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High resolution mass spectrometry: theoretical and technological aspects

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Abstract:

Over the past decade, Time of Flight (TOF) and Orbitrap™ (Thermo Fisher Scientific) mass spectrometers have aroused a growing interest, and we observed a progressive replacement of low resolution mass spectrometers (LRMS) by high resolution mass spectrometers (HRMS). Thanks to high mass accuracy, they provide an unambiguous identification and allow structural elucidation of unknown compounds. Furthermore, they offer new powerful acquisition modes based on untargeted screenings dependent or independent of previous information. These advances are of particular interest in the field of toxicology, and we observed an increasing number of HRMS presentations at scientific meetings, corresponding original papers, and review articles dealing with application HRMS. The aim of this study is to compare HRMS with respect to LRMS in toxicology, to detail and explain the physical and technological principles on which the TOF and the Orbitrap mass analyzers are based, the data acquisition modes and data processing methods. Finally, three applications in toxicology are detailed to highlight the interest of this technology in toxicology.

Key words: High Resolution Mass Spectrometry; Orbitrap; Time of Flight; Chromatography; Toxicology

Introduction

Over the past 40 years, analytical toxicology has made huge advances thanks to technological evolutions, from gas chromatography coupled to mass spectrometry (GC-MS) to liquid chromatography coupled to mass spectrometry (LC-MS) and more recently with the advent of high-resolution mass spectrometry (HRMS). In 2010, HRMS was almost ignored even in the academic environment, but today thanks to easier handling and lower cost (equivalent to low-resolution mass spectrometry (LRMS) system) most toxicology laboratories have their own Orbitrap or Time of Flight (TOF) mass spectrometer. The move from LRMS to HRMS has not only improved the resolution of the measured *m/z* allowing the determination of elemental composition of parent compounds and fragments, but has also led to a profound change in the way mass spectrometers are used. TOF and Orbitrap mass analyzers operate in a different manner from the quadrupole, and they are mostly supplied as hybrid mass spectrometers (Q-TOF or Q-Orbitrap). In addition, technological developments have made it possible to produce more reliable and reproducible MS^2 spectra allowing the incrementation of large mass spectra libraries along with data processing software. These changes offered novel data acquisition modes more suitable for the analysis of complex matrices and paved the way for the development of applications in the field of toxicology (screening, quantitation, metabolism, biomarkers identification).

The aim of this review is, to compare LRMS and HRMS, to detail the operating principle of the Orbitrap and TOF mass analyzers, the data acquisition modes and data processing methods, and to present some applications highlighting the interest of HRMS in toxicology.

1. Low Resolution Mass Spectrometry versus High Resolution Mass Spectrometry

From the development of time of flight (TOF) mass analyzers in 1946 by Stephens, to the first triple quadrupole (TQ) systems in 1980's, or the Orbitrap mass analyzer in 2000 by Makarov (1), different technologies and systems have been developed with a quite similar number of application fields and possibilities of use. Nowadays, it can be fairly said that none of them can be considered as a universal system for unambiguous identification and accurate measurement of compounds of toxicological interest. High-resolution mass spectrometry (HRMS) and low-resolution mass spectrometry (LRMS) are rather complementary approaches, and one should always explore which is the most suitable, depending on the applications to develop. In this part, the authors discuss the main strengths and drawbacks of both approaches. In mass spectrometry, resolution is a measure of the instrument's ability to discriminate two peaks of slightly different mass-to-charge ratios ΔM in a mass spectrum (Figure 1). Measuring resolution with the "Full Width at Half Maximum" (FWHM) method allows us to distinguish modern LRMS systems (TQ), with a resolution around 2000 FHHM, and HRMS systems which are defined by a resolution up to 20 000 FWHM (2). This high selectivity offers a greater certainty of identification because it allows discrimination of isobaric compounds in MS^1 and spectrum identification in MS^2. Moreover, the acquisition of an MS^2 spectrum linked to a unique molecule also presents accurate masses of the product ions and their isotopic abundances.

This is a great advantage for structure elucidation and drug discovery. The application is particularly interesting for the description of new psychoactive substances (NPS) and their metabolites (3), for the discovery of new biomarkers (4) and active molecules from plants (5). Indeed, some HRMS systems benefit from a new approach in MS^2 which consists in an increasing range of collision energies (CE-spread) from low to high values (6). Using such an

approach, acquired MS^2 spectra are pooled to give an average ("hybrid") spectrum containing all the product ions.

Obtaining an accurate mass can be very useful for both targeted and untargeted applications. The main improvement for targeted analysis is the high level of selectivity which prevents the risk of false positives by excluding isobaric interferences. This was for example illustrated by Geib et al. who developed 2 methods for the measurement of 25-Hydroxyvitamin-D3 in human serum: one with a LRMS system (TQ) and another with a HRMS system (Q-TOF) (7). The LRMS method failed to measure 25-OH-vitamin-D3 because of a co-eluted isobaric interference. Only HRMS was able to discriminate 25-OH-vitamin-D3 (m/z = 401.3414) from this interference (m/z = 401.2666).

The added value for untargeted analysis is obviously the possibility of unambiguous and exhaustive identification of the compounds possibly present in a sample. Theoretically, and contrary to LRMS, HRMS can identify an unknown compound without any *a priori* information about its structure or fragmentation pathways. Indeed, in MS^1, using a full scan-HRMS, every compound potentially present in a sample is theoretically considered and its spectral information is registered (and can be searched) according to its exact mass. The derived MS^2 spectra also contain specific fragments of each pseudo-molecular ion. Intuitively, this tremendously decreases the uncertainty of the identification when comparing a candidate to a spectrum registered in a library (6). Additionally, having accurate information about every fragment is also very helpful for structural elucidation. Contrary to LRMS, a HRMS untargeted screening also enables more exhaustive and precise retrospective analysis of previous acquisitions where a former analysis was not able to identify a detected compound. This is an actual benefit for new emerging drug monitoring like new psychoactive substances (NPS) (2). Consequently, HRMS screening is applied in numerous fields: clinical toxicology with general unknown screening (GUS), environmental toxicology with the analysis of soil or

water samples (8), toxico-epidemiology with applications for the biomonitoring of human exposome (6) or measurements of drugs/illicit drugs in wastewaters (9).

First generation HRMS instruments were limited by a narrow dynamic range. Consequently, it was recommended to use them only for qualitative applications, while LRMS would have been the gold standard for quantitation in biological matrices (10). However, the latest instruments have a significantly improved sensitivity and much wider dynamic ranges. This offers better detection and resolution performances (10). Currently, multiple studies support the fact that HRMS systems can now challenge LRMS systems for quantitative applications. Indeed, Fedorova et al. in 2013 compared performances of Full-scan HRMS (Q-Exactive™, Thermo Fisher Scientific) and TQ SRM (Selected Reaction Monitoring) for the quantification of 27 illicit drugs (11). As a result, 15 compounds (63%) gave similar limits of quantification (LOQs) with both systems and 8 (30%) even presented lower LOQs with HRMS. In 2016, Grund et al. conducted a similar study with 15 molecules (proteases inhibitors, tyrosine-kinase inhibitors, metanephrines and steroids) (12). Among them, 18% presented lower LOQs in Full-scan HRMS, 60% similar LOQs and 22% higher LOQ (respectively 29%, 53% and 18% in HR-MS^2). Lastly, Geib et al. obtained similar LOQs for Q-TOF and TQ for the quantification of 25-hydroxyvitamin-D3 and Henry et al. highlighted comparable sensitivity for Orbitrap and TQ-SRM (7,13). Finally, it could be fairly said that sensitivity is not systematically a limitation for HRMS. A case-to-case analysis is necessary to evaluate whether HRMS is suitable or not for a quantitative application.

The theoretical exhaustivity of the HRMS approach produces a huge quantity of data (which implies increased storage capacities), up to 25 times more than those produced by a classical LRMS approach (4). This phenomenon can be even worse when sample preparation (cleaning) is not optimal. Consequently, a long and fastidious results processing by trained analysts is necessary (2). Besides, to keep mass accuracy performances and prevent false

negatives, frequent recalibrations of the system with reference ions are needed. This can be very time consuming. To avoid this, a so-called “internal mass calibration” can be proposed. It consists in calibrating during acquisitions, but ion suppression phenomena can occur because of competition in source between calibrants and samples (14).

2. High Resolution Mass Spectrometry

a. Fourier Transform Mass Spectrometer - Orbitrap

The concept of orbital trapping described by Kingdon in 1923 (15) was applied in the field of mass analysis by Alexander Makarov, a Thermo Electron Corporation’s scientist (now Thermo Fisher Scientific) (1). In this study, a new type of orbital electrostatic trap mass analyzer was introduced; this mass analyzer was subsequently commercially marketed under the Orbitrap trademark by Thermo Fisher Scientific. A specific design of the trap with two outer electrodes forming a barrel surrounding a central spindle-like electrode (Figure 2), allowed the production of a quadro-logarithmic electrostatic field with the following potential distribution:

$$U(r,z) = \frac{k}{2}\left(z^2 - \frac{r^2}{2}\right) + \frac{k}{2}(R_m)^2 ln\left[\frac{r}{R_m}\right] + C$$

Where r and z are cylindrical coordinates, C is a constant, k is field curvature, and R_m is the characteristic radius.

The application of a voltage between the inner and outer electrodes creates this linear electric field. When the ions are introduced tangentially into the Orbitrap mass analyzer, they experience both electrostatic attraction and centrifugal force arising from their tangential velocity. Ions are then squeezed towards the central electrode by decreasing its voltage. After the voltage decrease stops, ion trajectories become a stable spiral. Due to the shape of the

electrodes, in the axial direction, ions are forced to move away from the narrow gap towards the wider gap near the equator, inducing harmonic axial oscillations without the need for any further excitation (16). Because of the strong dependence of the rotational frequency on ions energies, angles and position, the ion packet rapidly spreads over the angular coordinate and forms a rotating ring (Figure 3). Lighter ions enter the Orbitrap mass analyzer earlier than heavier ions, so they form a rotating ring of a different ellipticity as compared to heavier ions. The movement of the ions within the Orbitrap mass analyzer is characterized by three frequencies: the frequency of rotation that describes the movement of the ions around the central electrode, the frequency of radial oscillations that reflects the motion of the ions between the maximum and the minimum radii around the central electrode, and the axial frequency that describes the harmonic oscillations of the ions along the central electrode and is used for the *m/z* determination. The axial frequency is independent of the initial velocity and position of the ions and is described as follows:

$$\omega = \sqrt{\frac{e}{(m\,/\,z)} \cdot k}$$

Where e is the elementary charge (1.602×10^{-19}C), m is the mass of the ion, z is the charge of the ion, and k is the field curvature.

