A Layman's Guide to High-Resolution Mass Spectrometry

Elizabeth McKenna, Priyamvada Sharma, Daniel L. McCurry, and David Hanigan

Key Takeaways

Mass spectrometry is a tool that allows researchers to identify and quantify molecules using their known mass.

The entertainment industry has made lab analysis seem easy, but identifying chemicals in a solution is very complex.

There are different types of mass spectrometry, each with variable analytical power and associated cost.

Understanding the basics of mass spectrometry helps water professionals realize the full capabilities of lab instruments and comprehend the conclusions and limitations of water research.

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With a tool like mass spectrometry, "Where's Waldo?" becomes "There's Waldo!"


KEY INSTRUMENTS
LPM AIR
10
8
6
4
2

The entertainment industry has led the public to believe that scientists are able to take a sample of anything and quickly identify specific toxicants that might be present. The popular television show *Crime Scene Investigation* includes a (darkly lit) forensics lab that quickly produces unequivocal identification of chemicals and poisons in blood or other random solutions. Until relatively recently, being able to identify an unknown compound in a complex mixture of other organic compounds was mostly a fallacy. However, the wider adoption of high-resolution mass spectrometry (HRMS) has permitted identification of unknown toxicants, bringing fiction closer to reality.

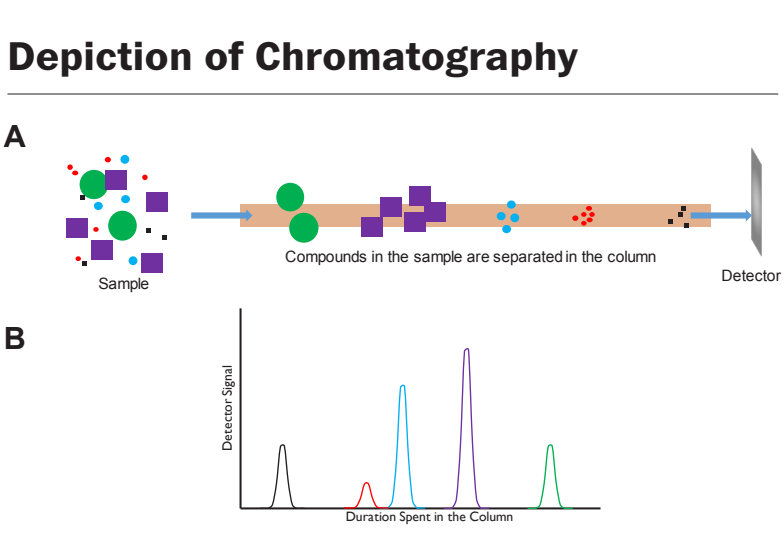
Although HRMS has been used in thousands of publications, one recent water-related example that follows the *Crime Scene Investigation* concept comes from the lab of Edward Kolodziej at the University of Washington, Seattle. Kolodziej's research group took samples of urban stormwater during rain events that resulted in coho salmon fish kills. Using HRMS, the researchers identified organic chemicals that co-occurred in storm events and runoff sources, showing that organic chemicals leaching from tire wear particles were a likely cause of the coho salmon mortality (https://doi.org/10.1021/acs.est.8b03287).

In this article, it is our goal to inform water industry practitioners—who may not be well versed in chromatography or mass spectrometry—how such forensic feats can be accomplished by the labs in their own backyards. This will help water professionals conceptualize what happens to their samples when they are sent for analysis, better understand the limitations of the data, and help troubleshoot problems with in-house instrumentation. The variety of techniques covered here will inspire others to consider the possibilities of what they can measure and think more critically about present water research.

Chromatography

Complex environmental samples are mixtures of hundreds or thousands of different chemical compounds; the goal of chromatography is to separate compounds across time to enable their separate detection. Nearly all HRMS systems first use either gas or liquid chromatography (GC or LC) to make identification simpler. In either case, the real work of chromatography occurs inside a chromatography "column," a flexible or rigid tube filled with a stationary phase. The stationary phase is composed of solid particles or film coatings with specific chemical properties that help separate chemical compounds. Figure 1, part A, shows the chromatographic separation of multiple compounds.

As the mobile phase (a liquid or gas) passes through the column, it flows through the stationary phase. The sample is injected into the mobile phase, and the chemical compounds stick to or interact with the stationary phase in which they are retained for longer or shorter periods of time based on their chemical properties. As an analogy, let's say we're looking for two specific people in New York City. It might be hard to find them among 8.6 million people, but if everyone in the city is made to run a marathon and we stand at the finish line looking for our two individuals, we'll have a much better chance of finding them because they'll be separated by their running speed and the time they have to run. In this analogy, the interaction with the mobile phase is the fitness level, and the



In part A, the sample passes through a column and is separated by its chemical properties. In part B, the black chemicals elute more quickly and thus appear first in the plot of signal versus time.

Figure 1

compounds that remain with the stationary phase longer are the slower runners who will inevitably finish the race (i.e., leave the column) later.

GC and LC are the most common chromatography techniques used for environmental applications. For GC, a sample is first heated to a vapor, and then an inert gas—usually hydrogen or helium—acts as the mobile phase, pushing the vaporized sample into a relatively low-temperature column (approximately 50 °C). Chemical compounds in the sample may stick to the stationary phase or even recondense in the column. An oven heats the column slowly, decreasing some chemicals' affinity for the stationary phase before others and causing them to separate over time by boiling point (i.e., low boiling point compounds will exit the column first).

