FT-ICR mass spectrometry in the drug discovery process

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The high mass accuracy and resolution of Fourier transform (FT)-ion cyclotron resonance (ICR) mass spectrometry are making it an increasingly useful tool in drug discovery and development. The basics of FT-ICR are described here, including modern ion sources and fragmentation methods. Although FT-ICR is not a high-throughput method in the traditional sense, previously difficult and complex problems are being efficiently approached using steadily improving instruments and magnets. Applications are surveyed in fields such as proteomics, metabonomics, natural product analysis and non-covalent complexes.

Modern drug discovery and development is heavily dependent on rapid and insightful analytical methods. Emphasis is increasingly placed on speed and high throughput, and Fourier transform (FT)-ion cyclotron resonance (ICR) mass spectrometry (MS) has traditionally not been widely used. Although modern trap and time-of-flight (TOF) instruments can measure many mass spectra per second, an FT-ICR spectrum can require seconds. However, the true goal can be lost in the pursuit of speed. Scientists are continually searching for information that leads to insight and decisions; insight about disease mechanisms and pathways, and decisions about drug targets, candidate molecules, efficacy and toxicity. By this standard, FT-ICR is increasingly attractive in the pharmaceutical industry because of its ability to deliver more information per measurement.

This information primarily takes the form of precise and accurate mass-to-charge ratios (m/z), which can often be immediately interpreted without acquiring further information. This can be contrasted with the need for tandem MS (MS–MS and/or MS^n, where n indicates the order of fragmentation) when the mass accuracy is not sufficient. However, FT-ICR also offers a rich repertoire of techniques to produce compositional, structural and thermodynamic information on molecules and complexes.

FT-ICR MS [1] is, in some respects, the highest performance MS method currently available. FT-ICR provides ultra-high mass resolution and mass accuracy, non-destructive detection, high sensitivity and multistage MS^n. It has undergone rapid development and is now applied in many fields [2]. Examples of biomedically related achievements include characterization of low attomole proteins (8–29 kDa) with resolution of 60,000 and a mass accuracy that is less than 1 amu [3]; resolution of 8,000,000 for bovine ubiquitin (8.6 kDa) [4]; and MS–MS sequencing of ubiquitin with over 900,000 resolution [5]. High mass accuracy ensures rapid protein identification with high confidence based on single peptide mass measurements [6].

Principles of FT-ICR

The principles of FT-ICR are comprehensively described in various reviews and books [2,7], therefore, only the fundamentals of the technique are covered here. Ions are trapped in a FT-ICR cell (Figure 1) by a combination of magnetic and electric fields. The magnetic

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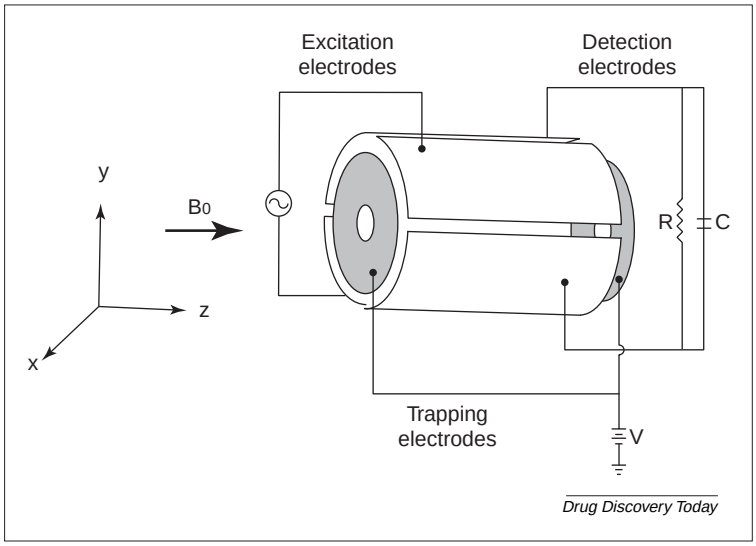

