Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometer as a New Tool for Organic Chemists

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new tools

This article is dedicated to Professor Zofia Kasprzyk for her 81 birthday

Abstract: Currently, mass spectral determination of molecular weights and elemental compositions of synthetic products is a common practice. It is aided by truly impressive developments in methods and instrumentation enabling to characterize great variety of compounds including even those of a relatively high molecular weight. Confidence in such measurements, however, depends upon selecting a suitable ionization method, unambiguous identification of a molecular-type ion, and the adequate precision of mass determinations. This review addresses major aspects of the ion formation and analysis with special attention given to the ion-cyclotron resonance. This powerful technique, different in its principle from most other methods of ion analysis, offers a superb combination of ion manipulation and measurement parameters and full compatibility with an array of ionization methods.

Key words: mass spectrometry, ultra-high resolution mass spectrometry, ion-cyclotron-resonance, soft ionization, stable isotopes, molecular weight determination.

1 Introduction

Mass spectrometry (MS) is the technique that renders mass-to-charge ratios (m/z) and relative intensities of ions formed (with exceptions), separated, and detected in the high vacuum. The remarkable analytical potentials of MS have been immediately recognized by its inventor J. J. Thomson and, in years immediately following the First World War, the detection and precise characterization of isotopes of almost all elements have been achieved employing the very first mass spectrographs and spectrometers.[1] With some delay, however, these seminal discoveries have been followed by a systematic effort to analyze organic compounds. Ever since, thanks to a steady progress in techniques and instrumentation, MS continues to serve in a variety of inorganic and organic applications. Moreover, by gaining truly astonishing capabilities in terms of ionization, mass range, sensitivity, and accuracy, MS methods cover classes of compounds that originally have been considered as entirely unsuitable for such an analysis.[2]

Still, as exemplified by the recent synthesis of two diterpenoids,[3] synthetic work can be carried out with the complete omission of MS. As a rule, however, MS data are used to confirm MW and to replace elemental analyses of the final reaction products. Only seldom, synthetic publications include detailed spectral interpretations and use more advanced MS techniques. This low visibility of MS contrasts with the structure elucidation of natural and metabolic products and, more recently, with the characterization and sequencing of linear biopolymers. Still, an early implementation of MS analysis in the synthetic practice may greatly facilitate structural assignments well before time and effort is invested in separation and purification of products. MS analysis, especially when combined with the on-line chromatography, provides an advantage in confirming identity and purity of substrates, in finding if the expected compounds are formed, in identifying intermediates and by-products and, finally, in guiding throughout their isolation and purification. Such an extensive use of MS methods, however, requires an access to instrumentation supported by the comprehension of novel and traditional methods with the full awareness of their inherent limitations.

This review, in an attempt to assess the relevance of MS in establishing MW, surveys selected methods of ion for-

mation and analysis. It gives special attention to Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometry (FT-ICR-MS), an advanced method providing an unprecedented ultra-high mass resolution, high sensitivity, and versatile MS/MS, but still only seldom used in a routine characterization of organic compounds. Easier access to FT-ICR-MS instruments, by offering gains analogous to those of now routine use of the high field NMR, should help in traditional and combinatorial synthesis aimed at targets of ever increasing MW and architectural complexity.

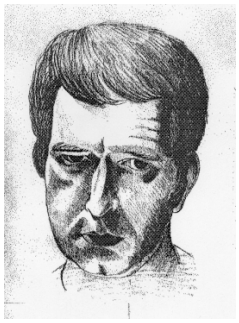
2 Ion Analysis

2.1 Types of mass analyzers

Selection of an **ion analyzer** (**Table 1**) results from specific analytical tasks and compatibility with the mode of sample introduction and ionization. For every mass spectrometer, configuration of its three principal parts, **ion source, ion analyzer,** and **ion detector**, determines **mass range**, **scanning speed**, **mass resolution**, **mass accuracy**, **sensitivity,** and the **dynamic range** of ion intensity measurements. As exemplified by interfacing MS detection with chromatographic methods, some of these parameters can be compromised, especially in instrument dedicated to a single task. Thus, for gas chromatography-mass spectrometry (GC-MS), mass analyzers capable of fast scanning to only about 1,000 u and resolving power separating only the adjacent ions (**unit resolution**) may be quite adequate. Such instruments may operate only with EI (**Section 3.2**) but require good sensitivity and broad dynamic range defining ions that differ in their intensity by 2-3 orders, in components that may differ by another 2-3 orders. For a comparison, LC-MS utilizes greater variety of ion sources and in some configurations must work with interfaces handling a considerable flow of solvents. Moreover, for techniques such as ESI (**Section 3.7**) mass analyzers of an extended mass range (2,000-4,000 u) and resolution depicting multiply charged ions are highly desirable. Although magnetic-sector instruments usually furnish all such necessary parameters, quadrupoles and ion traps[4] prevail as detectors in chromatographic applications. Newer solutions to LC and GC interfacing come from the recent developments in time-of-flight (TOF) instruments offering very high rate of spectral repetitions and good resolution. Still, registering spectra at medium and high resolution, rather than a unit resolution, provides richer structural information and considerably improves specificity of the detection in analytical applications. While a simple, on-line MS detection characterizes compounds by their chromatographic mobility and spectra, tandem-MS (MS/MS) measurements (**Section 2.4**) provide another dimension that delineates specific parent-daughter (or according to PC nomenclature, "precursor-product") ion relations. This information considerably augments selectivity in the detection of selected targets whereas for novel compounds may help in their structure elucidation.

Recent developments are aimed primarily at the emerging applications of soft ionization methods pertinent to medium and high MW biological compounds. There is particular attention paid to the TOF and ion trap instruments, mostly because of their great simplicity, and to FT-ICR, mostly because of its superb parameters. Yet, high resolution magnetic-sector instruments still suit well all major ionization methods including those that are most frequently used in the organic MS and, in terms of resolution and dynamic range, provide benchmark spectra. Even older sector instruments upgraded with new data systems and new ion sources may still offer very good performance.

Biographical Sketch



Jan Pyrek, born in 1942 in Jaslo - a subcarpatian town, was educated in Krakow and Warsaw. He participated in Polish Chemistry Olympiads as early as 1957. He studied and worked in the Biochemistry Department of Warsaw University (1960-7) under the direction of Prof. Zofia Kasprzyk. Subsequently, he obtained his Ph.D. degree from the Institute of Organic Chemistry working with Prof. Osman Achmatowicz Jr. on structure and synthesis of quinone antibiotics (1968-72). Then, still in the same Institute and while visiting laboratories of Professors G. Jommi (University of Milan) and E. Wenkert (Rice University), he continued to work on natural products. In 1979, he joined the faculty of the University of Texas at Houston, initiating new interests in steroids, bile acids, and biomedical application of mass spectrometry subsequently pursued in the MS Laboratory of the Department of Biochemistry of Rice University (1984-9). Currently, as a faculty in the College of Pharmacy, he directs the MS-Facility at the University of Kentucky. He has authored over 90 publications concerned mostly with structure, metabolism, and synthesis of various natural products. Apart from science, he has a long interest in art and, while living in rural Kentucky with his wife Marta and sons Adam, Sebastian, and Amadeusz, he is learning how to grow tobacco and raise cattle.

These large instruments, however, usually require skillful operators and must be re-configured and re-tuned to every ionization method prohibiting an immediate access. Thus, in spite compromised parameters, smaller but fully dedicated instruments may provide an advantage in circumstances where speed is valued over quality. A real competition to sectors, however, comes from instruments that render entirely new features.

Table 1 Mass analyzers

Low accelerating potential (<100V):

Quadrupole ion guide: confines ion beam by alternating electrostatic field perpendicular to its direction; movement of ions controlled in two dimensions; no m/z selection.

Quadrupole mass filter: alternating electrostatic field superimposed on a ramped potential; movement of ions controlled in two dimensions; selective ion transmission according to m/z.

Quadrupole ion trap: alternating electrostatic field; ions confined in three dimensions; selective storage and ejection of ions.

Ion cyclotron resonance: co-linear electrostatic and magnetic fields; ions confined in three dimensions; selective storage and ejection of ions.

High accelerating potential (>1,000V):

Time-of-flight: no permanent fields; sorts ions according to their velocity; in the **reflectron time-of-flight,** electrostatic mirror compensates for the energy spread of ions.

Magnetic-sector: fixed or ramped magnetic field (B) perpendicular to the direction of the ion beam; direction focusing deflects and disperses ions of different m/z in one dimension. Energy focusing obtained by the additional electrostatic sector (E), with curved electrostatic field perpendicular to the ion beam and perpendicular to the direction of the magnetic field; configurations: EB (normal geometry) or BE (reverse geometry).

Wien filter: mutually perpendicular and overlapping magnetic and electrostatic fields; ions dispersed in one dimension.

Parabola mass spectrograph: co-linear and overlapping magnetic and electrostatic fields; ions dispersed in two dimensions.

2.2 Compatibility of ion sources with mass analyzers

Ion sources, such as EI, CI, FAB, and ESI (**Sections 3.2, 3.3, 3.4,** and **3.7**), produce a continuos beam of ions fully compatible with scanning by quadrupoles or magnetic-sectors. Scanning, by transmitting to the detector ions of only a narrow m/z window, wastes most of the total ion current. For sector instruments, this window is determined by the width of the entrance and exit slits, magnet geometry, and other active elements such as non-scanning quadrupoles confining the ion beam. For scanning quadrupoles, ion transmission is determined by their specific tuning conditions. Because **mass resolution** can be defined as $M/\Delta M$, with ΔM representing peak width at its half-height (this is one of several possible definitions of the resolving power), it is increased by narrowing the observed mass window with a dramatic decrease in the ion transmission and hence, with much decreased sensitivity. Still, for measurements performed at the limited mass range, ion multipliers capable of counting even a single ion provide very good sensitivity. Sensitivity can be improved by focusing of sections of the entire mass range on an array detector in a step-wise manner. This way is analogous to the photographic registration employed in the early mass spectrographs and finds major applications at the extended mass range. Alternatively, a significant increase in sensitivity can be obtained by monitoring only a selected m/z (commonly employed in the isotope-ratio instruments) or a very narrow scanning (**selected ion monitoring**, SIM, frequently utilized in chromatographic applications).

With the high-resolution sector instruments, scanning of a narrow mass range serves also in the accurate mass determination. It is usually performed by the alternative scanning of the measured ion and one or two reference ions at a fixed, pre-selected magnetic field by switching of the precisely measured accelerating voltage. It compensates for fluctuations of the magnetic field and instabilities of power supplies. This "**peak-matching**" technique may provide single ppm accuracy of mass determination much better than a slow scan in presence of a reference compound. But with sector instruments, even short term, ppm accuracy can not be obtained in absence of a simultaneously measured internal mass standard.