The harmonic oscillation along the axis (the z-direction) of a coherent ring of ions, all with the same *m/z*, induces an alternating current on the outer electrodes. Its amplitude is proportional to the number of ions in the ring, and it has the same frequency as the axial oscillations of the ions in the ring. The outer electrodes act then as receiver plates that detect an image current of these oscillating ions, or transients. The signal is amplified, digitalized, and transformed from the time domain to the frequency domain using a Fourier transform (FT), like the approach used in Fourier Transform - Ion Cyclotron Resonance (FT-ICR) Mass

Spectrometry. This way, the sensitivity and the signal to noise levels obtained for the Orbitrap mass analyzer are similar to that in FT-ICR instrument, but without the need for magnetic fields. To be able to detect and differentiate ions with very close masses, the ions need to diverge over the time of detection, and this requires a large number of oscillations. Therefore, the resolution of the Orbitrap mass analyzer is proportional to the time of acquisition. The oscillations of the ion packets remain stable if they do not experience collisions with residual gases. The Orbitrap mass analyzer therefore works under ultra-high vacuum conditions, around 10^{-10} mbar, that will ensure transient times around a second or above, depending on the molecule size. The resolution of the Orbitrap mass analyzer also decreases proportionally to the square root of the *m/z* ratio, and therefore has a better performance for high masses as compared to the FT-ICR analyzers. The first Orbitrap mass analyzer from (1) achieved a maximum resolution of 150,000 for $^{56}Fe^{+}$, and a mass accuracy of 5 ppm for $^{23}Na^{+}$ which, in turn, set benchmark values towards future innovations.

Following the first application of the Orbitrap mass analyzer, a series of developments were achieved to improve its capabilities both in terms of performance, such as speed, resolution and sensitivity, and usability with hyphenation and addition of fragmentation techniques (16,17). The first developments consisted of using a quadrupole to accumulate the ions before entering the Orbitrap analyzer (18,19). These studies of ion accumulation later resulted in the creation of a curved rf-only quadrupole (referred to as the "C-Trap") that was designed to store, cool, and focus the ions into a tight thread. Using pulsed extraction orthogonally to the C-Trap axis, the resulting ion packet is pulled through lenses via different vacuum stages, and curved ion optics focuses them for capture and detection by the Orbitrap mass analyzer. The introduction of the C-Trap made it possible to interface Orbitrap mass analyzers with any other type of ion source or mass analyzer. This leads to the introduction of the first commercial instrument by Thermo Electron Corporation (now Thermo Fisher Scientific,

Bremen, Germany) and consisted of a linear ion trap coupled to the Orbitrap analyzer; this instrument was marketed under the name LTQ Orbitrap™ mass spectrometer (20). The instrument made use of a process named automatic gain control (AGC) that allows the fast determination of ion current to enable the storage of a fixed number of ions (named AGC target value) with the accurate control of injection times. The AGC of the ions gave a quantitative dimension to this new system, since it helped regulate the ion current to have enough sensitivity but also to deal with space-charge effects that appear in any trapping device. In the case of the Orbitrap mass analyzer, space-charge effects are observed with higher amounts of ions than in regular radiofrequency ion traps due to higher ion energies and the shielding action of the central electrode. The consequences of overcharging the Orbitrap mass analyzer could be observed as mass shifts, reduced accuracy, or the coalescence of ion packets with very close *m/z* (16). Both the C-Trap and the AGC process are still used in current instruments and have been improved for more flexibility and faster results.

Another important aspect concerning the Orbitrap analyzer is that even though fragmentation could be achieved inside the Orbitrap volume, this would adversely affect mass accuracy and sensitivity. Therefore, the Orbitrap technology has only been used as a mass analyzer. Using the linear ion trap, the instrument could achieve fragmentation by collision induced dissociation (CID) using resonant excitation. In the field of analytical toxicology, fragmentation is essential for confirmation of the presence of some analytes, and a rich MS^2 spectrum is needed including low-mass ions. Therefore, beam-type fragmentation (referred to as higher-energy collision dissociation (HCD)) in Orbitrap-based instruments is advantageous. This type of fragmentation was adapted to the Orbitrap mass analyzer with the introduction of a gas-filled quadrupole, called the HCD cell, located just behind the C-Trap (21). The fragmentation occurs when ions are transferred from the C-Trap and accelerated to the HCD cell where they collide with residual nitrogen gas. The HCD cell stores the resulting

fragments and transfers them back to the C-Trap that, in turn, directs the ions towards the Orbitrap mass analyzer for subsequent analysis. The addition of this fragmentation mechanism permitted greater flexibility towards instrument functionality and hyphenating a quadrupole mass filter with the Orbitrap mass analyzer gave rise to the Q Exactive™ MS series instrument portfolio (Thermo Fisher Scientific, Bremen, Germany) first introduced in 2011. This type of instrument has been widely accepted for its performances in the field of analytical toxicology. Current quadrupole-Orbitrap instruments, with the implementation of ultra-high field Orbitrap mass analyzers, can achieve resolution up to 480,000 (FWHM) at *m/z* = 200 for the high end Orbitrap Exploris™ 480 MS (Thermo Fisher Scientific, Bremen, Germany), and up to 120,000 (FWHM) at *m/z* = 200 resolution for the Orbitrap Exploris™ 120 MS (Thermo Fisher Scientific, Bremen, Germany). As for the cycle time that can be achieved with the ultra-high field Orbitrap mass analyzer, as mentioned before it will be related to the resolution of the instrument, and the number and type of scan events that take place simultaneously. As an example, for the Orbitrap Exploris 120 MS instrument, the lower working resolution is 15,000 at *m/z* = 200, with a scan speed of 22Hz for a full scan acquisition, and the highest resolution is 120,000 at *m/z* = 200, with a scan speed of 3Hz for a full scan acquisition. Cycle time is mass range independent and current quadrupole-Orbitrap instruments can operate with mass ranges starting at *m/z* = 40, and that can go up to 3000 for the Orbitrap Exploris 120 MS or 8000 for other quadrupole-Orbitrap instruments having a biopharma option included. The quadrupole-Orbitrap technology has also been adapted to gas chromatography (GC). Finally, the hyphenation, speed and resolution have been pushed even further with Tribrid™ instruments, combining a quadrupole, a linear ion trap and an Orbitrap mass analyzer, going up to 1,000,000 (FWHM) at *m/z* = 200 as an option for the Orbitrap Eclipse™ Tribrid™ mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The Orbitrap mass analyzers have been employed in the fields of clinical and forensic toxicology

for drug metabolism studies (22,23), drug pharmacokinetics and toxicokinetics (24,25), screening (26–30) and quantitative measurements (31–36).

Different scan modes available for use on quadrupole–Orbitrap instruments could be untargeted, such as full scan acquisition, and targeted such as single ion monitoring (SIM) or targeted MS^2 (37). For quantitative analysis, three different approaches can be used with increasing selectivity: full scan, SIM or targeted MS^2. It is possible to optimize quantitation by combining the three acquisition modes. Considering that for Orbitrap instruments, the ions accumulate in the C-Trap, and with very complex matrices, the full scan could sometimes be limited in sensitivity for compounds co-eluting with highly concentrated interferences. High resolution instruments will acquire important amounts of information as compared to triple quadrupole instruments. The size of a file will depend on many different parameters such as run time, number of experiments, experiments type, and acquisition type. Therefore, the more complex the acquisition experiment is, the larger the size of the corresponding file. As an example, for a 15 minutes run of full scan positive and negative with data dependent acquisition in profile mode, so typically the experiment performed for screening purposes, the size of a file is around 150 to 200 MB, while for a SIM experiment in profile mode with 15 compounds, the size of a file is around 8 to 15 MB. To increase sensitivity, it is possible then to use the quadrupole mass analyzer to extract the analytes of interest and perform a SIM analysis. Quadrupole – Orbitrap instruments have the capability of accumulating different ion groups in the C-Trap before sending them as a combined packet towards the Orbitrap mass analyzer to optimize cycle time of the analysis. This is referred to as multiplexing, and the current portfolio allows up to 20 species to be multiplexed for a single Orbitrap cycle. More details on acquisition types, and particularly on full scan acquisition will be presented further.

b. Time of Flight Mass Spectrometry (TOF MS)

~~Over the last decade,~~ There has been a significant growth in the use of High Resolution Time

of Flight Mass Spectrometry (TOF HRMS) for trace level detection in environmental, chemical, biological, and life science applications, including applications such as contaminants, impurities, pollutants, residual pesticides, metabolomics, proteomics, lipidomics, pharmaceutical, biopharmaceutical, and clinical diagnostics. In support of this growth, substantial improvements have been made in the analytical performance and functionality of today's mass spectrometers especially increases in instrumental resolution, sensitivity, and scan speeds. Simultaneous advances in software for post-acquisition data analysis have enabled targeted and untargeted analyses from a single MS acquisition.

Early TOF MS instruments used an inline flight tube as debuted by Cameron and Eggers (38) (Figure 4) and relied on the creation of ion packets by employing a pulsed ionization source or a beam deflector. In addition to highlighting key analytical directions for TOF MS, Cameron and Eggers also identified key design factors fundamental to the performance for all TOF instrumentation. ~~Their system incorporated key functions found in today's TOF instruments: electron impact ionization, a mechanism for pulsing the ion beam, an extended flight path, a flat detector, and high speed electronics, which at that time relied on an oscilloscope to display the spectra. Having analyzing dichloromethane, water vapor, and mercury from the diffusion pump, their results highlighted two key factors.~~ First, when the ion transit times were solved for velocity, the arrival times for the various *m/z* values followed the simple relationship of $E=1/2\ mv^2$. And second, it was important to keep the initial energy spread of the ions to a minimum to minimize the spread in arrival time at the detector plate, and hence to preserve mass spectral resolving power.