FIGURE 1

Schematic of a closed cylindrical ICR ion trap. Ions undergo circular motion around the magnetic field lines (B) and are held within the trap by voltages on the trapping electrodes. Radio frequency excitation is placed on the excitation plates to cause the ions to spiral outward in larger orbits. After this excitation is stopped, the orbiting ions induce voltages in the orthogonal detection plates at their rotation frequencies.

field forces ions to move in circular orbits (cyclotron motion) but does not restrict their motion in the axial direction along the field lines. Axial trapping plates (or rings) at a low potential (e.g. 1 V) are therefore used to keep ions in the cell, as in Figure 1. The ion motion in an ICR-cell is a superposition of three independent motions, namely the cyclotron motion, the magnetron motion and the trapping oscillation. Only the 'reduced' [2,7] cyclotron frequency is detected, by which the m/z of an ion is determined, as shown in Equation 1:

$$\upsilon_c = \frac{zB}{2\pi m} \qquad \text{[Eqn 1]}$$

where B is the magnetic field strength, and m and z are the mass and the charge of an ion, respectively. Equation 1 shows that for each m/z, a unique cyclotron frequency exists in field B. Cyclotron frequencies typically range from kHz to MHz.

A singly-charged 100 Da ion at room temperature in a field of 9.4 tesla has a cyclotron radius of 25 μm. The signal caused by such a small motion is undetectable. Multiple ions also do not move together, thus the average of their signals approximates to zero. To obtain a measurable signal, ions are accumulated in a small packet at the center of the cell, and are excited, as a packet, to larger radii. After excitation has stopped, the large diameter packet motion can be monitored by detecting the oscillating image charge induced at the detection electrodes as in Figure 1. If only a single ion type was present, the signal would be a slowly decaying sinusoidal wave. When ions of different m/z are in the trap, the signal is a superposition of multiple frequencies. To recover a mass spectrum, the signal versus time is Fourier transformed to obtain intensity versus frequency; the frequencies can be converted to m/z using Equation 1. Properties of the transform show that the resolving power is inversely proportional to m/z. The resolving power increases by increasing magnetic field. In addition, to measure the frequency as long as possible, the pressure must be low to avoid collisions, preferably 10^{-9} mbar or less.

Magnets

The defining component of FT-ICR mass spectrometers, in both cost and performance, is the magnet. The higher the field, the better the resolution and sensitivity [8], so superconducting magnets are widely used. Fields of 3.0, 4.7, 7.0, 9.4 and, recently, 12 tesla are common. Modern magnets are, in general, actively shielded.

Ion sources

FT-ICR has been combined with many ion sources. Ion generation is usually external, and ions are guided by various optics through differentially pumped regions to the cell. For biologically related applications, electrospray ionization (ESI) [9] is probably the most widely used technique. Matrix-assisted laser desorption–ionization (MALDI) [10] has become more popular since the high-pressure source (keeps kinetic and internal energies low) was introduced. To retain resolution, ions must be trapped in the ICR-cell at low voltages, thus it is essential that kinetic energy is kept to a minimum [11]: low internal energy reduces metastable decay in the cell. A combined ESI–MALDI source is also now commercially available [12]. Internal ion sources, such as electron impact (EI), chemical ionization (CI) and photoionization, for volatile compounds, as well as laser desorption–ionization (LDI) for non-volatile compounds, can also be combined with FT-ICR. Atmosphere pressure chemical ionization (APCI) and atmosphere pressure photoionization (APPI) [13] have also been applied.

Combination of FT-ICR with separation techniques

FT-ICR can be directly and indirectly coupled with different separation techniques. Using ESI, high-performance liquid chromatography (HPLC) [14] and capillary electrophoresis (CE) [3] can be online coupled to FT-ICR. Coupling LC with MALDI-FT-ICR is usually off-line. Recently, Brock *et al.* [15] have presented a fully automated LC-MALDI-FT-ICR platform for proteomics enabling peptide mapping, MS–MS and complex mixture analysis in a high-throughput fashion. Nano-HPLC combined with nano-ESI-FT-MS has been implemented for characterizing complex protein mixtures without pre-fractionation [16].