Sources such as PD and LD, including MALDI (**Section 3.5**), utilize a short, nanosecond pulse of the ionizing radiation and produce a single burst of ions that is incompatible with the usual scanning mode. Although this obstacle can be circumvented,[5] mass analyzers detecting all ions of a pulse by very fast analysis of all ions (TOF) or by storing ions (quadrupole ion trap and ICR-MS), provide a preferred solution. Ever since the incidental discovery of ^{252}Cf-PD (first method that revealed formation of stable, high-mass molecular ions,[6]), TOF has become the analyzer of choice for all ion sources producing pulses of ions. TOF, introduced in 1946 as ion velocitron,[7] provides simple construction at theoretically unlimited mass range. Recent improvements, such as addition of electrostatic mirrors, re-introduction of the "delayed ion extraction" or "time-lag focusing", and the availability of very fast electronics, enable mass resolution approaching 10,000 and very good mass accuracy.[8] In addition, the unique capability of the very fast ion analysis warrants new applications in combination with chromatography and as a component of tandem MS systems.

2.3 Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometer

2.3.1 Overview

ICR-MS finds its early beginning in the "omegatron".[9] At first, this method of ion analysis has been applied mainly to study ion-molecule reactions. However, the introduction of an ion-trapping cell,[10] Fourier transform for the ion detection,[11] and interfacing with different ion sources, has significantly improved its parameters. New FT-ICR instruments find an array of applications that take full advantage of the unprecedented combination of the ultra-high resolution, high sensitivity, and excellent accuracy of mass measurements. FT-ICR has a unique possibility to isolate, to store, to excite, and to reanalyze ions, enabling, amongst other features, to perform multiple tandem-MS experiments without any additional hardware. History, basics, and major aspects of FT-ICR-MS have been extensively reviewed.[12] Two recent articles are especially recommended for a lucid and comprehensive description of the ICR principles as well as good literature coverage.[13,14] The second article presents systematic overview of calculations of all major parameters pertinent to the trapping, excitation, and frequency measurement of ions moving in the ICR-cell.

2.3.2 Trapping ions in the ICR-cell

In ion-beam instruments, ion source, analyzer, and detector are spatially separated. Thus, as result of a limited ion velocity, there is only a short temporal (μsec) separation of the ion formation, analysis, and detection. In the ICR instruments, except for ions formed externally to the magnet, these three major events may be separated on the order of seconds or even minutes but still take place in the same ICR-cell. This cell, arranged from a set of electrodes in a form of a cube or an elongated box or a cylinder, is placed in a strong magnetic field now most frequently provided by super-conducting magnets. Homogeneity and stability of this field is crucial for the performance and, similarly as for spinning NMR sample, its small imperfections are averaged by the ion motion and, similarly as in NMR, an increased strength of the magnetic field decisively improves all measurement parameters.

In a cubical cell, positive (negative) ions are confined in the electrostatic potential well, which is formed between the two parallel electrodes that are oriented perpendicularly to the lines of a magnetic field and positively (negatively) biased. The combination of these electric and magnetic fields forces the trapped ions into a complex motion separable into three components: **trapping oscillations**, **cyclotron motion**, and **magnetron motion** (**Figure 1)**, the most crucial aspect of the ICR principle (compare ref. 14).

Square trapping electrodes of a cubical cell form an electric field deviating from the ideal one in which a charge oscillates along Z-axis between the two equidistant electrodes of the infinite dimensions. The frequency of this **trapping oscillation** ν_z is inversely proportional to the square root of m/q (m being mass and q the charge of an ion), proportional to the square root of the trapping potential V_t, and inversely proportional to the electrode separation a. In the equation below, parameter α is related to the cell geometry (for a cubical cell, it is equal to 2.774 whereas for the ideal cell it is equal to 4):

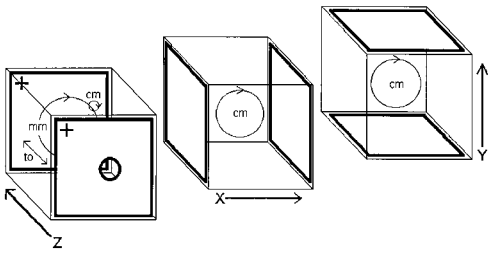


Figure 1. The cubical ICR-Cell: (1) positive ions injected on the Z-direction are trapped by the positively biased **XY** electrodes and "cooled" by the moderation gas; ions assume a complex trajectory composed of slow trapping oscillations (**to**), very slow magnetron motion (**mm**), and fast cyclotron motion (**cm**); (2) after attaining high vacuum, ions are excited by applying a complex frequency signal to the **YZ** electrodes assuming larger and coherent cyclotron orbits; (3) finally, their image current is detected on the **XZ** electrodes. For clarity, only the first figure shows directions of the magnetron motion, trapping oscillations, and cyclotron motion.

$$\nu_z = (1/2\pi)(2qV_t\alpha/ma^2)^{1/2} \text{ or } \omega_z = (2qV_t\alpha/ma^2)^{1/2}$$

The uniform magnetic field (B, in the Z-direction) bends the ion of a velocity v into a circular path in the plane perpendicular to the one determined by the directions of this field and the ion velocity v (Lorentz' force). As result, the angular acceleration v_{xy}^2/r is proportional to the strength of a magnetic field, charge and velocity of the ion in the XY-plane, but it is inversely proportional to the ion mass:

$$v_{xy}^2/r = qv_{xy}B_o/m$$

By substituting the angular velocity $\omega_c = v_{xy}/r$, this equation is transformed to:

$$\omega_c^2 = q\omega_c B_o/m \text{ or } \omega_c = B_o q/m$$

As evident from the above, the angular cyclotron frequency ω_c is independent of the ion original velocity and has the sole dependence on m/q and the magnetic field strength. This is in a sharp contrast to the situation of magnetic-sector instruments following similar equation but including r and the ion velocity (i.e. the acceleration voltage). In the real cell with finite dimensions, however, trapping oscillations contribute a radial component opposite to the Lorent' force. Thus, the corrected force acting upon the ion is equal:

$$m\omega^2 r = q\omega B_o r - qV_t\alpha r/a^2$$

This equation when rearranged into a simple quadratic form, is independent of the ion radius r:

$$\omega^2 - q\omega B_o/m + qV_t\alpha/ma^2 = 0$$

The two solutions of this equation can be expressed using ω_c and ω_z defined as above, and describe the **"reduced" cyclotron frequency** and **magnetron frequency** respectively:

$\omega_{\text{reduced cyclotron}} = \omega_{rc} = \omega_c/2 + ((\omega_c/2)^2 - \omega_z^2/2)^{1/2}$

$\omega_{\text{magnetron}} = \omega_m = \omega_c/2 - ((\omega_c/2)^2 - \omega_z^2/2)^{1/2}$

It is immediately apparent that the difference of ω_c and ω_{rc} is equal to the ω_m and with the frequency of trapping oscillations being much smaller than the cyclotron frequency, there is only a small deviation from a linear dependence holding for the "ideal" cyclotron frequency ω_c. This, nevertheless, requires the exact calibration of the frequency (i.e. mass) scale of the FT-ICR instrument, possibly under the same trapping conditions and for a similar number of trapped ions. For example, in a magnetic field of 4.7 Tesla, for ions at m/z 19 (H_3O^+) and 720 (C_{60}^+), the calculated cyclotron frequencies ν_c (calculated from the relation $\nu=\omega/2\pi$) are 3,798,615 and 100,241 Hz respectively. In a 1 inch cubical cell and 0.5 V trapping potential, their trapping oscillation frequencies are 23,518 and 3,820 Hz respectively resulting in the reduced cyclotron frequencies of 3,798,542 (-73 Hz, -20 ppm difference) and 100,168 Hz (-73 Hz, -720 ppm difference) respectively. For these two ions, their calculated magnetron motion frequencies are very close being 72.80 and 72.85 Hz respectively. In the same cell, for the same two ions at m/z 19 and 720, raising the trapping potential to 10 V increases the calculated frequencies of their magnetron motions to 1,478 and 1,457 Hz respectively. Another value that can easily be calculated for the above equations is the critical mass for which cyclotron motion is no longer stable, i.e. frequencies of the reduced cyclotron motion and magnetron motion are equal. In the 1 inch trapping cell, 0.5 V trapping potential and 4.7 Tesla magnetic field (i.e. as for the above examples) the critical mass is 248,000 u (Da). For other reasons, such as possibility to trap with an acceptable radius, such a high mass range may not be available.[15]

Space charge formed by the trapped ions is another important factor affecting the accuracy of mass determination and precluding ions of a very close m/z to attain discretely different cyclotron frequencies. Space charge restricts the number of ions that can be kept in the cell without effecting their perfect storage and detection and, consequently, limits the dynamic range. At low trapping potentials, ions can be lost from the ICR-cell by their ejection along Z-axis. However, a significant frequency and amplitude of the magnetron motion may also affect ion storage in the XY direction.

2.3.3 Detection of the trapped ions

Initially, ions are trapped at the increased pressure caused by a buffer gas admitted into the ICR-cell region as a short pulse. It causes ions to decrease their velocity and to attain a small (<1mm) radius of an incoherent cyclotron motion. The subsequent pumping (to ~10^{-8} Torr) prepares these "cooled" and trapped ions for the detection. For this purpose, the excitation signal is applied to a second pair of electrodes placed in the XZ-plane. It is either a short pulse or a complex signal containing all frequencies corresponding to a selected mass range applied at once or in a fast succession. At their resonant frequency, ions of the same m/z absorb energy increasing their linear velocity and assuming larger but now fully synchronized cyclotron orbits. These orbits have the same radius for ions of different m/z as, importantly, there is no dependence on the m/z in the excitation event. Ion packets, even as small as about two hundreds of singly charged ions,[16] are now able to induce a measurable **image current** on the third pair of electrodes parallel to the XY-plane (**dipolar detection**). Alternatively, an image current of the doubled frequency can be detected by both XZ and YZ electrodes (**quadrupolar detection**). The image current is independent of the magnetic field but is proportional to the charge and radius of the cyclotroning ions. Amplitude of the excitation signal must be well adjusted to prevent ion ejection (see below) why allowing to obtain sensitivity sufficient for detecting complex transient signals corresponding to all trapped ions. This signal, digitized and registered in the time-domain, is now ready for processing by the Fast Fourier Transform (or other methods) extracting all component frequencies allowed by the selected digitization rate. Signals are digitized either directly (**broad band acquisition**) or after subtracting a close, fixed frequency (**narrow band** or **heterodyne acquisition**, the mode used in FT-NMR). Limitations of electronics performing digitization as well as limitations in storing and processing large arrays of data disallow long transient to be acquired at very high digitization rates. Thus, under the condition of a broad band acquisition, its is impossible to take full advantage of the available frequency resolution. Narrow band acquisition, however, due to dramatic decrease in measured frequencies, enables both long acquisition and low digitization rate. As in NMR, FT process can include apodization and zero-filling affecting the apparent peak shape and resolution.