In an effort to increase resolving power Wiley and McLaren introduced the concept of space focusing (39) but at the time the energy spread of the ions still limited the final *m/z* resolution. ~~In their electron impact ionization instrument they realized that ions created at the rear of the ion source volume had a longer distance to travel to the detector than those that were created~~

~~closer to the front of the ion source volume. They demonstrated that with the appropriate increase in voltage at the back pusher plate within the ion source it was possible to significantly reduce the loss of resolution due to the origin of the ions within the source. This two-field acceleration leads to a planar focus of the ions far outside of the ion source (Figure 5) that is referred to as Wiley–McLaren Space Focusing. While the ions achieved space focusing, the energy spread of the ions still limited the final *m/z* resolution. An additional outcome of Wiley's work was the early development of a commercial instrument offered by Bendix in the mid 1950's (40).~~

To address the spread of energy, B. A. Mamyrin, while a doctoral student at the Russian Academy of Sciences (40), proposed using an ion mirror (referred to as a reflectron) in the flight path ~~to compensate for the spread in the initial energy of the ions~~. Mamyrin showed that when a proper reflecting field was established, ions with greater energy proportionally travel further into to the reflectron before being reflected with the effect that the arrival time of the ions at the detector, regardless of the energy spread, would be the same.

In 1989 Dawson and Guilhaus (41) expanded beam deflection and introduced an orthogonal pulsing approach. ~~the advantage with which it minimized the energy spread, a limitation noted even beginning with the very first TOF research.~~ The introduction of the orthogonal TOF geometry (oa-TOF, Figure 5) was central to adapting TOF for atmospheric pressure ionization sources, such as electrospray ionization, and the subsequent growth in TOF applications in LC/MS. The result of these achievements means today's TOF instruments can achieve resolution values ranging from 20,000 to 60,000 in real-world conditions. ~~Applications involving atmospheric sampling opened time-of-flight analyses to a very broad set of analytes that placed further demands on the electronics used to convert time to *m/z*. Subsequent performance and efficiency improvements have led to remarkable sensitivities in support of ultra-trace level measurements.~~

The flight time for each ion of particular *m/z* is unique. The flight time begins when a high voltage pulse is applied to the back plate of the ion pulser (Figure 3) and ends when the ions of interest strike the detector. The flight time (t) is established by the energy (E) to which an ion is accelerated, the distance (d) it has to travel, and its mass (strictly speaking its mass-to-charge ratio).

There are two well-known formulae that apply to time-of-flight analysis. One is the formula for kinetic energy, the energy of an object (or an ion) in motion, which is expressed as: $E=1/2\ mv^2$, which solved for m becomes: $m= 2E/v^2$. The second equation is the familiar velocity (v) equals distance (d) divide by time (t) or: $v=d/t$. Combining the first and second equations yields: $m=(2E/d^2)t^2$. This gives us the basic time-of-flight relationship. For a given energy (E) and distance (d) the mass is proportional to the square of the ion flight time.

The equation stipulates that for a given kinetic energy, E, smaller masses will have larger velocities, and larger masses will have smaller velocities. Hence, ions with lower masses arrive at the detector earlier, as shown in Figure 6.

In the design of an oa-TOF mass spectrometer, much effort is devoted to holding the values of the energy (determined by the high voltages) and the distance the ion travels constant, so that an accurate measurement of flight time will give an accurate mass value. As these terms are held constant they are often combined into a single variable, A, so: $m=A(t)^2$. This is an ideal equation, based on true flight times. In practice, there is a delay from the time the control electronics send a start pulse to the time that high voltage is actually present on the ion pulser plates. There is also a delay from the time an ion reaches the front surface of the ion detector until the signal generated by that ion is digitized by the acquisition electronics. These delays are very short, but not insignificant. Because the true flight time cannot be measured, it is necessary to correct the measured time, t_m, by subtracting the sum of both the start and stop

delay times which, when added together, are referred to as t_0: $t = t_m - t_0$. By substitution, the basic formula that can be applied for actual measurements becomes: $m=A(t_m-t_0)^2$.

To make the conversion from measured flight time, t_m, to mass, the values of A and t_0 must be determined, so a calibration is performed. A mixture of compounds whose exact masses are known with great accuracy is analyzed. Then a simple table is established of the flight times and corresponding known masses (Table 1).

Now that m and t_m are known for a number of values across the mass range, the computer that is receiving data from the instrument does the calculations to determine A and t_0. Using an intelligent algorithm, it tries different values of A and t_0 until the right side of the calibration equation, $m=A(t_m-t_0)^2$ matches as closely as possible the left side of the equation (m), for all seven of the mass values in the calibration mix.

While this initial determination of A and t_0 is highly accurate, it is still not accurate enough to give the best possible mass accuracy for time-of-flight analysis. A second calibration step is needed. After the calibration coefficients A and t_0 have been determined, a comparison is made between the actual mass values for the calibration masses and their calculated values from the equation. These typically deviate by only a few parts-per-million (ppm). Because these deviations are small and relatively constant over time, it is possible to perform a second-pass correction to achieve an even better mass calibration. This is done via an equation that corrects the small deviations across the entire mass range. This correction equation (typically a higher-order polynomial function) is stored as part of the instrument calibration. The remaining mass error after this two-step calibration method, neglecting all other instrumental factors, is typically at or below 1 ppm over the range of calibration masses.

Improvements in TOF instrumentation have led to the increasing use of MS in a broader set of applications which continued to place further demands on MS technology. TOF instrumentation is well suited to these demands, particularly with the large mass range

achievable by these devices; mass to charge ratios of 10 *m/z* to 20,000 *m/z* are achievable in modern TOF instruments. While the applied markets have long been serviced with targeted analytical workflows, the need to acquire full spectral data and be able to post-acquisition interrogate data sets for an arbitrary list of analytes has been steadily growing. This migration to full spectral data acquisition has grown in parallel to instrumental enhancements to scan speed and dynamic range. The complexity of the original sample matrices frequently poses challenges due to chemical similarity to the analytes and the presence of large amount of matrix with respect to the analyte. It was, and remains, not uncommon for LC/MS samples to contain matrix in large excess of the analyte, and in such fashion that the analytes of interest may be at concentration levels many decades lower than the residual matrix.

The initial growth of the use of high-resolution mass spectrometry for life science applications resulted in greater development effort being focused on Q-TOF instrumentation in support of LC and nano-LC separations as compared to gas chromatography (GC). Each of these separations techniques also impact instrument run times, thus it is worth mentioning the data file sizes generated by TOF HRMS. Approximately hundreds of megabytes (MB) are acquired for a thirty minute run but the size varies heavily on several instrument conditions. Despite largely liquid phase orientation, product commercialization and important application development took place in support of GC separations as well.

While the earliest reports of GC/MS took advantage of utilizing the ion source in a pulsed mode, acting as both ion volume and pusher directly at the entrance to the flight tube, in the late 1990's, as commercial systems became available, GC/MS applications also took advantage of the orthogonal geometry that become standard for LC/MS based systems.

The development and introduction of TOF systems for GC separation is significant for the direct support of many of the analytical challenges in pesticide residue, environmental, forensics and metabolomics analysis. Electron impact ionization (EI) and chemical ionization

(CI) are well suited for samples with high complexity and for chemical classes that are enabled for GC separation using analyte derivatization. The high peak capacity of GC separation, high retention time stability, and larger immunity to matrix suppression makes GC/MS ideal for many applications.

~~Over the last two decades, commercial TOF instrumentation has seen tremendous technological improvements in performance and increased applicability to solve today's most challenging analytical problems. In addition to the tremendous full spectral acquisition capabilities of TOF, modern TOF systems have achieved outstanding resolving power to address the needs of very complex sample sets. Significant gains in the increase in fundamental sensitivity have also been true for both LC/MS and GC/MS applications. Lastly, the challenge of ultra-trace analysis in complex matrices places one of its largest demands on dynamic range.~~

~~A fundamental strength of time-of-flight mass analysis is that the measurement of trace level analytes can still be achieved in the over-abundance of matrix, and in spectrum dynamic range is also the greatest for high-resolution measurement. Yet, with demands for improved quantitative and confirmatory results, future improvements are expected to continue to drive adoption of TOF instrumentation in these markets.~~

3. Data acquisition

A schematic representation of the four data acquisition modes is presented in figure 7.

a. Full Scan

For high resolution instruments, one of the basic acquisition modes is wide-mass acquisition, so called full scan. As compared to a triple quadrupole, where scanning in full scan will have an impact on sensitivity and speed, for high resolution instruments full scan is extremely

useful both in quantitative and qualitative analysis. With full scan acquisition, no selection is made prior to data collection, and there is no limitation on the number of analytes that can be studied. In fact, any compound that ionizes and is found within the mass range of the full scan will be detected. This provides significant advantages towards screening purposes and could be used to perform retrospective analysis, opening up the possibility to study emerging drug trends in forensic toxicology and sport doping, as well as studies into metabolic pathways. Also, for quantitation, full scan analysis has proven to be as selective and sensitive as single reaction monitoring (SRM) analysis with triple quadrupoles when resolution is above 25,000 and small extraction windows are used (12,13,42). With high resolution full scan acquisition, the isotopic pattern of a compound can be investigated, and can be used for structural elucidation and therefore for identification purposes (43). Also, full scan in high resolution acquisition has the advantage of generating two identification points, which is helpful for confirmation (44). Therefore full scan gives simultaneously quantitative and qualitative information. When using full scan acquisition, method development is much simpler with no need to infuse individual standards. The selection of the molecules to be analyzed is made after the acquisition step, offering significant advantages for high throughput laboratories and workflows that require urgent analysis, such as emergency toxicology. The data processing consists in using a narrow extraction window around the exact mass of the ion of interest, and this is obtained easily by having the formula of the compound. This gives the user the possibility to increase the list of analytes as needed in one single method.

b. Data dependent acquisition

For screening purposes, as well as for confirmation analysis, acquisition may include MS^2 data. One acquisition type that generate fragmentation data is Data-Dependent Acquisition (DDA). In this acquisition mode, the presence of a compound of interest either in full scan or

in SIM scan can trigger its fragmentation. The triggering can be targeted, based on a list of compounds to be fragmented, or untargeted, and then based on the signal intensity of the ions in the full scan.