Features of FT-ICR

For high mass accuracy, internal mass calibration is the typical approach. Some instruments provide internal calibration by mixing calibrant and analyte ions in the ESI emitter, which causes ion suppression and reduces the effective dynamic range. A new approach with independent ionization, introduction and transmission control of

analyte and calibrant species has shown much better performance for MALDI [17] and ESI sources [18]. Control of trapped ion quantities avoids space charge effects in the ICR cell, which shifts cyclotron frequencies [2]. With internal calibration and 'automatic gain control' (AGC) (Thermo Finnigan), the number of identified tryptic peptides increased tenfold in comparison with the external calibration and fixed external ion accumulation.

The triple quadrupole-FT-ICR hybrid enables data-dependent ion preselection, external fragmentation (MS^n) and data-dependent MS^n, and can be online coupled with HPLC [18]. Combining linear ion traps (Thermo Finnigan) or triple quadrupoles (Bruker Daltonics and IonSpec) with FT-ICR makes it possible to perform automated ultra-high resolution, high mass accuracy and MS^n in biological applications.

The ion cyclotron radii can be increased until ions impact the walls. Because such ejection can be frequency tailored to reject or retain ions selectively, specific ions can be isolated for various purposes, such as MS–MS. After isolation they are brought back to smaller radii, usually by collision with gas. Selected parent ions in an ICR cell can be fragmented by collision-induced dissociation (CID) [19] [also called collision-assisted dissociation (CAD)], surface-induced dissociation (SID) [20], one-photon ultraviolet photodissociation (UVPD) [21], infrared multiphoton dissociation (IRMPD) [22], blackbody infrared radiative dissociation (BIRD) [23] and electron capture dissociation (ECD) [24]. Two complementary variants of CID are sustained off-resonance irradiation-CID (SORI-CID) [25] and multiple excitation collisional activation (MECA) [26].

With the exception of UVPD and ECD, the daughter ions reflect thermal internal energy distributions. ECD is a non-thermal fragmentation technique well adapted to FT-ICR. It selectively breaks bonds in the near vicinity of the charges, not only the weakest bonds. A valuable current application is backbone cleavage of peptides and proteins, without loss of non-covalent bonds or post-translational modifications (PTMs). A combination of ECD (non-thermal) with IRMPD (thermal) fragmentation gives a great deal of structural information [27,28].

Target identification and validation

Proteomics

The sequencing, identification and quantitative or qualitative profiling of proteins (proteomics) has led to a host of biomedical applications relevant to drug discovery [29], particularly for target identification and diagnostic profiling [30]. MS is an indispensable tool for proteomics and FT-ICR is gaining in importance because of the unique possibilities it presents (Box 1) [31,32].

For the identification of proteins by MS, fragmentation is generally necessary because the intact mass alone is not sufficient. In addition, mass resolution and accuracy degrade at high m/z. There have been two approaches to protein fragmentation [33]: (i) 'top-down', where fragmentation takes place in the mass spectrometer; and (ii) 'bottom-up', where proteolysis is part of the sample preparation. Top-down has the advantage of starting with the intact protein, including any PTMs. However, it can be difficult to observe enough fragments for reliable identification or adequate sequencing. Electron capture dissociation (ECD) is a particularly appropriate fragmentation technique for 'top-down' proteomics, because it yields more fragments than thermal methods (e.g. CID and IRMPD), but usually does not fragment or cleave the PTMs [34]. Recent strategies have been developed to enable automated top-down protein analysis including database searching for protein identification [35].

BOX 1

FT-ICR in proteomics

Unmatched mass accuracy (~1 ppm versus ~10 ppm) and resolution (>10^6 versus ~10^4) lead to:
- Higher confidence in protein identification.
- Measurement of a fewer number of peptides required for protein identification.
- *De novo* sequencing is possible with only few fragment ions.
- Facilitation of protein structure experiments.