As stressed above, ion kinetic energy does not influence cyclotron frequency, which can precisely measured with resolution increasing proportionally to the length of a transient signal. Thus, in absence of ion/neutral-collisions and other mechanisms leading to the ejection of ions from the ICR-cell or disturbing their coherent movement, undistorted trapping of ions lasting for seconds or even minutes is achievable for small and medium ions.

As mentioned above, applying a complex frequency signal to the cell causes the trapped ions to absorb energy at their specific resonance. Thus, a broad range of frequencies is applied to cover the measured mass range with amplitude and duration adjusted so as to put all ions on the undistorted cyclotron orbits within the cell dimensions and to give an optimal image current. Such excitation allows also ejecting of all ions from the cell if their orbits reach the electrodes. It is possible, however, to construct a complex signal that misses one or more frequency windows of a predetermined width (**stored waveforms inverse Fourier transform, SWIFT**).[17] Applying such a complex signal to the trapped ions causes ejection of all ions with exception of a selected cluster of isotopic ions or even a single ion. This and similar modes are used prior

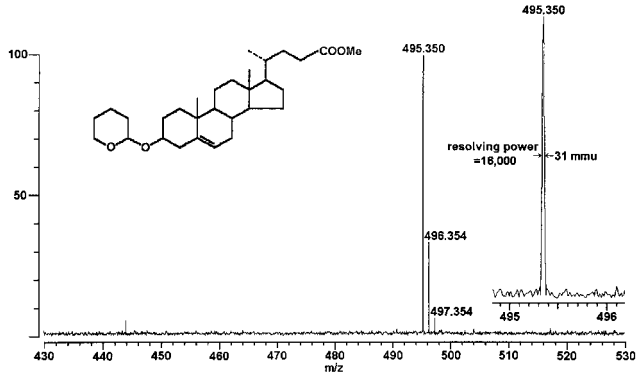


Figure 2. Externally calibrated broad-band MALDI-FT-ICR-MS of a bile acid THP ether obtained in 2,5-dihydroxy-benzoic acid (DHB) spiked with sodium iodide. This spectrum was obtained at 2 MHz sampling rate with 512 k data points processed. The instrument was calibrated about one week earlier. The calculated exact mass of MNa^+ ion is 5 mmu lower.

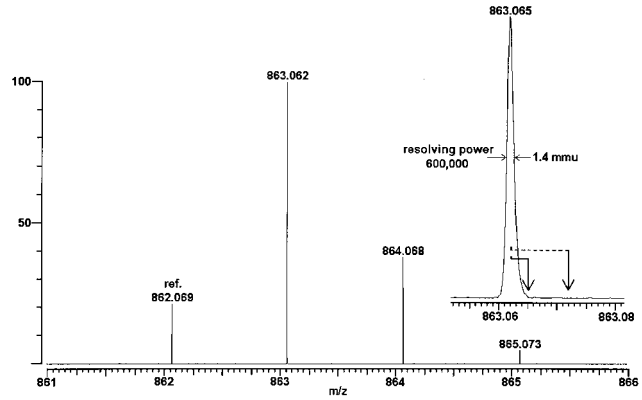


Figure 3. Narrow-band, negative MALDI FT-ICR-MS of the boron compound shown also in Figure 8. In spite very high resolution, the excessive buildup of a space charge prevented separation of the two isotopic ions $^{11}BC_{32}H_{12}F_{24}$ (calculated 863.065) and $^{10}B^{13}C^{12}C_{31}H_{12}F_{24}$ (calculated 863.072, expected intensity ~8%) and shifted their positions from that expected (see arrows). Mass scale for this spectrum was set by taking the monoisotopic ion $^{10}BC_{32}H_{12}F_{24}$ (calculated 862.069) as the reference. This provided good agreement with the observed positions the two other ions $^{11}B^{13}C^{12}C_{31}H_{12}F_{24}$ (calculated 864.068), and $^{11}B^{13}C_2{}^{12}C_{30}H_{12}F_{24}$ (calculated 865.072). An analogy can be made to a large group of runners occupying track of a limited width. In such a situation, it is difficult for each individual to retain its own speed, especially if the difference is minimal. Slightly slower or faster individuals are forced to run with the major group and affect the averaged speed.

to further manipulation of ions, including tandem-MS measurements (**Section 2.4.2**).

FT-ICR provides a non-destructive mode of the detection that may be repeated many times for the same ion packet. After each cycle, this mode of **ion re-measurement** employs ion re-axialization in the center of a cell followed by another excitation and acquisition.[18] As result, similarly as for other spectral methods using signal averaging, there is an improvement of the signal-to-noise ratio proportional to the square root of a number of individual measurements. In this respect, **quadrupolar axialization** allows converting of the magnetron motion into cyclotron motion and improves not only ion re-measurement but also many other important parameters of FT-ICR.[19]

The following spectra, obtained at the field strength of 4.7 Tesla with the instrument equipped with the two separated MALDI and ESI units (IonSpec),[20] illustrate major features of FT-ICR-MS measurements. For compounds below 1,500 Da (singly charged ions), the broad-band acquisition with the 2 MHz sampling rate, 512k data points (0.262 second transient) provides mass resolution of about 20,000 (**Figure 2**). It can still be doubled by going to 1024k points. In a narrow band mode, for transients lasting almost a minute, the resolution may reach 2,000,000 (**Figure 3**). Such ultra-high resolution, but only with the optimal trapping conditions, allows to separate ions that differ only by a few mmu (**Figure 4**). Stability of

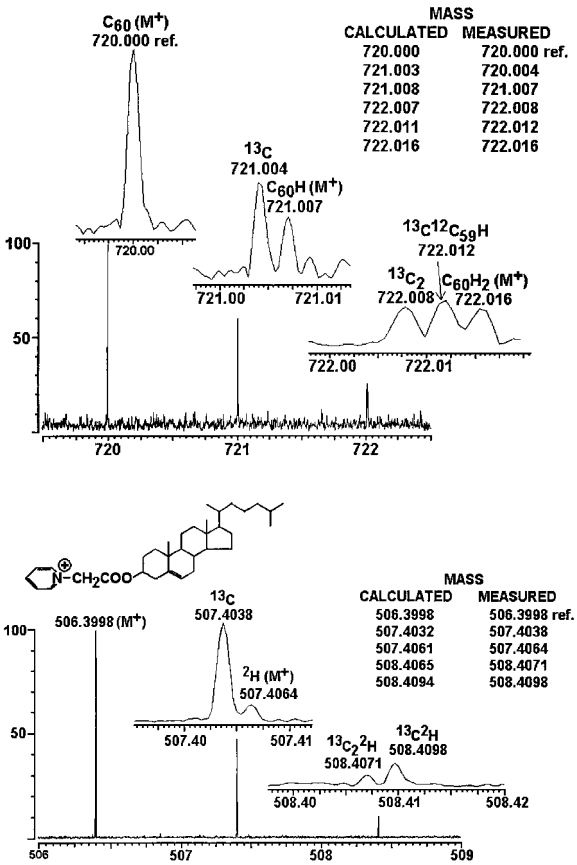


Figure 4. (**Top**) Ultra-high resolution, narrow-band positive MALDI-FT-ICR-MS of cholesterol betaine-type ester obtained for the mixture of the unlabeled and 4-mono-deuterated cholestrol in a ration of about 10:1 measured in DHB as a materix. In spite a lower resolution (~340,000), if compared to that in Figure 3, trapping a smaler ion population allowed to separate two pairs of isotopic ions at m/z 507 $^{13}C^{12}C_{33}H_{52}O_2N$ and $C_{34}{}^2H^1H_{51}O_2N$, and at m/z 508 $^{13}C_2{}^{12}C_{32}H_{52}O_2N$ and $^{13}C^{12}C_{33}{}^2H^1H_{51}O_2N$. (**Bottom**) Narrow-band MALDI FT-ICR-MS of partly hydrogenated C_{60} (courtesy of Prof. Mark Meier, University of Kentucky) measured in 9-nitroantracene as a matrix. Resolving power of about 500,000 (for m/z 720) allowed differentiating all expected isotopic ions at mass 721 and 722 (J. St. Pyrek and A. C. Harms, unpublished data).

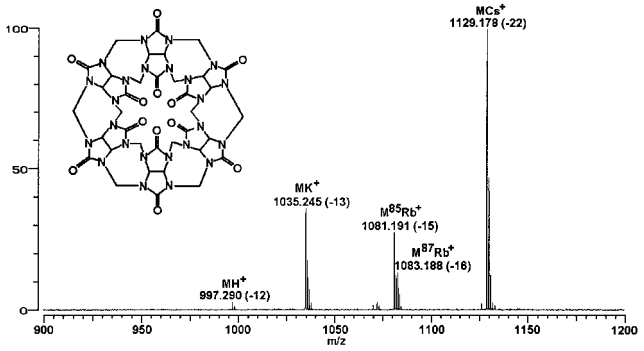


Figure 5. Externally calibrated positive MALDI-FT-ICR-MS of cucurbituril measured in DHP as the matrix. Although the mixture of halides of lithium, sodium, potassium, rubidium, and cesium was added to the matrix, this compounds shows the preference to large alkali metal ions. With a smaller ion population in the ICR-cell, only major ions were observed but the mass measurement errors dropped to 2-8 mmu. This sample was generously provided by Prof. William L. Mock, University of Illinois at Chicago

the super-conducting magnet, both short and long term, is another extremely valuable characteristics of this system enabling mmu accuracy of mass measurements even in the absence of internal calibration reference (**Figure 5**). Ion isolation, required prior to MS/MS experiments, can be achieved by applying suitably adjusted frequency signal that retains only one isotopic component of an ion cluster (**Figure 6**).

2.3.4 Getting ions into the ICR Cell

In the ICR system, ions may be formed directly in the cell by a laser pulse focused on a suitably positioned probe. Furthermore, the ICR-cell may directly serve as the EI source with the analyzed compound admitted either continuously or, preferably, via a pulse valve. In such a source, filament located on the Z-axis emits electrons toward the cell along the lines of the magnetic field. Cyclotron motion puts electrons on a spiral trajectory, similarly as in the conventional EI sources employing small permanent magnets, thus increasing their path and the ionization efficiency. Because ionization and detection are separated in time, the electron beam can be switched off during detection eliminating interference with the space charge of ionizing electrons. The same source can produce CI spectra if the ions trapped are allowed to equilibrate with their parent molecules via protonation/deprotonation (self-CI). Alternatively, pulse of a reagent gas admitted to the cell region produces conditions resembling CI in a continuous-beam ion source but at a comparatively low pressure. With a long time available for ions derived from the reagent gas to collide with neutral molecules, a sufficient number of product ions can be accumulated for detection.