Untargeted DDA is only based on full scan acquisition before the data-dependent triggering of the MS^2 spectra. For this acquisition mode, the instrument will investigate the full scan mass spectrum and select the most intense ions. Then, in the next scan event, those selected ions will be filtered by the quadrupole, fragmented, and analyzed generating high resolution MS^2 spectra. For the Orbitrap instruments, the method can be configured to select how many ions will be fragmented between each full scan event, and there is also a possibility to multiplex MS^2 spectra. The advantage of the untargeted DDA method is that it can give some specific MS^2 information for untargeted compounds. However, untargeted DDA is limited when it comes to low abundance ions since the dependency relies mainly on the intensity of the compounds in the full scan. Different strategies can be used to improve the results of this untargeted DDA consisting mainly in excluding the less interesting compounds from the fragmentation list. The exclusion can be simply defined in an exclusion list where the mass and eventually retention time window of the compound to exclude is defined. This exclusion list remains targeted and needs to be configured in the instrument method. The exclusion can also be dynamic during the run, where the masses that have just been fragmented can be excluded for a period defined in the instrument method. Some of the latest developments for untargeted DDA include the AcquireX™ (Thermo Fisher Scientific, Bremen, Germany) deep scan acquisition workflow (45). The AcquireX acquisition workflow uses a blank injection to establish an initial exclusion list used for the studied unknown sample. The sample is then injected first with this exclusion list, and then it is reinjected with an increased exclusion list generated by the previous injection. This process is iterative and allows the coverage of a large range of compounds for fragmentation.

Another way to handle low abundance of certain ions in the full scan is to use targeted DDA. In this case, the triggering of MS^2 spectra acquisition requires that the compound of interest gives a specified signal intensity, in a certain retention time window, and with a specific mass accuracy. The drawback of targeted DDA is that it will be limited for unknown compounds, and it requires a large inclusion list to be able to handle the diversity of drugs, metabolites, and other compounds required for screening purposes. A commercial high-resolution accurate-mass (HRAM) library and database was generated based on the DDA approach with over 2000 parent drugs and over 3000 of their metabolites (46). This approach has generated a lot of interest for screening purposes (28,47), and is today the basic approach for routine HRMS screening workflows.

c. Data independent acquisition

Data-Independent Acquisition (DIA) is a method of molecular structure determination in which all ions within a selected *m/z* range are fragmented and analyzed. DIA has become an established technique in MS-based 'omics' research (48) and is increasingly used for the screening of xenobiotics e.g. drugs, drug metabolites, pesticides, toxicants (48-52).

For many years, tandem mass spectrometry (Triple Quad and QTRAP®) with data dependent acquisition (DDA) was the gold standard for Forensic Toxicology screening analysis (53). But, multiple reaction monitoring on triple-quadrupole mass spectrometers is not easily adapted to identify large panels of compounds because it relies on targeted method development. High-resolution mass spectrometry (HRMS), including Quadrupole - Time of Flight (Q-TOF) and orbitrap technologies, has shown usefulness in general unknown screening (GUS) for untargeted compound identification, only achievable at high-resolution accurate mass. DDA, which performs a nontargeted screen of precursor ion masses and

selects the most abundant ones (often 10 or 20 per acquisition cycle) for secondary fragmentation and subsequent identification from a spectral library, has limitations for samples in which compounds may be in low abundance. Understanding this limitation of DDA led to seek alternative methods of unbiased data acquisition for HRMS toxicology screening.

Nowadays, a shift towards HRMS including the possibility of DIA (54) has been observed. Several manufacturers offer different solutions for DIA on the market, including the most up to date namely vDIA from Thermo Fisher Scientific (55), MS^E from Waters (56), all Ion MS/MS (Agilent Technologies), Broadband CID (Bruker), MS/MS-DIA (Shimadzu) or variable window SWATH® from SCIEX (57). Non-targeted screening with these new DIA methods is bias free (no inclusion list or prior target list is needed). They offer the possibility to detect all ionized analytes in a sample and to acquire product ion spectra for every precursor, and allows retrospective data analysis as an additional option.

In the vDIA approach (not available in the United States of America), a full scan is followed by two to twenty wide-isolation fragmentation scans, which together cover the same isolation range as the preceding full scan. The vDIA approach bridges the gap between full scan-data-dependent MS2 (FS-ddMS2) experiments and full-range fragmentation scan modes such as AIF. FS-ddMS2 experiments, where MS2 scans are performed on targets of interest (present on an inclusion list) upon their detection in the full scan, are known to be very selective and sensitive with respect to the fragment ion information obtained. Retrospective FS-ddMS2 data analysis for additional compounds of interest, however, is limited to full-scan quantitation by accurate mass without confirmation of identity by MS/MS. To maintain scan speed, this method has to work with dynamic exclusion, so only one survey MS2 scan is triggered per compound. As a result, no elution profiles can be extracted for the confirming fragment ions. Full-range fragmentation experiments like AIF, where fragments from all species present in

the full scan are detected in a single MS2 scan, have the advantage of collecting all possible full scan and MS/MS information for the sample. Thus, they are fully suitable for retrospective data analysis. However, dynamic range, selectivity, and achieved detection limits are limited as the number of ions fragmented per species is lower due to the combined nature of the analyses. The vDIA experiment combines the advantages of both approaches by using smaller isolation windows for the fragmentation scans while still fragmenting all precursors from the preceding full scan. With this, a complete record of MS and MS/MS data is kept, so all compounds of interest can be processed even in retrospective manner and full elution profiles of all confirming ions for all components can be extracted (58).

The MS^E permits the seamless collection of a comprehensive catalog of information for both precursor and fragment ions in a single analysis. It collects low and high energy spectra alternatively during the same acquisition. The first function acquires data in a low energy state providing the exact mass of precursor ions. The second function, acquired in an elevated energy state, provides the exact mass of product ions for additional confirmatory purposes. It allows detection/rapid screening for compounds by means of searching the exact mass of the compounds in the full spectra generated using low collision energy. Confirmation with reference standards, or tentative identification, could subsequently be performed searching for exact masses of fragment ions in the acquired spectra at high collision energy, where fragmentation of the compounds is promoted (59-61).

MS/MS-DIA associates full-scan and fragmentation events within specific “mass windows”. For small molecule analysis applied to forensic toxicology a full scan cycle of 100 msecs is typically used over the mass range of interest (*m/z* 100-1000) to generate high sensitivity. Precursor isolation windows are typically 20 Da for masses below *m/z* = 500 and increase to 35 Da above *m/z* = 500 with a cycle time of 20 msecs for each mass scan and a collision energy spread of 5-55 V. Using a collision energy spread generate complete MS^2 spectra

containing both the precursor ion (low CE) and the product ions. Fragmentation spectra may be chimeric in nature given the wide precursor ion isolation width which often requires a deconvolution routine to support library searching. Using a collision energy spread of 5-55V results in precursor and corresponding product ion spectra in each DIA mass scan which can be used in searching both internally generated libraries and third-party resources.

Sequential window acquisition of all theoretical fragment ion spectra (SWATH®) untargeted data acquisition works by analyzing "mass windows" in which all precursor ions within a small Q1 mass range undergo fragmentation and detection with no spectral data lost. This window then shifts to the next sequential range and repeats the process until the desired mass range is covered for that cycle (Figure 7). The complexity and amount of data collected from mass windows poses a number of challenges. The primary limitation is drugs of the same class with shared product ions that fall within the same mass and retention time window (e.g., amphetamine and methamphetamine). Additionally, compound-rich windows may be problematic owing to the complexity of spectra that must be deconvoluted in data analysis. To solve this problem, it's possible to use variable custom-sized mass windows down to 5 Da (61). In many cases, tighter Q1 windows (up to 200) can provide improved specificity by reducing the interferences from co-eluting analytes that have a similar m/z and produce similar mass fragment ions. Product ions are generated (up to 100 spectra/sec) using rapid cycling of low, medium and high collision energies between two set points (as known as collision energy spread), allowing library search capabilities of comprehensive and detailed mass spectral data for definitive analyte identification of novel compounds and their metabolites. In addition to all potential interesting compounds in a sample thanks DIA even at lower abundance, it allows also to quantify at the same time by MS/MS the rising number of medicinal drugs, drugs of abuse, as well as new psychoactive substances (NPS) (52).

HRMS workflows with non-targeted acquisition provide clear advantages to identifying new substances like NPS in a more timely manner, and the increased specificity gained through acquisition of fragment data is necessary (62).

d. Parallel Reaction Monitoring – Multiple Reaction Monitoring

The most selective acquisition mode is targeted MS^2, also called parallel reaction monitoring or PRM (Thermo Fisher Scientific), MRM (Agilent, Shimadzu), Tof-MRM (Waters) or MRM^{HR} (SCIEX), where the quadrupole selects a parent mass in a small mass window that is then fragmented with a specified collision energy in the HCD cell (Orbitrap) or collision cell (QTOFs), and for which all fragments are then detected in the mass analyzer (63-65). This is similar to product ion scan mode in triple quadrupoles with two main differences: the fragments are acquired simultaneously and at high resolution, so the analysis is even more selective, and the sensitivity is much higher, closer to single reaction monitoring (SRM) or MRM scan modes with Triple Quadrupoles. HR-MS instruments also allow the multiplexing of targeted MS^2 experiments. And finally, it's even possible to combine two workflows in one injection, like SWATH® and MRM^{HR}. The high sensitivity and selectivity of MRM^{HR} allows accurate quantitation of drugs and drug metabolites while SWATH® acquisition enables generation of high-quality MS/MS spectra and accurate identification of the analytes using spectral library searching (66).