Thermal and non-thermal MS^n enables:
- Top-down proteomics.
- Only protein backbone is cleaved – PTMs remain intact.
- Location of PTMs.

The 'bottom-up' approach is more widespread and is the classical proteomics method. In its original form, proteins are separated by 2D polyacrylamide gel electrophoresis (2D-PAGE). The gels are stained, spots excised and the proteins digested. The resulting peptides can then be analyzed by MS. For identification by database searching, accurate mass measurement is extremely valuable. The higher the mass accuracy, the fewer peptides of a protein are necessary for protein identification. In an example from Clauser *et al.* [36], with a mass tolerance of 50 ppm, a peptide of 1 kDa matches 323 proteins; while only seven hits result with tolerance of 10 ppm. A direct comparison between TOF and FT-MS for protein identification by peptide mass fingerprinting is given by Horn *et al.* [37].

Because of the high mass accuracy in FT-ICR, it is quite often possible to identify proteins by the detection of only one peptide [6]. In lower organisms, in particular, it is common that tryptic peptide fragments with a given exact mass can come from only one protein. In *Caenorhabditis elegans*, only 4% of the 2.5 kDa peptides are unique with a mass accuracy of 10 ppm (typical for MALDI-TOF). If the mass accuracy is 1 ppm (typical for FT-ICR), then 50% of all 2.5 kDa peptides are unique.

Bottom-up proteomics need not use laborious 2D-PAGE separation. All proteins can be digested together, and the complex peptide mixture analyzed. The high peak capacity (resolvable peaks per m/z unit) of FT-ICR enables such digests to be analyzed directly [38]; the spectra contain

thousands of peaks. Ten proteins in cerebrospinal fluid could be identified in one example [38]; however, this was limited by ion suppression effects. This problem is often overcome with a separation step before MS. On-line capillary electrophoresis doubled the peaks observed in one case [39], and 30 proteins were identified.

Simultaneous identification of many proteins in bodyfluids is demanding because of the enormous concentration range, stretching over more than 10 orders of magnitude in serum, for example [40]. The number of identified proteins can be significantly increased if the most abundant proteins are removed. Commercial affinity filters are available, depleting the six most abundant proteins, to leave 10–15% of the initial protein mass. In cases where dynamic range is less problematic, HPLC-FT-ICR can yield impressive results. For example, ~1000 proteins were identified from 100,000 detected peptides in a single measurement of a yeast digest [41].

The limits of sensitivity are also being expanded with nano-LC-FT-ICR. Recently, it has been possible to identify peptides from 75 zmol of a bovine serum albumin digest within the complex digested proteome of *Deinococcus radiodurans* [42]. Protein identification was also demonstrated with 0.5 pg of whole proteome extract (~100 times less than with an ion trap MS–MS) [43].

Although FT-ICR is capable of resolving compositionally different molecules with the same nominal mass, it cannot distinguish isomers. Hence, a peptide containing Glu and Asn, for example, cannot be resolved from a peptide in which these two amino acids were exchanged for Gln and Asp. If all possible peptides differing by only one, two or three amino acids are compared, there are a surprising number of chemical isomers with the same exact mass. For one variable amino acid, 10% of the possible sequences have the same mass. For two and three variable amino acids, the corresponding fraction is 29% and 53%, respectively [44]. Therefore, for an absolute identification, it is often necessary to perform MS–MS experiments, as in an example using LC-MALDI-FT-ICR to identify 111 proteins from yeast [45].

De novo sequencing of peptides and proteins is an analytical challenge in its own right. Usually, the task is approached with extensive MS–MS experiments. Spengler [46] has introduced a faster strategy that relies on exact mass measurements of the peptide and a few fragment ions, followed by comparison with all possible peptides of the same nominal mass. Only FT-ICR provides the mass accuracy required for this to succeed.