Limited size of a horizontal bore of a super-conducting magnet puts constrains on the vacuum chamber and makes difficult for probes and GC-MS interfaces to reach the ICR-cell located in the center of a magnet. For the

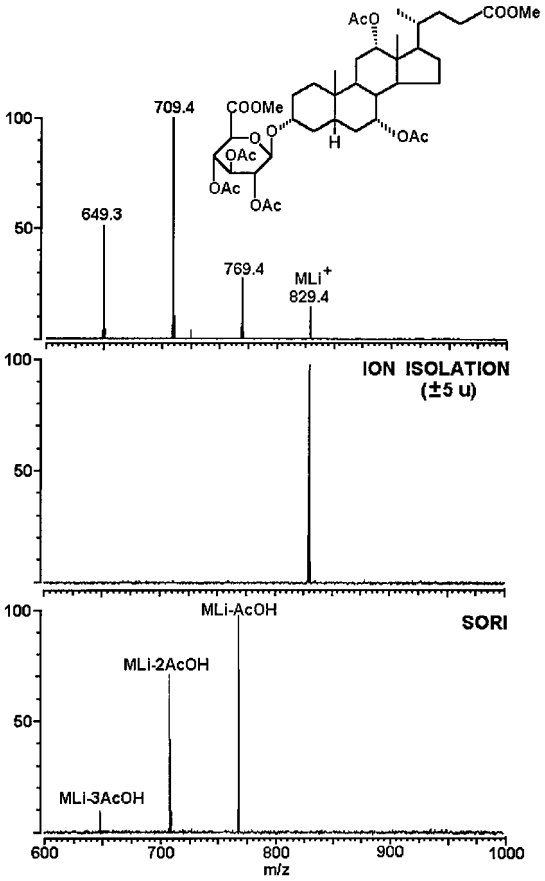


Figure 6. Positive MALDI-FT-ICR-MS of methylated and acetylated cholic acid 3-O-β-D-glucuronide in the DHB matrix spiked with lithium iodide followed by the isolation of MLi^+ ion and the subsequent SORI experiment.

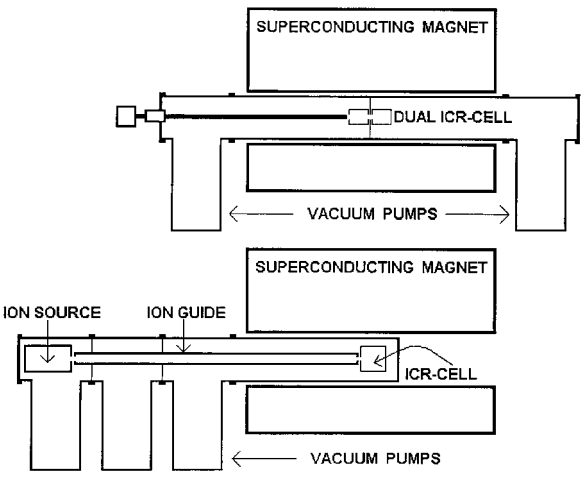


Figure 7. Two configurations of the FT-ICR-MS instrument: dual-ICR-cell with the internal location of the ion sources (top) and single ICR-cell with the external location of ion sources and the ion quide (bottom). There are two differentially pumped regions in the first configuration, usually with the lower vacuum on the source side. The second arrangement enables several stages of pumping before reaching the high vacuum region housing the ICR-cell and allows for a much greater flexibility.

same reason, vacuum pumps can not be positioned close to the ICR-cell and internal ion sources. One solution comes from arranging two ICR-cells back-to-back in the two separately pumped vacuum chambers communicating by a small opening for transfer of ions (**Figure 7**). One such cell serves as the ion source and, due to pressure limitations, ions trapped there can be measured at lower resolution whereas for better resolution, ions can be transferred to the second cell remaining at much better vacuum. A remarkable performance of such a system, in terms of resolution and ion manipulation, has been demonstrated with several ionization methods including GC-MS.[21] Although GC-FT-ICR-MS is still not able to compete with other mass analyzers in providing fast spectral repetition, possibility to perform high-resolution measurements without major sacrifice in sensitivity constitutes the major advancement. This technique may furnish both precise mass and MS/MS information for components that are already characterized by a unit resolution spectra helping in the structure elucidation of reaction/natural/metabolic products prior to their tedious purification.[22] Additional developments of this technique increase sensitivity, dynamic range, and mass measurement accuracy.[23]

Major improvements in interfacing FT-ICR-MS with different ionization methods comes from the introduction of ion guides allowing externally formed ions to enter the ICR-cell placed in a center of a strong magnet. Such guides utilize either non-scanning quadrupoles or electrostatic lenses allowing separating the external source by several pumped regions before ions reach pressure $<10^{-8}$ Torr (**Figure 7**). Currently, especially to implement analysis at high MW, interfacing of the external ESI and MALDI sources with instruments build around superconducting magnets of field strength of 7.0-9.4 Tesla is actively pursued.[24]

2.4 Tandem mass spectrometry

2.4.1 Tandem mass spectrometry with magnetic-sectors and quadrupoles

With many fragmentation pathways possible for a particular ion structure, balance between their kinetic parameters and the internal energy of an ion determines if it survives until detected or fragments before. Magnetic instruments may detect ions that decompose after full acceleration but before reaching the first magnetic or electrostatic sector (in the "first field-free region"). At a fixed accelerating voltage (and fixed potential of the electrostatic sector in the two sector instruments), such **metastable ions** (M*) are registered as broadened signals with the apparent value of m/z equal to $M^*=(M_d)^2/M_p$. Due to partial loss of kinetic energy, such product ions are focused between the parent (M_p) and daughter/product ions (M_d) that have been formed in the ion source attaining full acceleration. Usually, the presence of metastable ions in spectra results from a spontaneous, unimolecular decompositions of energetic ions. Nevertheless, it is useful in confirming a daughter-parent relation of the selected pair of ions. In order to better utilize this feature, an increased fragmentation can be brought about by collisions with neutral gas molecules (**collision activated/induced dissociation**, CAD/CID; several other methods, such as **surface-induced dissociation**, are also available). Collisions change ion kinetic energies into their internal energy, which is channeled into different fragmentations. Consequently, the enhanced formation of daughter ions enables to perform more efficient MS/MS (or **tandem-MS**) experiments and depict fragmentation sequences serving as the source of structural information. In addition, monitoring of characteristic fragmentation processes can be employed in the analysis of mixtures increasing selectivity and sensitivity of the detection (**Section 2.5**).

Normal scanning mode of a magnetic instrument does not provide good resolution (and thereby sensitivity) in detecting metastable ions. Two-sector instruments enable much improved resolution by employing so called "**linked-scans**" in which electrostatic and magnetic fields are varied simultaneously according to simple rules. Thus, respectively, by keeping constant either B^2/E, or B/E, or $B(1-E)^{1/2}/E$, precursor ions of a specified fragment or fragment ions formed form a specified precursor or ions formed by a specified loss of a neutral fragment can be detected. Alternatively, by combining three, four, or more sectors (for example: BEB, EBBE, EBEB, EBEEBE) allows to increase mass resolution in selecting the precursor ion and in monitoring the daughter/product ions. These configurations eliminate the lack of selectivity of the linked-scan and provide "true" MS/MS measurements. In an early demonstration of the power of the EB-EB system, two close ions of phenyl isocyanate (M-CO, C_6H_5H, 91.042) and toluene (M-H, C_7H_7, 91.055) have been separated in the first EB part (resolution of about 12,000) to give different daughter ion spectra analyzed in the second.[25] Quadrupoles, TOF, and ion traps can also be added to sectors resulting in **hybrid-instruments** (for example EB-Q, EB-TOF). In all these systems, every sector/section performs only one function on the accelerated ion beam providing all three modes of the MS/MS analysis. Thus, precursor ions are detected by setting the second analyzer on a specific fragment while scanning the first one. Product ions are detected by setting the first analyzer on the specified precursor while scanning the second. Finally, for detection of ions formed by the constant neutral loss, both analyzers are scanned with the mass-offset equal to that of the neutral fragment.

Another simple but quite powerful arrangement comes from combining two quadrupole analyzers via a collision cell employing the third, non-scanning quadrupole (QqQ). Such "triple quads" provide a versatile tool especially as detectors for the on-line chromatography and the more complex systems (QqQqQ) are being developed.[26]

2.4.2 Tandem-MS with FT-ICR-MS

For every step of the parent ion selection or product analysis, tandem systems described above utilize separate

mass analyzers acting upon the ion beam (two analyzers are required to follow one reaction). However, in systems based on the ion storage (quadrupole ion traps and FT-ICR), the same analyzer enables to follow consecutive fragmentation steps by a flexible, software-controlled sequence of ion manipulations with the temporal separation of individual steps on the order of seconds. This allows to prepare the ICR-cell to different functions by switching on/off and by ramping voltages applied to the individual electrodes, by applying suitably designed frequency pulses, and by admitting gases through fast operating valves followed by fast pumping. As for the normal spectral acquisition, such a sequence usually starts with the removal of any remaining ions by polarizing the trapping electrodes to a relatively high, opposite voltage. Subsequently, in the external source configuration, ions are admitted the cell. This is done by firing a laser (for MALDI) that is synchronized with potentials of the trapping electrodes gating ions into the ICR-cell while sources producing constant beam of ions may require much longer time for admitting. Following ejection, only the selected ions are retained in the cell, submitted to dissociation and analyzed for the product ions by the usual sequence of the excitation and measurement. If the isolation/dissociation steps are repeated, one can follow several successive decompositions with the number of repetitions limited by the number of ions left for the detection. Notably, such analysis of trapped ions detects only products whereas scanning analyzers perform MS/MS analysis in all three modes (**Section 2.4.1**).

In order to induce dissociation of the trapped ions either collisional or radiative energy can by applied. This includes different ways of exciting ions (to increase their kinetic energy) prior to or together with the CAD process (to increase their internal energy). One convenient way is to irradiate selected ions with a frequency close to their cyclotron frequency causing fluctuations of their cyclotron orbits (in a mode analogous to the interference of the two very close radio-transmitters). For the optimal performance, this method of a **sustained off-resonance irradiation** (**SORI**) requires the adjustment of the time, amplitude, frequency, and collision gas pressure.[27]

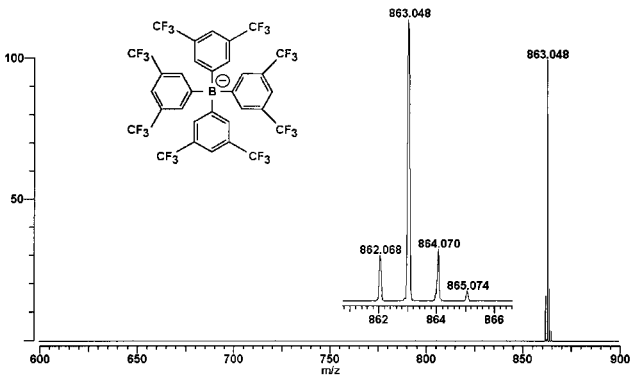


Figure 8. Negative MALDI-FT-ICR-MS of sodium tetrakis[3,5-bis(trifluoro-methyl)phenyl]borate (courtesy of Dr. Robert C. Haddon, University of Kentucky). This spectrum was acquired with the broad-band mode and 1024 k data points, however, only 512 k data points were processed to give the spectral resolution of about 10,000. Compare Figure 3.

