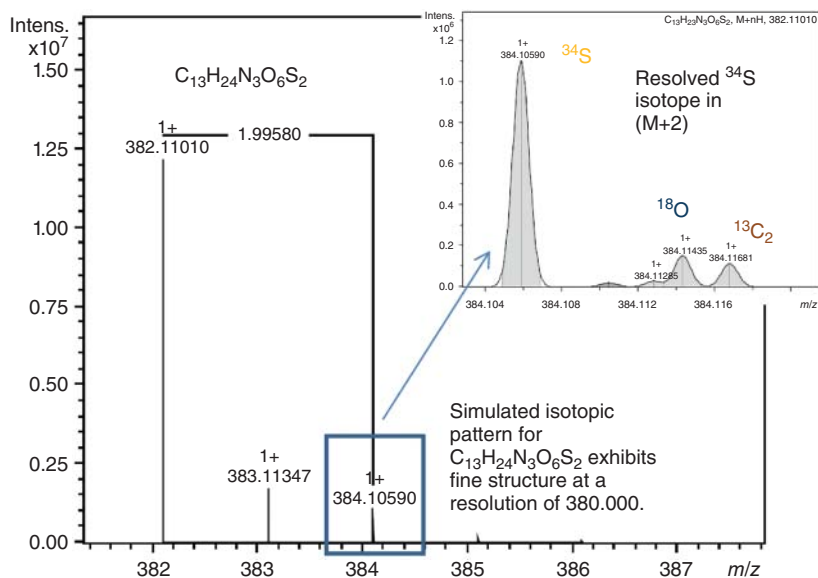


Figure 1.2 Demonstration of high-resolution mass spectrometry. Simulated isotopic pattern for an unknown compound with $C_{13}H_{24}N_3O_6S_2$ in an onion bulb with isotopic fine structure exhibited at

a resolution of 380 000. (Reprinted with permission from Prof. Kazuki Saito (RIKEN Plant Science Center, Yokohama, Japan) and Bruker Daltonics Application Note # LC-MS 85, ©2013, Bruker Daltonics, 1822187.)

a low m/z and a high m/z within a preset period of time (mostly ≤ 1 s). Obviously, the information content of the spectrum depends on (i) the selected ionization technique, (ii) the resolution of the instrument, and (iii) data system parameters. The mass spectra are acquired in continuous or profile mode, that is, with a number of data points per m/z value. For unit-mass-resolution instruments, ~ 10 data points per m/z suffice, whereas in HRMS far more data points per m/z are required to provide the appropriate resolution and mass accuracy. Either the profile data are saved by the data system, eventually after some data reduction such as apodization to reduce the data file size (see, e.g., [10]), or centroiding is performed, where only a weighted average of the mass peak is saved [4]. The latter greatly reduces the data file size. Post-acquisition data processing tools may require either profile or centroid data.

Some mass analyzers (see Table 1.1) can also acquire data in the selected-ion mode, which means that the mass analyzer is programmed to select a particular m/z for transmission to the detector during a preset period (the so-called dwell time, typically 5–200 ms) and to subsequently jump to other preselected m/z values; after monitoring all selected m/z values, the same function is repeated for some time, for example, during (part of) the chromatographic run time. Thus, compared to the full-spectrum mode, the MS has a longer measurement time of the selected ion, and thus provides enhanced signal-to-noise ratio (S/N). The data can be displayed in terms of XICs. This acquisition mode is especially applied in targeted quantitative analysis. With HRMS instruments not capable of a selected-ion mode, improved S/N and targeted quantitative analysis can be achieved post-acquisition in narrow-window XICs (see Section 1.2.1).

For a proper understanding of the possibilities and limitations of MS, one should be aware of the fact that a mass spectrometer can generally perform only one experiment at a time. However, various experiments can be performed consecutively. Functions may be defined to perform various experiments repeatedly. As outlined in Section 1.6.1, decisions for the next experiment may be based on the data acquired in the previous experiment (data-dependent acquisition, DDA). The time required for individual MS experiments very much depends on the type of instrument used (and its purchase date). Because of the huge progress in faster electronics, modern instruments can perform faster than older instruments.

1.3

Analyte Ionization

1.3.1

Introduction

More than 50 analyte ionization techniques are available for MS. An ionization technique has to generate gas-phase analyte ions, either in (high) vacuum or transferable from atmospheric pressure into high vacuum, to enable