PTMs are common in mammalian cells, having a central role in modulating protein function. PTMs are therefore highly relevant to the search for drug targets and diagnostic biomarkers. The dissociation methods available for FT-ICR enable a unique approach to the investigation of PTMs. ECD can localize the site of the modification, whereas IRMPD can then help to determine the structure of the modification; this is particularly useful for glycosylations [47]. A specific application of FT-ICR to glycoproteins was the development of new biomarkers for Alzheimer's disease (AD) [48], where structures of glycans in three proteins from cerebrospinal fluid were compared between AD patients and controls. ECD has also been used successfully to determine the sites of phosphorylations – modifications associated with cell signaling [49]. In some cases, PTMs can be identified merely by the high mass resolution of FT-ICR. The degree of deamidation in peptides can directly be determined, because ^{12}CO and ^{13}CNH (0.0193 Da) can be resolved even in multiply charged peptides [50].

Because structure is correlated with function, proteomics also includes the determination of the structure of proteins. Although MS cannot yield high resolution structures, unlike crystallography or NMR spectroscopy, FT-ICR has been used to generate low-resolution 3D protein structures using chemical cross-linking [51]. After linkers have covalently bonded to the protein surface, the protein is denatured and digested. FT-MS analysis identifies which amino acids were linked. Because of its relative ease of use and low sample consumption, FT-MS could prove useful in combination with molecular modeling of protein structures. In a more general sense hydrogen–deuterium exchange can also generate information on the 3D structure of proteins. Only protons on the protein surface can be replaced by a deuterium [52]. Subsequent fragmentation and sequencing in the spectrometer identifies the locations at which exchange occurred.

Noncovalent complexes

ESI-FT-ICR has proven to be a powerful tool in the study of noncovalent complexes [53], particularly because the method is well-suited for high-throughput affinity screening, and various types of complexes have been analyzed. For example, RNA has been used as a target for drug discovery in an assay called multitarget affinity–specificity screening [54].

Synthetic and natural product libraries are screened in a high-throughput and robust manner by taking advantage of the high-resolution and high-precision mass measurements of intact RNA–ligands complexes. With this assay, the identity of the small molecules that bind, the RNA target to which it binds and the compound-specific binding affinity can be determined in one set of rapid experiments. Other examples of RNA binding include the determination of stoichiometry and affinity with HIV-1 nucleocapsid protein [55] or multitarget affinity screening with natural products [56]. Protein–ligand complexes are important, with ligands including oligosaccharides [57], other proteins [58], peptides [59], antibiotics [60] and many others. The study of these interactions can also provide information on the folding [61] or conformation [59] of the proteins. To support drug development, a highly sensitive method (solution concentration of 10^{-9} M without the use of separations before ESI) for bioaffinity characterization by FT-ICR has been reported [62]. Despite its strengths, FT-ICR has some