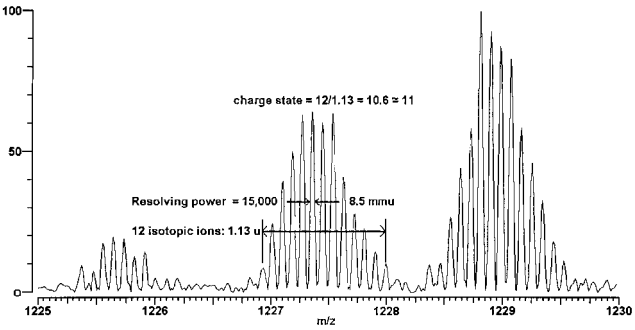


Figure 9. The expanded broad-band ESI-FT-ICR-MS of a peptide mixture. At the resolving power of about 15,000 (calculated from the half-width of peaks), base-line resolution of isotopic ions can be obtained and the charge-state can be derived from the intervals between individual ions measured in mass units. Molecular weight of the parent compound requires the identification of a mono-isotopic ion (for the mono-isotopic mass, which may or may not be present) or finding the center of mass from ion intensities (for the averaged mass). Moreover, one must confirm that the ion in question is charged exclusively by protonation or, possibly, the total charge is due to protonation as well as cationization.

3 Ion formation

3.1 Overview

In the MS ion source, ions are formed either by the removal/addition of an electron - leading to molecular ions $M^{+\cdot}$/M^{-}, or by protonation/deprotonation - leading to pseudo-molecular ions (or, more correctly, protonated/deprotonated molecules) MH^{+}/$(M\text{-}H)^{-}$, or by addition of another small ion - leading to cationized molecules such as MNa^{+}. For salts (e.g. ammonium, phosphonium, carboxylates, and sulfonates), ionization/desorption processes frequently produce gas phase ions that directly correspond to charged moieties and their counter-ions (**Figure 8**). While most ionization methods produce singly charged ions recognized by one mass unit spacing of ^{13}C isotopic components, some methods lead also to multiply charged ions. For such ions, ^{13}C isotopic components are spaced by $^{1}/_{2}$unit (**Figure 9**).

As a rule, identification of the molecular-type ions is the first step in interpreting mass spectra. First, possible origin of the ion of the highest m/z as either a fragment or an addition product must excluded; second, the types of molecular ion represented in the spectrum must be correctly recognized. Molecular ions and protonated/cationized molecules contain all original atoms of the analyzed compound and, consequently, their abundant presence, on one hand, and their correct identification in spectra, on the other, makes the determination of MW straightforward. This simple notion motivates the search for and the pre-

ferred use of ionization methods that lead to stable ions of a molecular-type or, conversely, methods that produce spectra in which these ions dominate over fragments. Kinetic parameters of all possible cleavage and rearrangement processes open to a specific ion structure, on one hand, and the internal energy available to its individual bonds, on the other, determine the temporal stability of an ion during mass analysis. As result, for ions with the same amount of the internal energy those with more atoms and more bonds (for instance for a series of homologues) are less likely to fragment.[28] Notably, for soft ionization methods involving an increased pressure in the space of the ion formation (including methods that desorb ions from liquid or solid phases), collisions with neutral molecules remove an excess of the internal energy (a "cooling" effect). Such soft methods are best exemplified by **field desorption** (**FD**), **californium-252 plasma desorption** (**PD**), **fast atom bombardment** (**FAB**), **liquid secondary ion mass spectrometry** (**LSIMS**), and **laser desorption** (**LD**). On this list are also **matrix assisted laser desorption/ionization (MALDI**) and **electrospray ionization** (**ESI**), two powerful methods that gain general acceptance. In spite limited understanding of the exact ways of ion formation,[29] the use of all these methods tremendously expands the realm of MS analysis with the most spectacular results obtained for biological compounds of a relatively high MW.[30] Notably, these ionization methods also cover many groups of organic compounds that are either difficult or entirely incompatible with **electron impact** (**EI**) and **chemical ionization** (**CI**), the two principal methods of a traditional organic MS.

3.2 Electron impact ionization

The process of electron impact ionization (EI) involves a unimolecular interaction of gas phase molecules with electrons accelerated to about 10-100 eV. As a primary product, EI forms an excited odd-electron molecular radical-ion $M^{+\cdot}$. These ions are additionally destabilized by having an unpaired electron and, usually, are capable of entering complex processes of high-energy demand. Although stability of molecular ions may be improved by lowering energy of the ionizing electrons (thus leading to their increased proportion in spectra), for most of organic compounds such ions fragment extensively. Consequently, the presence of molecular ions in spectra is an exception and not a rule. Some structural characteristics, as those favoring the charge localization, may have a dramatic effect on fragmentation and may increase the relative stability of high-mass ions and channel fragmentation processes as for pyridine containing Schiff base derivatives of aliphatic aldehydes.[31] Similarly, long chain aliphatic alcohols show only barely detectable $M\text{-}H_2O$ ion while low-mass fragments prevail. This contrasts with spectra of their trimethylsilyl ethers (TMS ethers) showing a prominent M-Me ion consistent with the charge at the TMS group.

As has been nicely exemplified for a series of normal hydrocarbons, the intramolecular vibrational and rotational super-cooling of molecules in a **Supersonic Molecular Beam** (**SMB**) leads to an impressive increase in the stability of molecular ions formed by EI.[32] With the conventional EI, normal hydrocarbons show only barely detectable $M^{+\cdot}$ whereas such ions dominate in spectra obtained with SMB. In another approach of **Metastable Atom Bombardment** (**MAB**), gas phase chemi-ionization (not chemical ionization) can be brought about by metastable atoms of noble gases. Employing one of five of these elements (with the excitation energy decreasing from 20 to 8 eV, when going from helium to xenon) one may control the energy transferred to the ionized molecules. For example, ionizing with metastable atoms of xenon minimizes fragmentation and allows very selective ionization.[33] It may be predicted that much broader utilization of MAB and SMB-EI may augment significance of EI ionization, the method that is still well suited to many important classes of organic compounds.

3.3 Chemical ionization

Chemical ionization (CI) is the gas phase process involving ion-molecule reactions with species derived from a reagent gas (or their mixtures) that is first ionized by EI at high electron energy. Typically, the even-electron pseudomolecular ion/protonated molecule MH^+ or $(M\text{-}H)^+$ ions are formed. The efficient CI process, in case of ion sources producing a constant beam of ions, requires relatively high pressure of the reagent gas (~1 Torr) but in instruments that trap ions for an extended time, ion sources may work at much lower pressure. Notably, collisional cooling of the nascent ions by molecules of the reagent gas warranties the relative softness of the CI process whereas specificity of ion-molecule reactions (usually of the acid/base character) affects ionization efficiency and impacts upon the type of ions formed. Very efficient ionization leading to M^- ions can be obtained for compounds of high electron affinity. As result, with a suitable derivation, many compounds can be analyzed at the exceptionally high sensitivity by GC-MS with the negative CI detection.

In addition to ions mentioned above, other high mass ions may result from the association with species derived from the reagent gas or even from "true" chemical reactions. Such ions are exemplified by $M(C_2H_5)^+$, $M(NH_4)^+$, and $M(NO)^+$, formed with methane, ammonia, and nitric oxide respectively. The relation of such ions to the "unknown" parent molecule may not be discernible and their presence may obscure clarity of mass spectra. In such cases stable-labeled reagent gases may greatly help in their identification. For example, the use of a mixture of $^{14}NH_3$ and $^{15}NH_3$ leads to the labeled adduct ions $M(^{15}NH_4)^+/M(^{14}NH_4)^+$ but the protonated ion MH^+ remains unlabeled.[34] Furthermore, with a suitable reagent gas (e.g. ND_3 or D_2O), the in-source deuteration of exchangeable positions can be obtained. Such an exchange can be car-

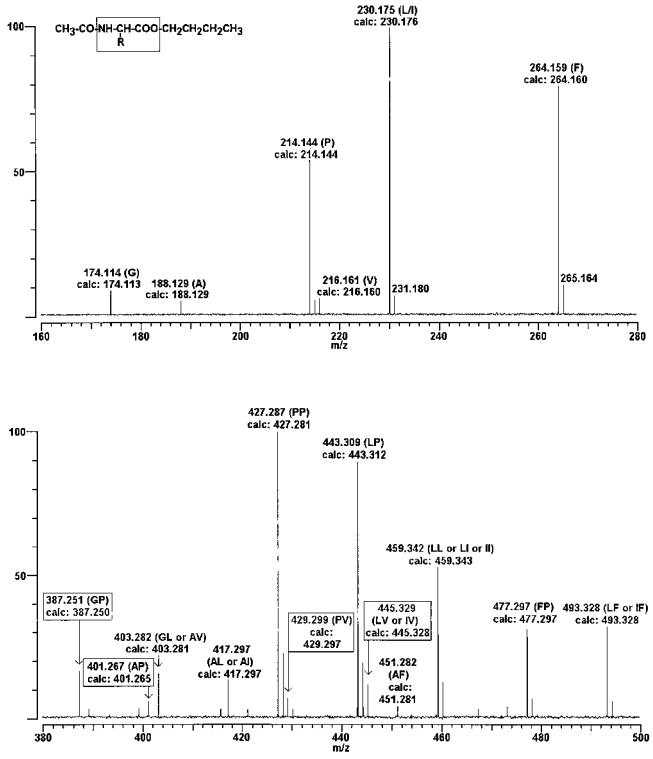


Figure 10. Externally calibrated, positive APCI-FT-ICR-MS of a mixture of amino acid n-butyl esters N-acetyl derivatives (**G** glycine, **A** alanine, **P** proline, **V** valine, **L** leucine, **I** iso-leucine, and **F** phenylalanine taken in an approximately equimolar amount. The **top spectrum** shows protonated molecules (MH^+) of all compounds included in the mixture with dramatically different ionization efficiencies observed for individual derivatives (resolving power about 50,000). The **bottom spectrum**, registered at about 5-times higher concentration (resolving power about 30,000), in addition to ions listed above, shows the set of ions corresponding to 11-12 proton-bound-dimers ($M_1M_2H^+$). There are 21 expected dimers for these 7 amino acids as I and L can not be differentiated based on MW. This feature, although interesting from a point of the apparent selectivity (prevalent dimers were observed for P, V, and L/I), complicates the overall spectrum and requires the accurate mass determination for making the unambiguous identifications. As indicated by this example, formation of such multiple ions (frequently observed in CI, APCI, and FAB) may hinder spectral interpretations. Obviously, such dimeric ions can be recognized more easily for pure compounds, especially for compounds with the known MW.

ried out for an extended time (5-30 minutes!) for ions trapped in the ICR-cell.[35]

Atmospheric Pressure Chemical Ionization (**APCI**), increasingly important in combination with liquid chromatography, involves high temperature nebulization of solutions in presence of a corona discharge or β-emitter while solvents or their additives (for example ammonium acetate) play the role of a reagent gas (**Figure 10**). Ions formed at the atmospheric pressure are transferred to the high vacuum part of a mass spectrometer via several stages of pumping and mass analyzed.[36] A version of CI process, LD-CI and MALD-CI, leading to the attachment of silver ions, can also be obtained with laser desorption. Recently, this method combined with FT-ICR-MS has been applied to the analysis of non-polar polymers.[37]

3.4 Fast atom bombardment

Both EI and CI, capable of ionizing only gas phase molecules, are limited to compounds with some vapor pressure in vacuum and, if higher temperatures are required for volatilization, with a sufficient thermal stability. This contrasts with soft ionization methods not posing such a restriction. Soft methods may efficiently ionize compounds that are entirely involatile either because their high polarity or presence of a charge(s) or high MW or all these factors combined. These methods, however, display selectivity and usually require liquid or solid matrices selected for the reasons of solubility and the most efficient ionization. Matrices, in turn, introduce additional ions resulting from complex clustering processes that may involve impurities and additives. Thus, if MS is used to establish and not just to confirm the anticipated MW, deciphering obscure spectral features may be quite perplexing.