4. Data processing

a. Compound identification

For untargeted screening purpose, compound identification is realized using software tools and compound databases. A compound database is a collection of chromatographic and mass spectral information on compounds that may be useful in software searching and information retrieval for identification purposes. HRMS provides important information for compound structural elucidation, such as accurate mass, fragmentation pattern (product ion masses and their relative abundances), isotope distribution, retention time (Rt) when associated with a separative method, and collision cross section (CCS) when ion mobility technology is used. The combination of those orthogonal proprieties measured and their interpretation by comparison to experimental or predicted databases permit to annotate features. A screening approach employing accurate mass libraries was first described by Gergov et al. in 2001 (67). Authors built their in-house library based on orthogonal proprieties recorded with authentic reference substance and used those proprieties to identify xenobiotics in samples of interest. In the case of unavailability of reference substances (and so missing orthogonal proprieties), a lack in reliability of identification could occurs, possibly leading to false positive identifications as experimented by Ojanperä et al. They found a rate of 39% of false positive by screening five authentic urine samples and employing isotopic pattern and accurate mass matching to a library of 735 compounds (68). Indeed, depending on strategy used for feature identification, different levels/degrees of confidence could be listed. A consensus on those levels were proposed by the Chemical Analysis Working Group (CAWG) of the Metabolomics Standards Initiative (MSI) (69). Although the authors have an interest in metabolomics, the paper contains much useful information which can be applied in the practice of toxicology screening. The level 1 is the highest level of confidence, defining a confident and definitive identification of a compound. This level is achieved when two or more orthogonal proprieties are matched with authentic standards analyzed in same analytical conditions. It must be noted that some analytes require specific caution of unambiguous

structural identification, as isomers configurations. Levels 2 (high level) and 3 (medium level) are putative annotations of a compound or a class of compounds respectively. Here, annotation is based on comparison of data with public libraries without confrontation to authentic chemical standards under same analytical conditions. As described previously, those no-standards driven methodologies could generate a high number of errors of annotation and therefore a high number of false positives. The lowest level of confidence (level 4), gather unknown compounds. Although unidentified and unclassified, these metabolites can still be differentiated and quantified based upon spectral data.

Actually, the first step in feature annotation is based on data comparison with existing in-house or external databases. If no match is possible, a de novo structure elucidation could be performed based on analyte isolation associated with *in silico* orthogonal properties predictions.

i. In-house libraries

The construction of an in-house library consists in selecting and purchasing commercially available standards, and acquiring their orthogonal data in predefined in-house experimental conditions that will be used for sample analysis. Even if this workflow theorically permits to annotate compounds with the highest level of confidence, it could be quickly expansive and time consuming when considering the large panel of compounds that could be targeted. However, this local database can be easily completed with new compounds using authentic standards solution. In a sample of interest, identification is then achieved by comparing experimental results to the results that were obtained from authentic standards and stored in the in-house library.

ii. External libraries

By contrast to in-house libraries, external ones are only gathering experimental and/or predicted mass spectra information as well as accurate mass. For this reason, retention time cannot be used for compound identification. Few examples are presented in Table 2. They are extensively used as they are time-gain tools. They could be consulted from open-access libraries in community databases, with unrestricted access to the data, or from commercially available resources. The latter could appear more costly, but users could benefit from easy and ready-to-use workflows, as well as integration with proprietary software from instrument vendors. Both types co-exist and could be a complementary resource, that's why some users combine them to increase the analyte coverage. In both cases one have to take deep care on conditions of databases generation, as closest conditions to the selected experimental conditions should be preferred. For example, databases could permit annotation from full scan data (as PubChem (70), Chemspider (71) among others), or from MS/MS or MSn data (as Massbank (72), mzCloud from Thermo Fisher Scientific). By performing annotation with external libraries, a matching score of orthogonal data could be generated based on compared expected *vs* observed data, permitting to rank candidates if necessary.

iii. *In silico* prediction

Some features could remain misidentified, because of unknown or novel metabolite identification (73). In the context of metabolites listed in molecular structure databases but without recorded reference spectra in spectral libraries, computational approaches could be performed. *In silico* prediction of fragmentation, Rt (74), CCS (75) could be helpful. When possible, the novel metabolite identity needs to be confirmed by synthesis of standard and its analysis under the same analytical conditions. But in some cases, new substance could be not already listed in any database (76). One can perform an isolation of the substance or

metabolite of interest by NMR analysis for structural elucidation (77), LC-MS/MS analysis combined with *in silico* prediction of orthogonal proprieties (78), or multiple-stage tandem MS (MS^n).

In toxicology analysis, one should keep in mind that metabolites of xenobiotics could be of great interest, and also need to be correctly identified. In these cases, some software as MetaSite, Metabolite Predict or MetWorks software for example, are able to predict *in silico* metabolism (79). This prediction is based on principal metabolism enzymatic reaction (hydroxylation, dehydrogenation, demethylation…). Theoretical spectra are generated for each metabolite and then compared to the one obtained for unidentified compound. This approach could benefit from interpretation tools as metabolic networking tools for interpretation of complex metabolization processes and placing identified components in a biological context.

iv. Metabolic networking (MN)

MN is a computational strategy that may help visualization and interpretation of the complex data obtained from MS analysis. MN is able to identify potential similarities among spectra within the dataset and propagate annotation to unknown but related molecules (80). This approach is based on the hypothesis that structurally related molecules produce similar fragmentation patterns, and therefore they should be related within a fragment network (81). In MN, HRMS data are represented in a graphical form, where each node represents an ion with an associated fragmentation spectrum. Links among the nodes indicate similarities of the spectra. By propagation of the structural information within the network, unknown but structurally related molecules can be highlighted and successful dereplication can be obtained (82).

b. Applications in toxicology

i. An exogenous purpose

Since 2014, various articles have been published on different aspects of screening for detection of pharmaceuticals or drugs of abuse (83–88). Patteet et al. (2015) focused their review on screening and quantification of antipsychotic drugs and stated that HRMS may be promising in situations when a combination of known or unknown screening and quantification is required (85). Among the applied techniques, LC–HRMS was discussed to be best suited for multi-component target methods and for general unknown screening. Another advantage is the flexibility of HRMS to quickly add metabolites to the existing screening approaches simply by use of their accurate molecular masses from the HR full scan. An excellent and comprehensive review of HRMS applications in meds, drugs of abuse, and doping substances screening will be extensively discussed in the following articles.

ii. Metabolomics in toxicology

Metabolomics is the science concerned with the profile of identified and / or quantified metabolites in biological fluids, cells and tissues. It aims to characterize and identify metabolites, i.e. the end products of cellular metabolism. Although known for its role in the discovery of endogenous biomarkers, metabolomics can also be used to identify metabolites that can mechanistically explain alterations of the phenotype of a cell type or an organism (89). Metabolomic fingerprints (or metabolomic profiles) can be obtained from any type of matrix, in particular those that can be taken in the fields of toxicology such as biological fluids or cell samples. The "omics" sciences, including metabolomics, are currently finding their place in the fields of clinical toxicology and forensic medicine to provide answers to

various questions that are sometimes unresolved (90, 91). Numerous studies have previously described metabolomics application in different areas of toxicology. In fact, Olesti et al. demonstrated that rat's neurotransmitters' profile was disturbed after exposition to cocaine, heroin, THC, MDMA or methamphetamine with association between pharmacological effects and metabolic perturbations. Furthermore, based on this observation, they also could predict pharmacological effects of JWH-18 (92). On another side, numerous authors have previously described that environmental factor disturbs metabolism. For example, authors found endogenous biomarkers of mercury exposition and associated them to mercury intoxication symptoms (93). Additionally, these biomarkers have been described disturbed with mercury concentration lower to toxic threshold. Finally, metabolomics studies also demonstrated their potential in forensic for different application. For example, Bohnert et al studied 41 human CSF samples collected post-mortem during an autopsy between 2 and 13 days after death (94). By comparison of metabolomic profiles, the authors were able to differentiate deaths caused by trauma to the head with survival time <2 h in 11 subjects compared to deaths of cardiovascular origin for 10 subjects, by multiorgan failure for 10 subjects, by torso trauma without head trauma for 10 subjects.

iii. Combination of endogenes and exogenes

The combination of untargeted metabolomics and identification of toxic substances lead to additional information. In the publication of Chen et al, authors used an untargeted metabolomics workflow associated with a library including toxic substances and endogenous compound on 4 children plasma to identify the cause of their neural and cardiac symptoms (95). Thirteen xenobiotics were found significatively higher in the intoxicated group than in control group. However, endogenous metabolome profiling revealed that acyl-carnitines were

among the dysregulated endogenous metabolites. The dysregulation of acylcarnitines is associated with many pathological conditions with mitochondrial dysfunction, including neurotoxicity (96). Finally, in the list of xenobiotic compounds detected, only penfluridol has pharmacological effects on the central nervous systems. Combining these sources of information, penfluridol was considered most likely to cause these intoxications and its concentrations in plasma samples were then estimated using MRM. According to authors, measured concentrations were higher than ones found from adults after oral administration (almost 12 ng/mL at peak concentration). Considering that the blood samples of the children were collected 2 days after intoxication, it was speculated that the children were suffering from the effect of relatively high levels of penfluridol for those 2 days.

Conclusion

High-resolution mass spectrometers are complex machines, relying on cutting-edge technologies, allowing to perform analysis faster, more sensitive and with increased resolution. The narrow dynamic range, one of the main limitations identified no longer exists, and many quantitative methods have been developed over the last decade illustrating the ability of HRMS to perform quantitative analyzes in addition to screening. New powerful acquisition modes based on untargeted screenings dependent or independent of previous information offer the possibility of detecting and characterizing almost all the ions of interest in a sample. However, these technologies do not make it possible to get rid of analytical interference, in particular on complex biological material, emphasizing the interest of coupling these tools with efficient separation methods based on GC or LC.

The limits are gradually moving from mass spectrometers to data reprocessing, which requires high-performance software, innovative approaches and large spectral libraries. The following

reviews will focus on HRMS techniques for research and application in clinical toxicology and forensic toxicology.