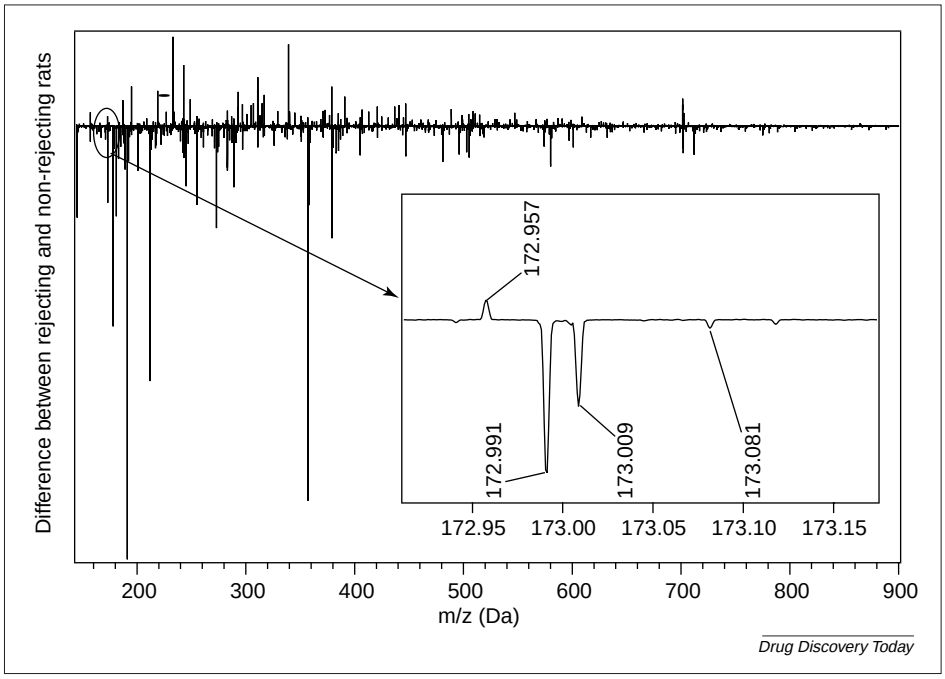

FIGURE 2

Differential FT-ICR spectrum of rat urine samples, between animals accepting and rejecting a transplanted organ. Many metabolites are upregulated or downregulated. The inset shows the value of ICR resolution, sensitivity and peak capacity.

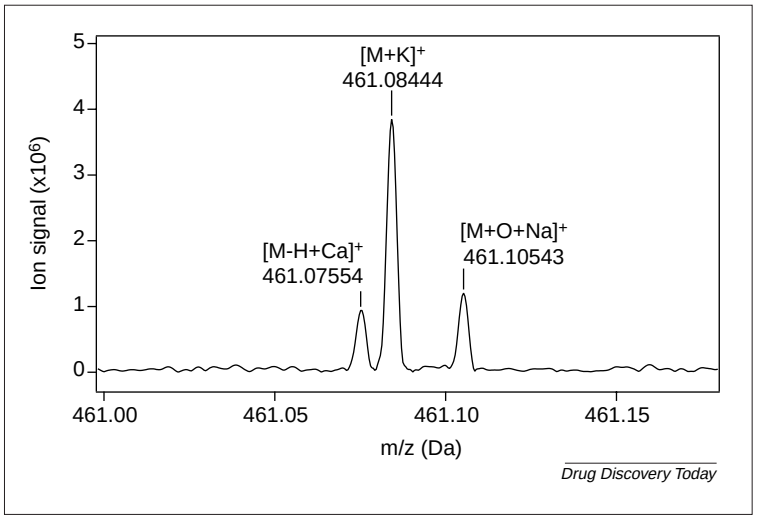

FIGURE 3

FT-ICR spectrum of a natural product. The substance was detected as cation adducts. In addition to the expected species, a derivative containing one extra hydroxyl was also found. The mass accuracies for the species shown are 0.07, <0.01 and <0.01 ppm, from left to right.

limitations for the study of noncovalent complexes, particularly when the ion signals of the complexes (perdominantly multiply-charged ions) exceed a few thousand m/z and/or form large adduct clusters.

Metabonomics

Metabonomics (often called metabolomics) is one aspect of modern systems approaches to biological complexity. It is an effort to observe global changes in the metabolic response of an organism to disease, treatment or other change. Metabonomics was initially developed with NMR [63], but metabonomics with MS is rapidly growing [64]. Because the goal is to detect as many species as possible simultaneously, the high peak capacity of FT-ICR is advantageous (Figure 2).

An attraction of metabonomics is that the number of (abundant) metabolites is a few thousand, versus tens of thousands of genes and up to hundreds of thousands of proteins. Patterns of metabolite changes are characteristic of large-scale pathways and so provide broader-scale functional information than proteomics or genomics approaches. However, the problem of rapid substance identification and pathway mapping has yet to be fully resolved. Many metabolites are in a mass range where FT-ICR can give immediate elemental composition, enabling direct identification without MS–MS, by comparison with public or locally generated databases.