In case of FAB or LSIMS, ions are formed by bombarding solutions with fast atoms or fast ions respectively. Measured compounds are dissolved in a solvent (matrix) of low volatility in vacuum such as glycerol, m-nitrobenzyl alcohol, and triethanolamine. In the routine characterizations of synthetic organic compounds, FAB is still one of the most frequently used methods together with EI and CI. Certainly, its broad acceptance can be attributed to the relative experimental simplicity, especially if compared to FD[38] that has preceded FAB. This is in spite FAB relatively low sensitivity (not especially important in the analysis of synthetic products), low-mass background of strong ions derived from the matrix ("chemical noise"), and a possibility of various reactions with matrix molecules.[39] As for other soft ionization methods, molecules with the inherent charge display especially abundant ions whereas many other compounds show protonated (MH^+) and cationized molecules (**Section 3.6**). Still, some organic compounds are ionized by a simple loss of an electron and produce molecular ions $M^{+\cdot}$.[40]

At the time of its introduction, there has been a great fascination with FAB inspired by a possibility to analyze polar compounds with no need for their derivation.[41] Possibility to introduce moieties carrying a fixed charge, however, extends applicability of FAB and other soft ionization methods. As demonstrated for bile acids,[42] steroids,[43] and peptides,[44] such derivation increases sensitivity and enables analysis of otherwise refractive compounds (**Example 1**). In FAB, complex surface phenomena may cause strong suppression effects leading to spectra showing only certain components or spectra that dramatically change with time.[45] To some extent, these features are obviated by a constant flow of a solution containing a few per cent of matrix applied to the FAB target. This Continuos Flow or Frit-FAB approach, by exposing constantly refreshed surface to the fast atom beam, allows also a successful coupling with LC.[46] Recently, however, this technique and thermo-spray ionization is superseded by APCI and ESI, two methods that enable efficient on-

line coupling with LC and electrophoresis (**Section 3.3** and **3.7**).

3.5 Matrix assisted laser desorption/ionization

Direct laser desorption (LD) is effective only if the λ_{max} of the analyzed compound matches the laser output. Matrix Assisted Laser Desorption/Ionization (MALDI) does not pose this restriction and provides good ionization efficiency at the high-mass range.[47] In MALDI, the light energy is first absorbed by an excess (usually >1,000) of a crystalline matrix with the absorption maximum close to the laser output. Specially formulated liquid matrices may also be used to form ions with a laser pulsing at a frequency of 10~20 Hz. This makes MALDI source compatible with a slow scanning sector instrument.[48] As a rule, however, a short burst of ions formed by a single laser pulse in LD or MALDI, similarly as ^{252}Cf-PD, is followed by non-scanning methods of ion analysis (compare **Section 2.2**).

In a simplified model, matrix evaporation forms excited and ionized species that in turn ionize other components and the type of ions formed parallels those observed in FAB (**Example 1**). As in case of other soft ionization methods, neutral species present in a plume before its dissipation in vacuum provide a cooling effect contributing to the stability of ions formed. Many compounds have been tested as matrices for MALDI but only some work well. For different classes of compounds, matrices show striking differences in the ionization efficiency and the difference in their suitability for different method of ion analysis. For instance, matrices working well with MALDI-TOF may not be suitable for MALDI-ICR. Still, MALDI may show lack of reproducibility resulting from the matrix selection, matrix-to-compound ratio, form of matrix crystallization, and possible presence of impurities including those formed by a degrading matrix (**Example 2**). MALDI and other soft ionization methods are highly selective and subject to strong suppression effects. Although this selectivity may facilitate detection of compounds with well recognized ionization characteristics but, at the same time, it may create an uncertainty in characterizing novel compounds as well as in evaluating purity. Suppression effects may completely prevent some or all components from producing ions and may lead to a situation in which ions observed represent only the most easily ionized components and not those that are quantitatively important. Consequently, for all soft ionization methods, the removal of salts, organic buffers, and detergents is important in avoiding sample-unrelated ions and suppression effects.

Example 1

Free cholesterol, unsuitable for the ionization by FAB, MALDI or ESI, can be transformed into a charge bearing betaine esters by reacting with the excess of 2-fluoro-1-methylpyridinium p-toluenesulfonate or N-chloroformyl betainyl chloride. These esters produce very intense spectra but strong ions derived from the reagents are also present.[49] We have prepared similar derivatives by a quantitative reaction of cholesterol chloroacetate with aliphatic tertiary amines, pyridine, picolines, and lutidines. Upon evaporation, these simple transformations lead to charged products that provide clean MALDI (**Figure 4**) and ESI spectra.

Example 2

In our experience with MALDI-FT-ICR-MS, several trace ions are frequently encountered. Two such ions at m/z 550 and 522 are suspected as a contamination. Their accurate masses are very close to the calculated for $C_{38}H_{80}N$ (550.629) and $C_{36}H_{76}N$ (522.598) and, most probably, represent the quaternary ammonium detergent (Aerosurf, $CH_3(CH_2)_{17}N^+(CH_3)_2$ and its lower homologue $CH_3(CH_2)_{15}N^+(CH_3)_2$ respectively). Another ion at m/z 273, found only occasionally, most probably represents a decomposition product of 2,5-dihydroxybenzoic acid used as a matrix (DHB, $C_7H_6O_4$). It accurate mass is very close to that calculated for $C_{14}H_8O_6H^+$ (273.040; two molecules of DHB minus two water molecules).

3.6 Cationization

Very early, formation of cationized ions has been recognized in FD and FAB proving to be very useful in the analysis of compounds that resist protonation.[50] Even traces of alkali metals, especially the ubiquitous sodium ion, may lead to full or a partial cationization but dissolving salts or hydroxides in a matrix allows much better control of this process. As tested for selected natural products, elements with well recognizable isotopic patterns, for example silver or thallium both with two naturally occurring isotopes (^{107}Ag 51.3% and ^{109}Ag 48.7%, ^{203}Tl 29.5% and ^{205}Tl 70.5%) produce easily discernible attachment ions.[51] Similarly, mixtures of alkali metal salts can produce well recognizable patterns (**Figure 5**) and are useful in the analysis of polymers, especially those containing ether bonds.[52] Moreover, to facilitate cationization, special derivatives incorporating moieties with the high affinity to alkali ions (e.g. crown ethers) can also be employed.[53] We have been able to observe efficient cationization accompanied by a minimal fragmentation for over 100 fully blocked methyl ester-acetates of glucuronides and glucosides of bile acids and steroids (**Figure 6**).[54] All these compounds, in matrices free from alkali metal salts, show only low-mass ions resulting from the extensive loss of acetic acid and sugar moieties while only the simplest produce informative EI spectra, especially at the low eV.[55]

Example 3

In EI, THP ethers of steroids and aliphatic compounds usually produce no significant molecular ion but a prominent fragment ion at m/z 85 and weak ions of a higher mass. These useful intermediates, however, can be characterized by FAB and MALDI as alkali metal attachment ions (**Figure 2**).[56]

3.7 Electrospray ionization

Electrospray ionization (ESI), being one of the softest ionization methods, produces ions directly from solutions by fast evaporation of charged micro-droplets that are obtained at the flow rates of about µl/min.[57] This evaporation starts at the atmospheric pressure and continues in the evacuated, hot interface until the droplet size reaches the point preventing the solvation of holding ions together ("Coulomb explosion").[58] Obtaining optimal spraying condition is the most important experimental aspect of ESI and, apart from the voltage applied to the spray orifice, is determined by the surface tension of a solvent or solvent mixture and the presence of electrolytes. To enhance the spray, a supporting flow of a liquid or a nebulizing gas can be applied and, interestingly, even very efficient nebulization in the absence of the high electric potential still produces ions ("Sonic Spray").[59] Most notably, very sensitive analysis of µl-size samples is obtained by reducing the flow rate to nl/min ("Nano-Spray"). This beneficial effect is explained by the substantial improvement in the ionization efficiency resulting from the formation of smaller droplets and their easier and much faster desolvation.[60]

Typically, ESI spectra of large and polar molecules are distinguished by the presence of multiply protonated/deprotonated molecules such as MH_z^{z+} or $(M\text{-}zH)^{z-}$. A series of such consecutive ions differs by a single charge directly allowing to calculate the "charge-state" (i.e. the value of 'z') and the averaged MW from a series of linear equations. This is possible even if the isotopic structure of these ions remains unresolved. If ions corresponding to two or more components are not resolved, such calculations might not be easy or even fully reliable. However, if every ion is discerned and resolution allows identifying isotopic components (**Section 4.1**), one can obtain an unambiguous determination of both the charge-state and MW justifying the need for the high resolution (**Figure 9)**.