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E.Z. PhD Student, partially financed by Shimadzu Corporation

V.T. Employee in Thermo Fisher Scientific Corporation

P.C. Employee in Agilent Technologies

K.P. Employee in SCIEX

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Table 1. TOF mass calibration and corresponding flight time

Calibrant compound mass (m)	**Flight time (µsec) (tm)**
118.0863	20.79841
322.0481	33.53829
622.0290	46.12659
922.0098	55.88826
1521.9710	71.45158
2121.9330	84.14302
2721.8950	95.13425

Table 2: A list of databases and libraries for annotation, identification, and interpretation from

Database	**Web address**
ChemSpider	http://www.chemspider.com/
NIST	https://www.nist.gov/srd
ChEMBL	https://www.ebi.ac.uk/chembl/
PubChem	https://pubchem.ncbi.nlm.nih.gov/
mzCloud	https://www.mzcloud.org/
Molecular network	https://ccms-ucsd.github.io/GNPSDocumentation/networking/
MassBank	https://massbank.eu/MassBank/
HMDB	https://hmdb.ca/
Kegg	https://www.genome.jp/kegg/

HRMS analyses

Figure 1. In a mass spectrum, resolution (R) can be defined by the width at 50% of the peak height (full width at half maximum: FWHM). Systems with R > 20 000 and mass accuracy < 5 ppm are called high-resolution mass spectrometry (HRMS), in opposition to low resolution mass spectrometry (LRMS)

Figure 2. A cut-out of a standard Orbitrap analyzer (Reproduced with permission of Thermo Fisher Scientific)

Figure 3. Diagram of the high-field Orbitrap mass analyzer showing a stable spiral trajectory

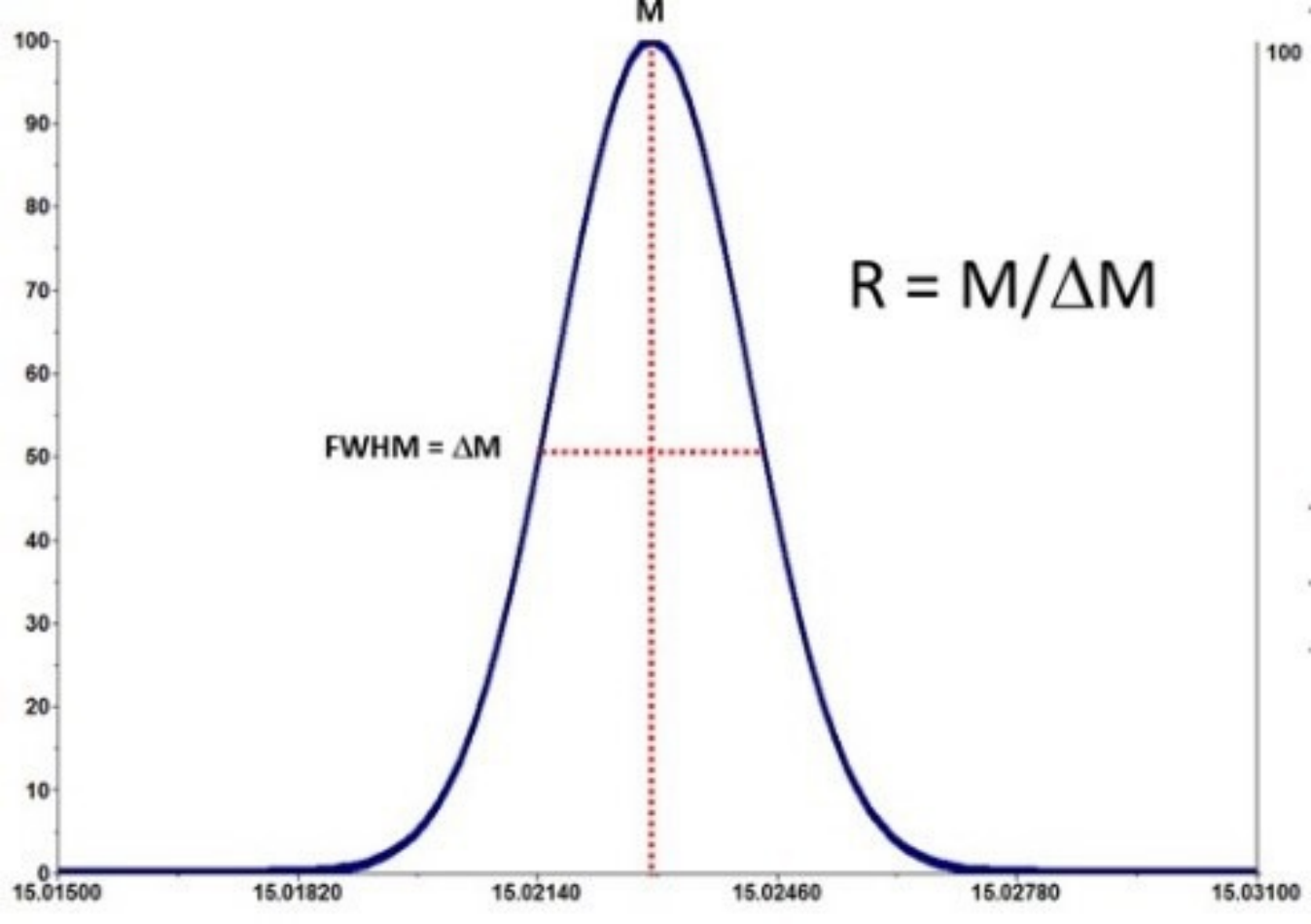
Figure 4. Schematic representation of a velocitron with key aspects highlighted in bold. Adapted with permission from reference 38 © 1948, AIP Publishing LLC

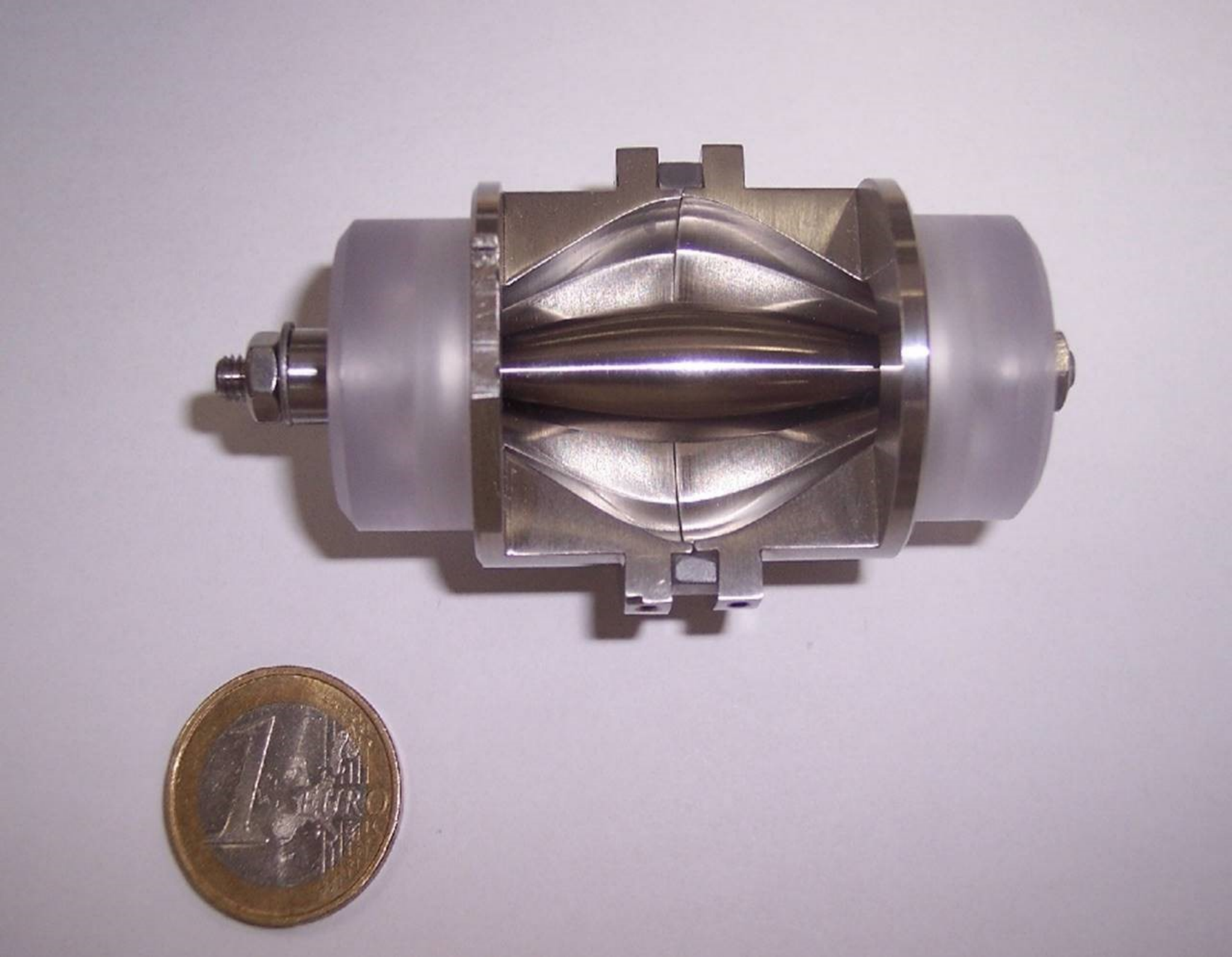
Figure 5. Illustration of space focusing, axial and off-axis energy nature of an ion beam formed using an orthogonal pusher from a continuous ion source, and energy focusing achieved using an ion mirror (reflectron)

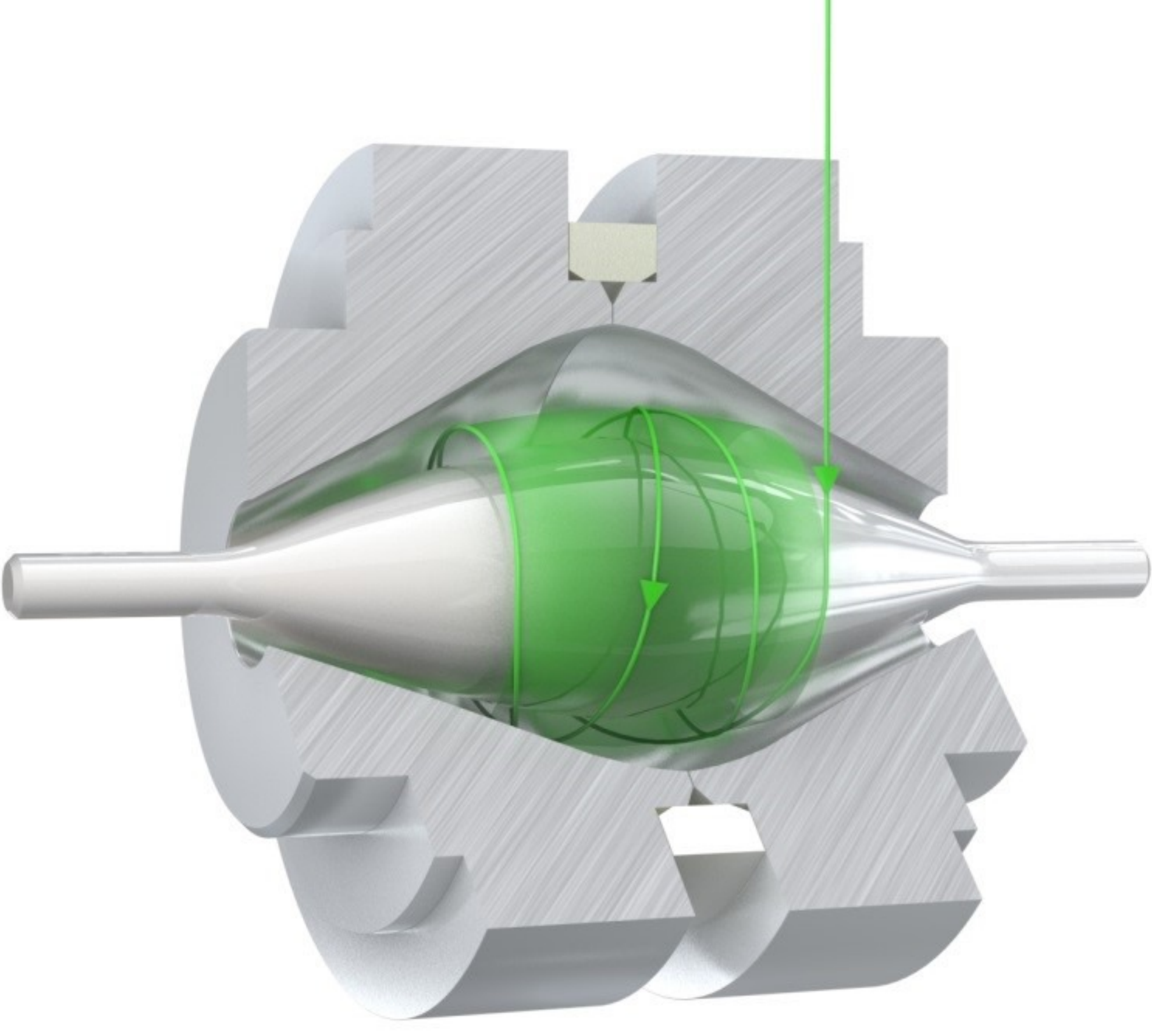
Figure 6. Orthogonal axis time of flight mass spectrometers with the configuration on the left showing linear geometry and the configuration on the left incorporating an ion mirror