Lead identification and optimization

Combinatorial libraries

The large peak capacity of FT-ICR is an advantage for the characterization of combinatorial libraries [65]. FT-ICR has been used to characterize mucin-2 antigen peptide libraries [66], or to study target antigens related to AD for the design of vaccine peptides [67]. FT-ICR was also coupled to capillary liquid chromatography to enable the screening of libraries with more than 1000 different peptides in less than 20 min [68]. The screening of substrate libraries by enzymes using combinatorial libraries in combination with FT-ICR has been reported [69]. FT-ICR was also utilized to identify the coding tags attached to a single-bead used in combinatorial chemistry [70], or to analyze single beads from peptide-encoded combinatorial libraries with MALDI-FT-ICR in HTS [71]. In further applications, FT-ICR was applied to identify RNA-binding ligands through multitarget affinity–specificity screening assay [72].

Natural products

Natural products are a rich source of medicinal compounds. Nearly half the major drugs on the market are derived from natural products. FT-ICR is heavily used for structure elucidation or verification, particularly for antibiotics [73]. FT-ICR with electron impact ionization has been used to establish the structure of a tumor inhibitor isolated from dried *Euphorbia pubescens* [74]. FT-ICR has also been applied as a unique method to validate the structure of saccharomycin antibiotics [75] with IRMPD MS–MS, and to elucidate the structure of unknown muraymycin antibiotics by means of SORI-CID FT-ICR with up to five stages of

BOX 2

Current applications of FT-ICR in drug discovery

- Biomarker discovery and analysis.
- Natural product structure elucidation.
- Metabonomics.
- Noncovalent complexes (including thermodynamics).

BOX 3

Developing applications of FT-ICR

- Mass spectrometry imaging.
- Elucidation of macromolecular 3D conformations.
- Molecular recognition.
- Spectroscopy of trapped ions.
- Combination with prefilter stages, such as traps.

MS (MS^5) [76]. FT-ICR also contributes to the dereplication and chemical fingerprinting of natural products, such as in bioactive marine macroorganisms [77]: FT-ICR analysis determined the elemental composition of the natural product in Figure 3 to be $C_{20}H_{22}O_{10}$. As well as the major compound of interest, a second species with one extra hydroxyl group was also observed. In this example, the resolution was ~170,000, thus enabling a clear separation of the calcium and potassium adducts, which have a mass difference of less than 9 mDa.

Conclusions

The high mass accuracy and resolution of FT-ICR are making it an increasingly useful tool in drug discovery (Box 2). Effectively used, this technology can lead to higher information content per spectrum and higher information output per unit operator time, although FT-ICR is not a high-throughput method in the traditional sense. Coupled with steadily improving instruments and magnets, this fact has led to a minor resurgence of the method in the industry.

The questions confronting drug discovery are rapidly becoming more complex because the 'low hanging fruit' of well-understood, readily treatable diseases are believed to have been largely 'picked'. Therefore, it seems probable that FT-ICR will become more valuable because of its ability to assist in the solution of complex questions. From protein activity modulation via PTMs to intermolecular complexes and binding sites to high-order structures to biomarker discovery and pathway recognition, FT-ICR has capabilities that are not readily matched in other instruments.

All technologies continually advance, and TOF and trap instruments, in particular, have improved dramatically in recent years. Nevertheless, in some performance categories, and for a range of analysis techniques, FT-ICR will probably retain its lead for the moment. In addition, a trend toward less complex control software will reduce the barrier to routine use.

New applications are likely to be developed by combination of FT-ICR with other techniques (Box 3), or by development of ingenious methods to observe systems not previously accessible. Macromolecular complexes, molecular conformations and intermolecular interactions of all kinds are currently developing particularly rapidly. Sensitivity and resolution will continue to be pushed to new extremes, via new ion sources and interfaces, and improved ICR cells, magnets and electronics.

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