As in case of other soft ionization methods, some additives can be employed to help in the analysis of difficult compounds by ESI (**Section 4.2**). Due to this exceptional softness of ESI, weak attachment processes may lead to ions that either obscure spectral clarity (e.g. attachment of salts) or allow detecting the non-covalent interactions.[61] Strong response of molecules with the inherent charge, encountered with ionization methods desorbing ions form liquid and solid matrices, gives an attractive possibility to observe charged reaction intermediates.[62]

4 Mass spectrometry and molecular weight determination

4.1 Stable isotopes and molecular ions

The number of atoms of elements with two or more isotopes contributes to the complexity of the ion pattern in a strictly predictable manner. Although for large, multi-element compounds such calculations may be quite lengthy,[63] for a small ion, even a simple comparison of the relative intensities and the odd/even position of ion signals, directly indicates the number of atoms of multi-isotopic elements (e.g. the well known nitrogen rule and counting of Cl and Br atoms). With the increasing number of atoms, the center of gravity (**averaged mass**) shifts toward ions containing heavier isotopes (**isotopic ions**) and the ion containing only the lightest isotopes (**monoisotopic ion**) becomes less and less significant. Thus, for compounds with high MW, the numerical difference (but not the relative!) between these two numbers becomes more apparent. Carbon clusters provide a simple illustration of calculating the relative intensities of isotopic ions from the binominal distribution. It includes the number of atoms in an ion as an exponential factor and the partial abundance of the two carbon isotopes as the contributing probabilities (**Example 4)**. It shows that even very small variation of the natural abundance impacts upon the pattern of isotopic ions. By measuring the averaged mass with the ppm accuracy, such small differences can be detected even for ions of large molecules. Such measurements, however, are reliable only if other close ions (e.g. M-1 or M+1 at the low mass or MNa^+ at the high mass) do not overlap.

Example 4

For C_n, only two isotopes are involved in calculations. For C_{60}, at 98.889% ^{12}C, the molecular ion cluster can be calculated as the individual terms of the binominal $(0.98889 + 0.01111)^{60}$. By taking new value for carbon ^{13}C (another end of the approximate spread encountered in Nature) it changes to $(0.98931 + 0.01069)^{60}$. **Figure 11** presents the comparison of such calculations performed for C_{60} and the two hypothetical clusters C_{600} and C_{6000}. **Figure 12** shows spectra of a series of carbon clusters obtained upon the high power laser desorption of the homogeneous sample of C_{60}. Evidently, as detected by FT-ICR-MS, C_{60} reorganizes with the formation of even carbon species that are up to about 180 atoms. One needs to note that all these ions are free from the interference of **isobaric ions** (the same nominal mass but different isotopic composition). Similar situation, i.e. all isotopic ions have unique composition occurs for perfluoro-hydrocarbons (PFK) used as mass standards in EI and CI (fluorine is monoisotopic). On a contrary, for all compounds that are build of two or more multi-isotopic elements only the lowest mass, monoisotopic ion has no isobars.

With the increasing number of atoms in the ion, complex isotopic clusters represent only more sampling points available to the MS measurement reflecting statistical distribution of all possible isotopic combinations. For instance, by selecting 10,000 carbon atoms as a representative sample, one needs to count ^{13}C atoms in one $C_{10,000}$ molecule whereas ten thousand C_1 molecules would be needed. Counting C_1 molecules is experimentally realized in the determination of ^{13}C content after combustion to CO_2. In view of the fact that FT-ICR-MS allows detecting single ions with a multiple charge, looking at the

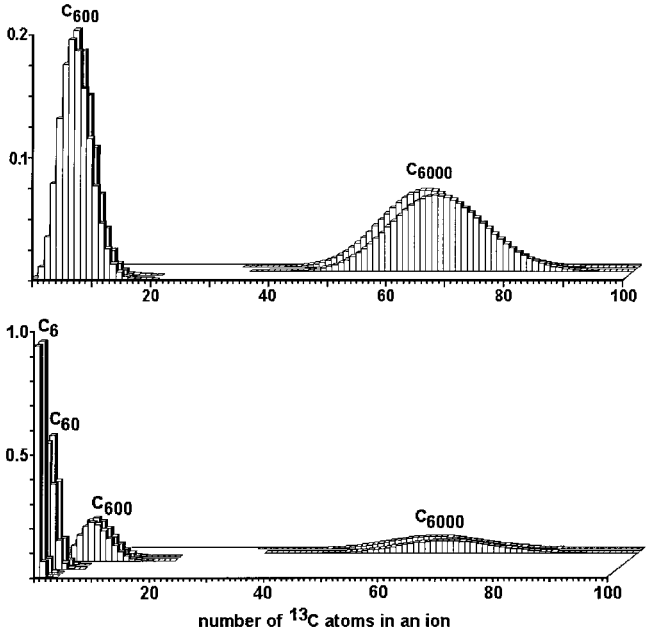


Figure 11a. Distribution of isotopic ions of four hypothetical carbon clusters, C_6, C_{60}, C_{600}, and C_{6000} calculated for the two different natural abundances of ^{13}C. First row corresponds to 1.111 and second to 1.069 % ^{13}C respectively. For the two larger clusters, shift of the average mass becomes more apparent only as result of more points available for the calculation. Still, the averaged masses of these four clusters constitute multiplicities of the averaged mass of carbon, i.e. 12.01115 and 12.01073 respectively. Their determination requires very accurate measurement of the relative intensities of all isotopic ions. Monoisotopic ions are significant only for C_6 and C_{60} (compare Figure 12)

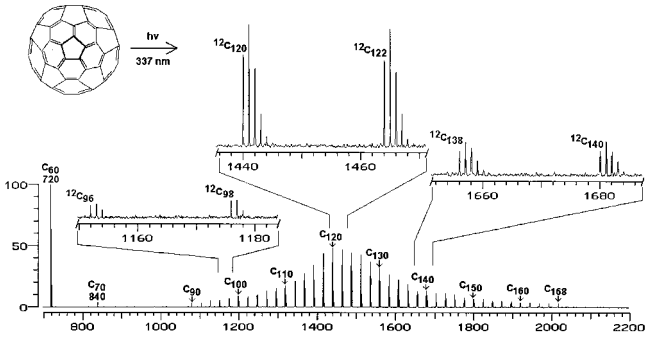


Figure 12. A single shot laser desorption FT-ICR-MS obtained from the film of a pure sample of C_{60} at no attenuation of the nitrogen laser (337 nm) and the maximum ion transmission set at about 1400 u. At the low laser power, C_{60} is the only component observed.

individual molecule is not only an intellectual exercise. This "single-ion-approach" may provide an alternative in characterizing ions of very large molecules by a very prolonged observation of only several trapped ions. Not only their m/z could be precisely measured, but also the charge 'z' could be deduced from its changes occurring during the storage time.[64]

Except for 22 monoisotopic elements, most are composed from 2-10 natural isotopes (**Table 2**). Three elements essential for organic compounds (H, N, O) show very low natural abundance of heavier isotopes while for others (C, Si, S, Cl, Br) heavier isotopes are more significant. Still, for many elements that are or may be used in organic synthesis, the lightest isotopes are not equivalent with the most abundant ones.

The following definitions are used in determining MW by MS for small and large molecules.[65] The **nominal ion mass** represents "the mass of an ion with a given empirical formula calculated using the integer mass numbers of the most abundant isotope of each element"; the **monoisotopic ion mass** represents "the mass of an ion for a given empirical formula calculated using the exact mass of the most abundant isotope of each element"; finally, the **averaged mass** corresponds to "the mass of an ion for a given empirical formula calculated using atomic weight of each element". An unclear definition of **monoisotopic** and **nominal mass** as "mass calculated by summing the atomic masses of the lightest (most abundant) isotopes, but including the mass defect … (… the exact-mass equivalent of the nominal mass)" can also be found.[66]

These definitions, although seem to be unquestioned, pose formal and practical problems. Thus, the term **empirical formula** (that "expresses in simplest form of relative number of the kind of atoms in a molecule") is used incorrectly and should be replaced by **molecular formula** (signifying "the actual number and kind of atoms in a chemical entity (i.e. molecule, group or ion)"; according to definitions in ref.[67]). Although there is no contradictions for compounds composed of either monoisotopic elements or elements with the most abundant isotope being also the lightest (such as H, C, N, O, F, P, S, Cl, Br, and I), for elements listed in columns 4-6 (**Table 2**) 'monoisotopic ions' are not free from isobars (for example boron containing ion, **Figure 8**). Replacing the term "most abundant isotope" with "the lightest isotope" eliminates the formal and practical inconsistencies in selecting ions for measurements both at low and high mass. The original definitions, evidently, date to the time when the range of mass measurements has been limited. Now, at much extended mass range, one needs to deal with spectra in which monoisotopic ions are entirely insignificant.

4.2 Isotopic depletion and substitution

Stable isotope label and its detection by MS represents one of the principal tools in chemistry and biochemistry and its applications precede that of radioisotopes.[68] This area is still actively pursued with exciting new methodological and instrumental developments.[69] Detection of mass shifts caused by isotopic substitution enables to follow stable label and finds multitude of applications such as isotope dilution for quantification, metabolic transformations, reaction mechanisms, or even clarification of structural ambiguities. At the high enrichment level, stable label can be detected by the "full-scan" or partial mass spectra (SIM, **Section 2.2**), whereas at the low enrichment, precise isotope-ratio methodology is indispensable. In this area, new developments include the "on-line" com-

bination of chromatographic separations with isotope-ratio measurements performed on low MW products obtained by chemical conversion (e.g. on-line combustion)[70] or reaction in the microwave plasma.[71] This approach makes the detection of label independent of spectral features such as fragmentation and the presence or absence of a molecular ion.

The alternative use of stable labeled substrates in synthesis may be beneficial in clarifying structural ambiguities. For example, complexes with one or more atoms of multi-isotopic elements (e.g. ruthenium, **Table 2**) produce very complex patterns of isotopic ions and, especially when analyzed by FAB, are frequently obliterated by matrix derived ions. A parallel synthesis performed with an enriched isotope could not only improve the clarity of spectra but also increase signal-to-noise ratio. A recent report of ESI spectra of $Ru_3(CO)_{12}$ and similar compounds of Os and Re cationized by silver ions[72] exemplifies such a case in which the ion pattern would be greatly simplified by enrichment of the Ru component.

Isotopic depletion is another viable alternative. Recently, first example of a small protein biosynthesized from precursors containing only the light isotopes of carbon and nitrogen has been reported (**Example 5**). Certainly, this approach, by lowering contribution of isotopic ions with an increase in the relative intensity of monoisotopic ions, augments sensitivity and precision of the accurate mass measurements should gain importance in characterizing bio-molecules. Obtaining narrow isotopic clusters should be useful in characterizing mixtures, analyzing fragmentation, and counting exchanged deuterium atoms. This approach, by simplifying spectra, may find use for low MW microbial and plant metabolites. Furthermore, the synthesis of medium and large polymers and supra-molecules could also be accomplished from a few simple precursors depleted in heavier isotopes. One needs to remember that the complementary approach, i.e. the use of substrates/precursors enriched in heavier isotopes, may provide lucid spectra only at the relatively low MW. For large molecules, only compounds depleted in minor isotopes may be obtained with very high purity required to eliminate isotopic ions.