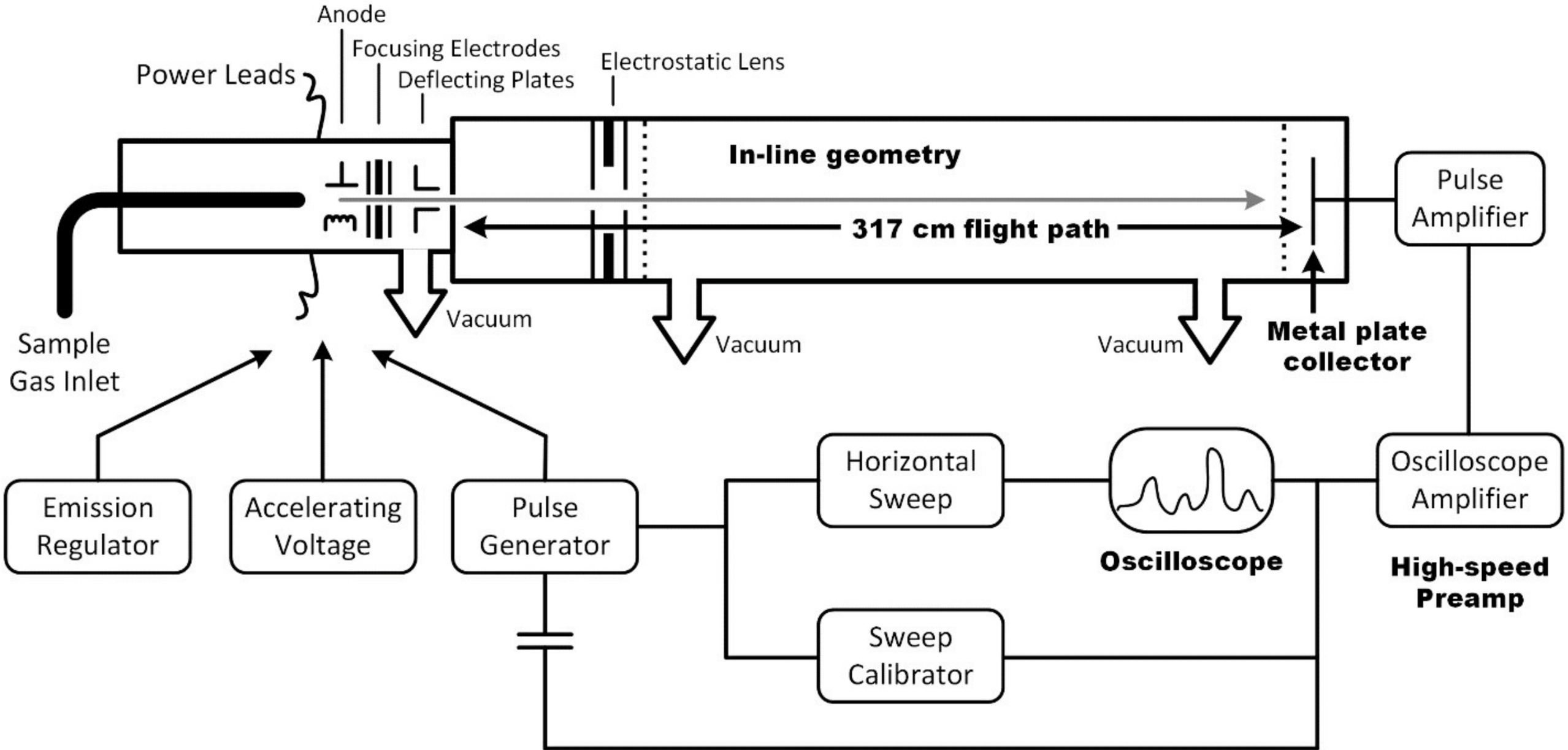
Figure 7. Schematic representation of the four main data acquisition modes: Full Scan, Data Dependent Analysis (DDA), Data Independent Analysis (DIA) and Parallel Reaction Monitoring (PRM).

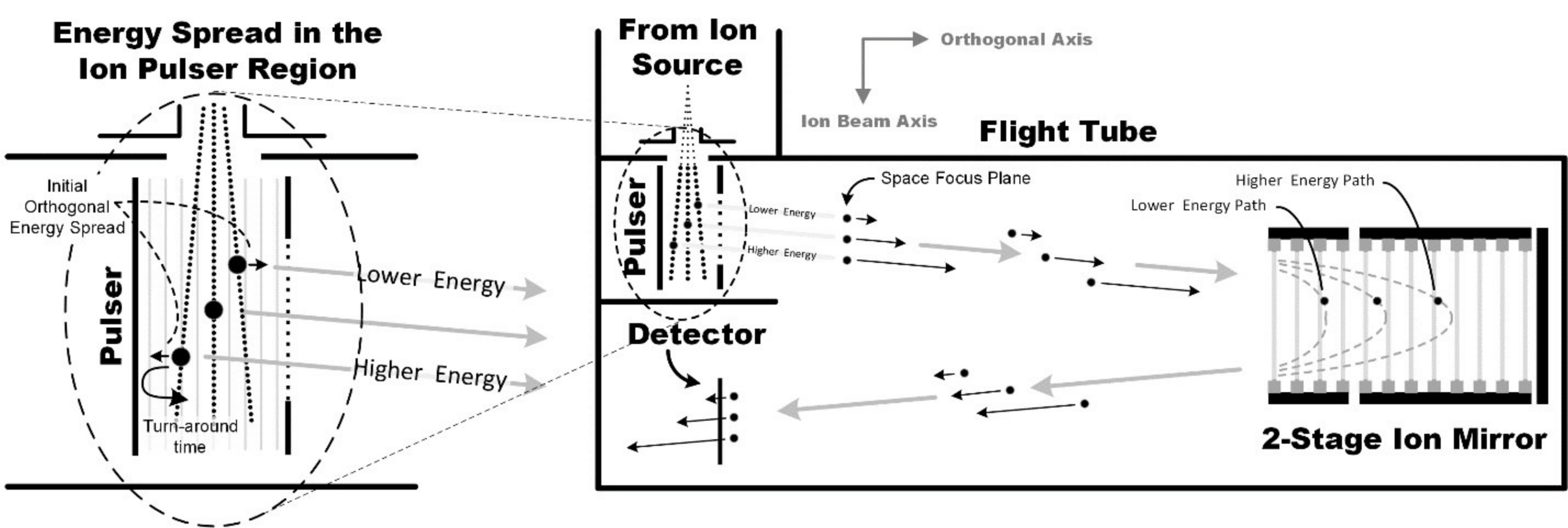
Figure 8. Investigating Q1 window sizes for SWATH Acquisition. When performing SWATH Acquisition, the Q1 isolation windows are stepped across the mass range, and high resolution MS/MS is acquired for a specific accumulation time at each step. When optimizing a method for a SWATH Acquisition experiment, one must optimize the Q1 window size, accumulation time and cycle time to strike a balance between sensitivity and specificity

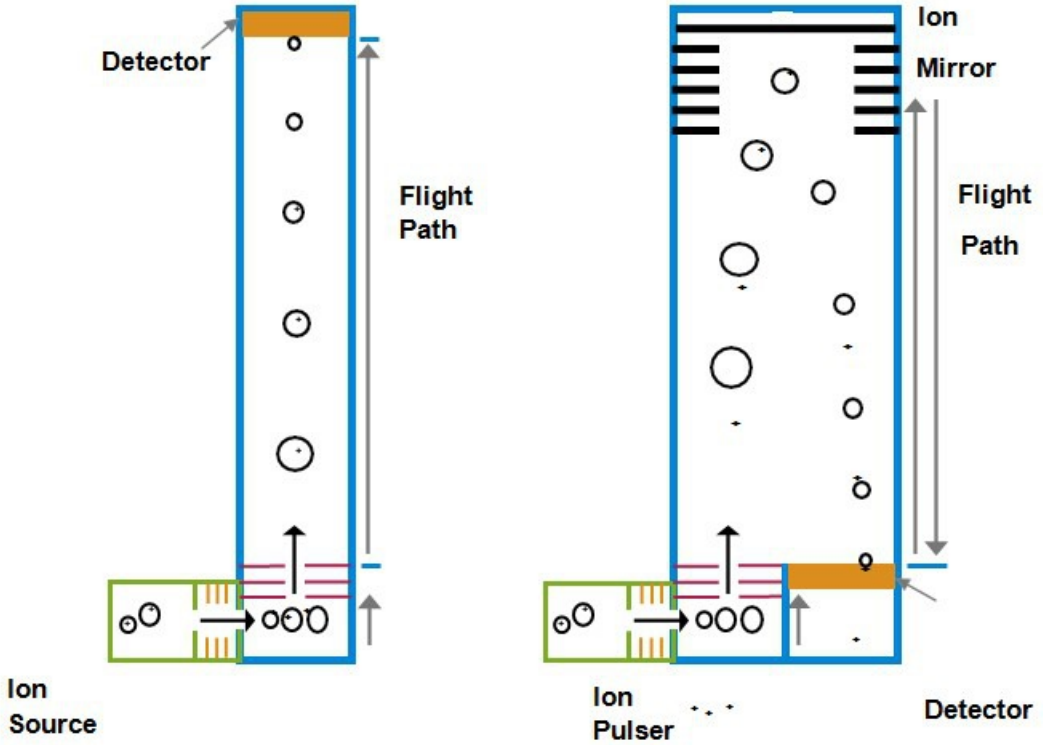

M
100
90
80
70
60
50
40
30
20
10
0
100
FWHM = ΔM
R = M/ΔM
15.01500
15.01820
15.02140
15.02460
15.02780
15.03100






Anode
Focusing Electrodes
Deflecting Plates
Power Leads
Electrostatic Lens
In-line geometry
317 cm flight path
Pulse Amplifier
Vacuum
Vacuum
Vacuum
Metal plate collector
Sample Gas Inlet
Emission Regulator
Accelerating Voltage
Pulse Generator
Horizontal Sweep
Sweep Calibrator
Oscilloscope
Oscilloscope Amplifier
High-speed Preamp


Energy Spread in the Ion Pulser Region
Initial Orthogonal Energy Spread
Pulser
Lower Energy
Higher Energy
Turn-around time
From Ion Source
Orthogonal Axis
Ion Beam Axis
Flight Tube
Pulser
Space Focus Plane
Lower Energy
Higher Energy
Higher Energy Path
Lower Energy Path
Detector
2-Stage Ion Mirror


Detector
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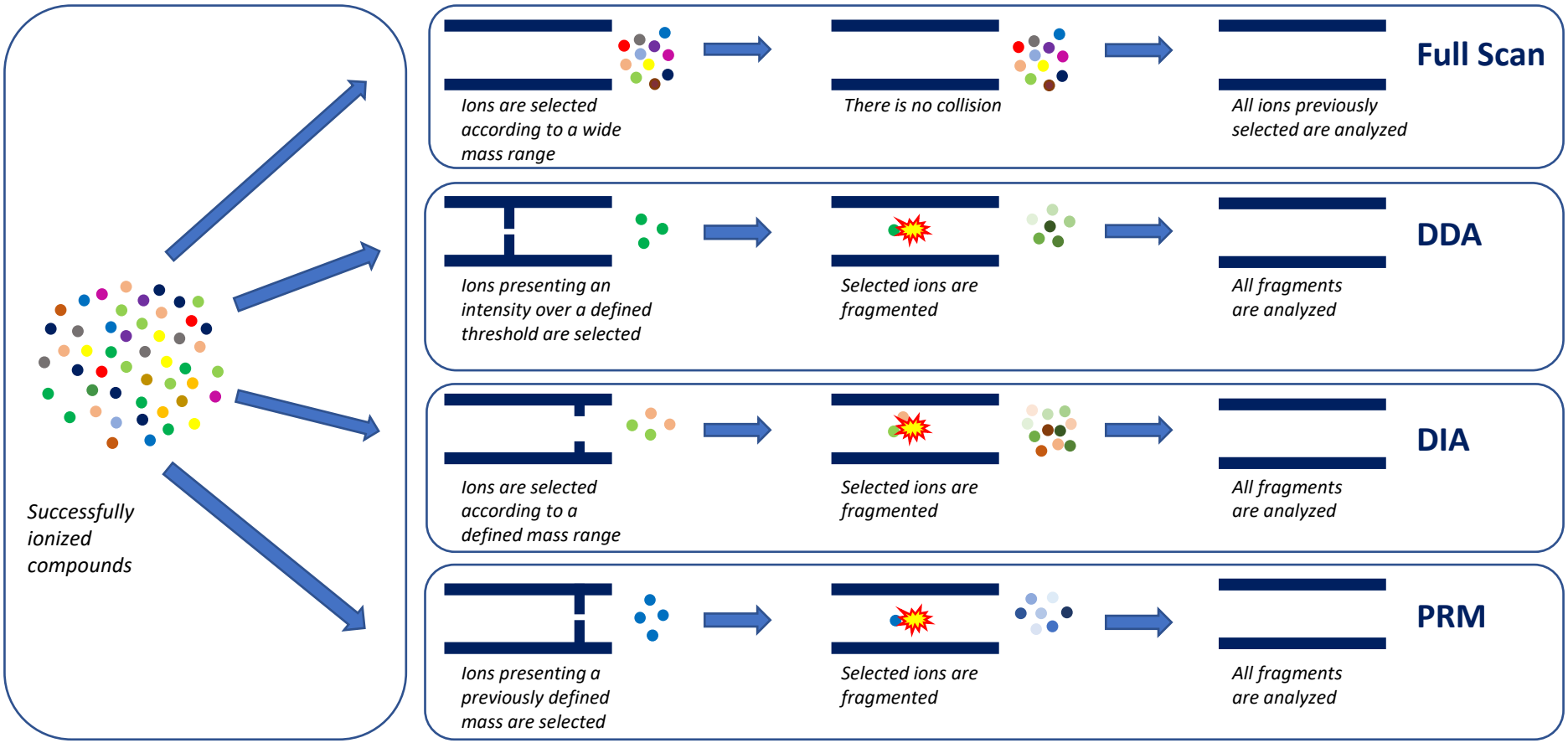
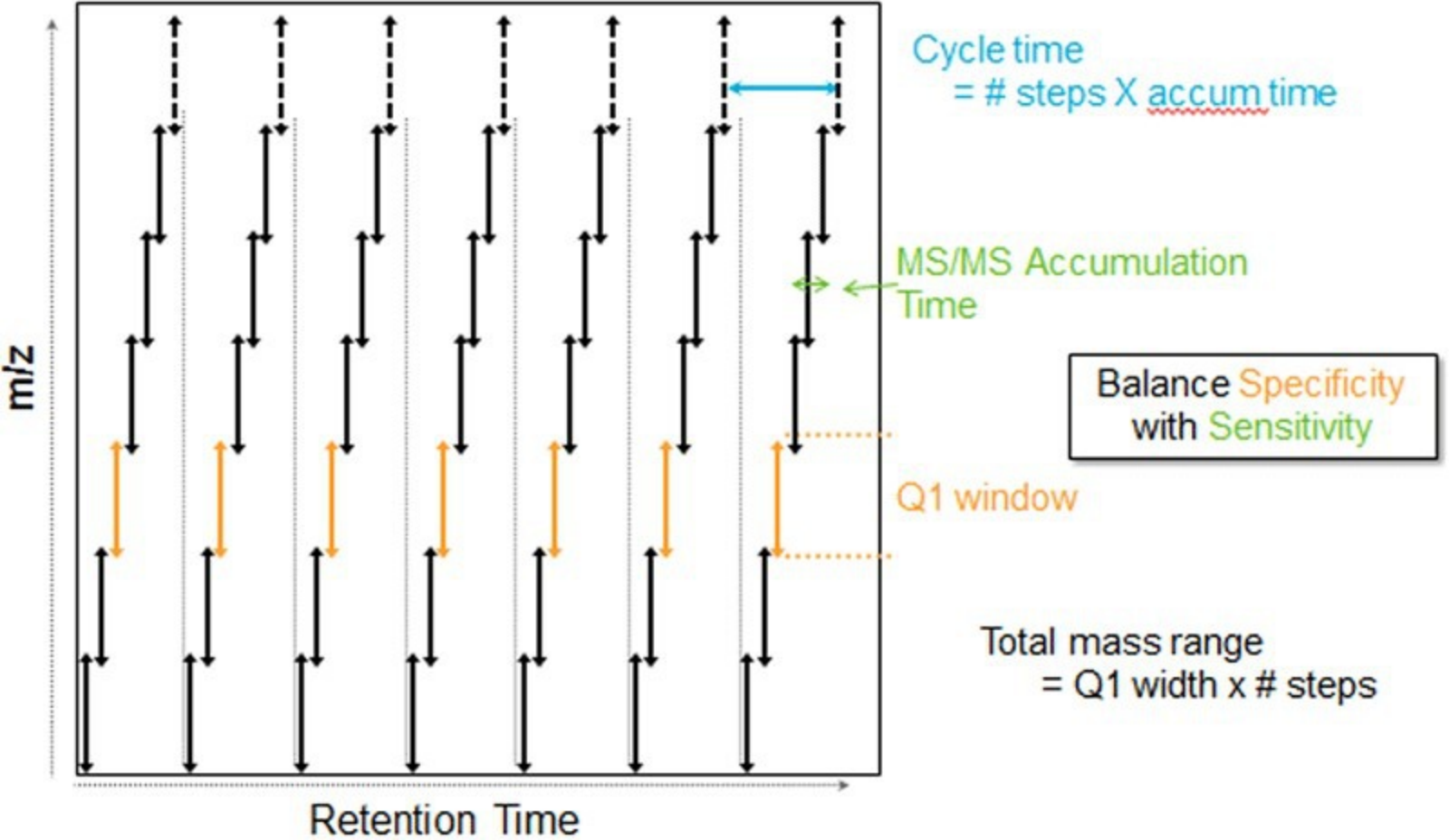
Detector


Q1
Collision cell
HRMS
Successfully ionized compounds
Ions are selected according to a wide mass range
There is no collision
All ions previously selected are analyzed
Full Scan
Ions presenting an intensity over a defined threshold are selected
Selected ions are fragmented
All fragments are analyzed
DDA
Ions are selected according to a defined mass range
Selected ions are fragmented
All fragments are analyzed
DIA
Ions presenting a previously defined mass are selected
Selected ions are fragmented
All fragments are analyzed
PRM


Cycle time
= # steps X accum time
MS/MS Accumulation Time
Balance Specificity with Sensitivity
Q1 window
Total mass range
= Q1 width x # steps
m/z
Retention Time