Example 5

Well-characterized FK506-binding protein has been isolated from *E. coli* grown on 99.95% glucose $^{12}C_6$ and 99.99% ammonium sulfate $^{14}N_2$.[73] This 107 amino acid protein has the elemental composition $C_{527}H_{830}N_{146}O_{155}S_3$, corresponding to the monoisotopic mass 11,780.07 with the calculated relative intensity only 0.65%. Its ESI-FT-ICR spectrum obtained at the natural abundance shows major ions at M+6 to M+9 whereas the spectrum of the same protein obtained on the depleted medium shows the monoisotopic ion as the most intense peak at m/z 11,780.01 (-5 ppm error!). Weaker ions corresponding to M+1 and M+2 represent mainly the contribution of oxygen and sulfur isotopes. Recently, a similar approach has been used to improve signal-to-noise ratio in the CID spectra of depleted cystatin, also investigated by FT-ICR-MS.[74]

4.3 High or ultra-high resolution?

For a singly charged ion at m/z 1,000, the difference of a mmu requires mass resolution of 1,000,000. The use of such high resolving power is illustrated here for a singly deuterated cholesterol derivative. It enables distinction of the two isobaric M+1 ions containing either one ^{13}C or one ^{2}H and differing by only ~3 mmu (**Figure 4**). A similar approach could be helpful in defining frequently encountered cluster of ions such as $(M-H)^+$, $M^{+\cdot}$, and $(M+H)^+$. In this case, at m/z 500, base-line separation of $^{12}C^1H$ and ^{13}C isobars, with 4.5 mmu difference, requires resolution of over 100,000. At medium resolution, one should measure the accurate mass of the $(M-H)^+$ ion only. For the already

Table 2

Naturally occurring isotopes with the lowest atomic mass

Total number of isotopes	Number of elements	Main isotope has the lowest atomic mass	Main isotope does not have the lowest atomic mass		
			Relative amount of the lowest mass isotope (%)		
			>1	1<>0.1	<0.1
1 monoisotopic elements	22	^{9}Be, ^{19}F, ^{23}Na, ^{27}Al, ^{31}P, ^{45}Sc, ^{55}Mn, ^{59}Co, ^{75}As, ^{89}Y, ^{93}Nb, ^{103}Rh, ^{127}I, ^{133}Cs, ^{141}Pr, ^{159}Tb, ^{165}Ho, ^{169}Tm, ^{195}Au, ^{209}Bi, ^{232}Th			
2	22	^{1}H, ^{12}C, ^{14}N, ^{35}Cl, ^{63}Cu, ^{69}Ga, ^{79}Br, ^{85}Rb, ^{107}Ag, ^{121}Sb, ^{175}Lu	^{6}Li, ^{10}B, ^{113}In, ^{151}Eu, ^{185}Re, ^{191}Ir, ^{203}Tl	^{50}V	^{3}He, ^{138}La, ^{180}Ta
3	7	^{16}O, ^{20}Ne, ^{23}Mg, ^{28}Si, ^{39}K		^{36}Ar	^{234}U
4	5	^{32}S	^{50}Cr, ^{54}Fe, ^{204}Pb	^{84}Sr	
5	6	^{58}Ni, ^{64}Zn, ^{90}Zr	^{46}Ti, ^{70}Ge	^{180}W	
6	7	^{40}Ca	^{102}Pd	^{74}Se, ^{78}Kr, ^{162}Er, ^{174}Hf,	^{190}Pt
7	10	^{142}Nd	^{92}Mo, ^{96}Ru, ^{144}Sm	^{130}Ba, ^{152}Gd, ^{168}Yb, ^{196}Hg	^{156}Dy, ^{184}Os,
8	2		^{106}Cd		^{120}Te
9	1			^{124}Xe	
10	1			^{112}Sn	
Total:	83	44	17	14	8

mentioned series of carbonyl complexes of ruthenium (7 isotopes), osmium (7 isotopes), and rhenium (2 isotopes) cationized by the silver ion (2 isotopes), unless the ultra-high resolution is applied, the selection of an ion suitable for the precise mass determination poses a formidable problem.

The charge state assignment in ESI spectra is also quite demanding (**Section 3.7**). Thus at m/z of 1,000 and ion with 50 charges, the resolving power of 50,000 is required to depicts the isotopic cluster.

Example 6
EI spectrum of molybdenum hexacarbonyl shows an abundant molecular ion cluster. With seven isotopes of molybdenum (92, 94, 95, 96, 97, 98, and 100 with the relative abundances of 14.8, 9.3, 15.9, 16.7, 9.6, 24.1, and 9.6 respectively) the original rule (**Section 4.1**) points to the ^{98}Mo ion at m/z 266 as the nominal and monoisotopic. This ion, however, has six isobaric components above 0.01% and its two major ones at m/z 265.8750 ($^{98}Mo(^{12}C^{16}O)_6$, 21.92%) and 265.8786 ($^{97}Mo^{12}C_5{}^{13}C_1{}^{16}O_6$, 0.587%) could be differentiated at the mass resolution of about 70,000. Thus, mass measurements performed routinely at a medium resolution, would be affected by only about 0.1 mmu (0.3 ppm). This small interference is due to accidentally low abundance of ^{97}Mo and the low number of carbon atoms in this molecule. True monoisotopic ion at m/z 259.8758 corresponds to the lightest isotopes $^{92}Mo(^{12}C^{16}O)_6$ (14.60%) and is suitable for the precise mass determination even at a relatively low resolution. The suitability of ions for precise mass determination requires case-by-case evaluation based on the expected elemental composition of the measured ion and the available resolving power. This, however, may no be feasible if the measured compound has an unknown composition.

4.4 Mass measurement accuracy

Recently, in view of the apparent contradiction in understanding of mass spectroscopists and editorial polices of organic journals (usually asking for HR-MS data to be within a small and pre-defined error), the accuracy required for the precise mass determinations has been discussed.[75] Except for very small ions, experimentally determined monoisotopic mass with a low mmu error is not adequate to deduce a unique elemental composition. Such measurements can distinguish, however, between several possible elemental compositions deduced from other spectral data or from independently obtained structural information. Only knowing the list of elements that may be present in the molecule and the approximate number of respective atoms, the required precision of mass determination can be evaluated.[76] In such calculations, the necessary constrains in the possible elemental composition may come from the number of carbon atoms calculated (or estimated) from the intensity of the M+1 ion, the number of carbon and hydrogen atoms counted from the number and multiplicity of carbon signals in ^{13}C NMR spectra (permitted by the lack of symmetry) plus the number of exchangeable hydrogen atoms bound to hetero-atoms determined from ^{1}H NMR. With the C_nH_m part established, complementing the elemental formula with hetero-atoms may be easier.

4.5 Can molecular weight be unequivocally determined by mass spectrometry?

Certainly, in the determination of MW, accuracy and sensitivity of MS are unmatched by other methods. Except for the electrophoretic characterization of polypeptides and oligonucleotides and characterization of synthetic polymers by the size-exclusion, MS has largely replaced the reliance on methods relying on bulk properties of compounds or their solutions. MS methods are capable of providing an immediate result with very small samples, and, frequently, it tolerates impurities such as solvents that are detrimental to other methods. Still, if the investigated compound is available in a pure form and quantities enabling spectral and chemical characterization, MS data are usually considered only as a confirmatory evidence for the predicted MW. Such predictions may come from different sources, the most important being the expected course of a chemical/metabolic transformation, derivation, and data obtained by other spectral methods. Notably, structural predictions also impact on the selection of a specific ionization method and, consequently, spectra are usually interpreted in a backward fashion. In a case of a good fit, MS results are accepted with little or no scrutiny, whereas if ambiguities or even contradictions appear, inadequacies of MS are first to blame. Still, for the entirely unknown compound or even for the unexpected reaction product, this approach may fail. Even in a simple case exemplified by a condensation expected to occur with the elimination of a small molecule 'E' (e.g. water, hydrogen halide), MS results can be misleading. Thus, the primary product, either due to the lack of such elimination or due to an unnoticed re-addition, may have its MW equal to the sum of MW of substrates but still may show only a prominent ion 'M-E'. If molecular or pseudomolecular ions are weak or absent, fragments (e.g. M-Me, M-water, M-C_4H_9 important for t-butyl-dimethylsilyl derivatives) can be extrapolated to the correct MW. The origin of such fragments, however, ought to be very well proven.

In spite a tremendous success obtained with novel ionization methods, MS is still not able to provide unequivocal MW information for every class of organic compounds. The confidence in such determination, however, can be reinforced if the ionization/fragmentation behavior of a particular class is well known, if consistent results are obtained with several different ionization methods (including experimentation with matrices and their additives), and if there is full consistency between spectra of well-chosen derivatives. Still, of a key importance is an agreement of MW established by MS with the conventional elemental analysis and, especially, with ^{13}C and ^{1}H NMR

data capable of providing the exact count of carbon and hydrogen atoms (if the parent compound is of relatively low MW and its molecule can not be divided into the entirely identical units). In absence of such data, it is crucial to select the most appropriate ionization method and to find ways of their verification. Such situation may result from a limited amount of the studied material encountered in the identification of minor components in complex mixtures or in the characterization of inseparable polymers. It may also result from a practical selection of MS as the sole source of information. This is exemplified by the need for automation in monitoring products of a combinatorial synthesis requiring streamlining of the MS analysis of a wide range of compounds to one method such as MALDI, ESI or APCI. To avoid compromised results, however, intermediates of a multi-step synthesis ought to be followed with ionization methods that accommodate increasing MW and changing ionization properties as new functional groups are added, blocked or deblocked.

Example 7

Cucurbituryl, a substance known since 1905, is a condensation product of glycoluril with formaldehyde and, apparently, has a polymeric nature. Cucurbituryl analyzes as $(C_6H_6N_4O_2)_n$ and shows high symmetry in its NMR spectrum. At the time of its 1981 reinvestigation, MS analysis has failed (this included FD) but its symmetrical structure has been established by X-ray crystallography of a calcium bisulfate complex.[77] **Figure 5** shows its MALDI spectrum measured in DHB spiked with the series of alkali metal iodides from lithium to cesium. This spectrum displays a series of ions that include MNa^+, MK^+ (dominant), MRb^+, and MCs^+ but, interestingly, MLi^+ ion is absent.

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References and Notes

(1) Rapid progress is not new for mass spectrometry. In a surprisingly short time, the detection of almost all stable isotopes completed by J. W. Aston and A. J. Dempster in middle thirties, has been followed by the construction of immense mass spectrometers named as calutrons. Calutrons have been employed for the separation of fissionable uranium for the Manhattan project and, subsequently, for the preparation of isotopes of many other elements (Yargey, A. L., Yargey, A. K., *J. Am. Soc. Mass Spectrom.*, 1997, *8*, 943). Possibility of the preparative use of MS for separation of isotopes has been suggested by Aston in 1919 and implemented by others in 1934. S. Glasstone "*Sourcebook on atomic energy*", Van Nostrand 1950. Mladjenovic, M. "*The history of early nuclear physiscs*" (1896-1931)" Word Scientific.
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