LC uses a liquid (like water) for the mobile phase instead of a gas. For LC, a sample is injected into the mobile phase stream, which then flows through the column. Valves allow the mobile phase to be slowly changed over time, usually 5–30 minutes during which the liquid is converted from 100% water to 100% of a less-polar solvent (e.g., methanol). The less-polar solvent changes the polarity of the mobile phase and thus slowly reduces the chemical compounds' affinity for the stationary phase. This causes them to leave the column at different times roughly according to polarity (i.e., more polar compounds will exit the column first).

Sensors at the end of the column detect chemical compounds as they exit. Figure 1, part B, shows a chromatogram of different compounds eluting across time. However, going back to our analogy, what if one of the two individuals we're looking for at the marathon finishes the race with a very large group of other runners? In practice, this would be like if we shifted the black peak on top of the red peak and would require us to be able to look for the black or red or both lines together. This situation occurs when two distinct chemicals have the same elution time, and for simple detectors it can make identifying one specific chemical problematic.

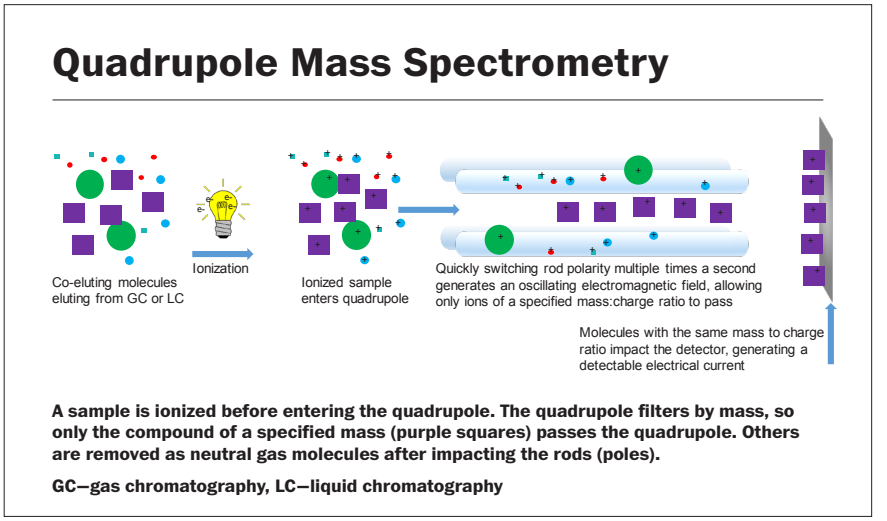


Figure 2

Quadrupole Mass Spectrometry

Running with the marathon analogy, let's say Waldo is one of the individuals we're trying to spot as he finishes the race. Waldo has a very distinctive appearance (skinny guy in red-and-white-striped shirt and hat, blue pants), yet he's difficult to find when there are many other people surrounding him. How can we differentiate him from every other runner? With enough time, we can search the post-race photos for Waldo's smiling face, which is akin to using quadrupole mass spectrometry in chromatography; however, we have only a short time to pick Waldo out from all the racers finishing at the same time.

The goal of high-resolution mass spectrometry is to measure the mass of molecules as accurately as possible.

After chromatographic separation, the mobile phase containing the now-separated chemical compounds is directed to a mass spectrometer. The use of a quadrupole mass spectrometer in tandem with chromatography is important in the water industry (and fairly common) because there are many compounds present in a water sample. The addition of a mass spectrometer helps identify low concentration compounds. The sample is ionized in the mass spectrometer, imparting an electric charge and

the charged molecules then enter a quadrupole, which selectively allows molecules through. A quadrupole is composed of four parallel rods (Figure 2) in a vacuum, which removes air and other interfering molecules that might be present. Electrical current is applied to the rods, producing four electromagnetic fields.

When the ionized molecule enters the quadrupole, it is repelled by like charges and attracted by opposite ones. The polarity of the rods is oscillated by reversing the current many times per second and thus an ion is pushed back and forth between the rods as it travels through them. Carefully tuning the oscillating electromagnetic fields ensures that only molecules of a specific mass-to-charge ratio (m/z) will make it the length of the four parallel rods without touching them. Molecules that are heavier or lighter than the target molecule are pushed harder or not hard enough to maintain a central path through the rods, so they impact the rods. Molecules that touch a rod gain or lose electrons from the rods themselves; first they lose their charge, then they are removed by the vacuum pump. Molecules that make it through without deviating from the central path impact the detector at the end of the quadrupole, which produces an electrical signal that is recorded by the computer. Importantly, the detector produces an electrical signal via the exchange of electrons with the ionized molecule for any ion that impacts it, so the mass filtering of the quadrupole is essential for discriminating the target molecules (that follow the central path) from other molecules (that impact the rods and are removed).

Referring back to the marathon analogy, quadrupole mass spectrometry (GC/MS or LC/MS) is like having Waldo tell us his weight before the race. To find him, we'll weigh all the runners as they cross the finish line. Anyone who doesn't have the same weight as Waldo will be directed to a special waiting area, and any runners with the same weight as Waldo will be pushed across the finish line; this gives us a much better chance of finding Waldo.

Until this point, we've used the analogy of a marathon, which has probable finishing times on the order of hours—but what if we make the timeline much shorter? A typical chromatography analysis, for example, is completed in less than 30 minutes, so it's conceivable that with millions of runners (or molecules), many will have the same weight as Waldo does and will finish at close enough to the same time that it's nearly impossible to find Waldo. We need another filter.

Triple Quadrupole Mass Spectrometry

Unfortunately, the next analogy is not very nice to Waldo. A quadrupole filters chemical species by mass, but there are many molecules with the same mass: for example, water (H_2O = 16 + 1 + 1 = 18 atomic mass unit [amu]) and an ammonium ion (NH_4 = 14 + 1 + 1 + 1 + 1 = 18 amu). Triple quadrupole mass spectrometry takes advantage of three quadrupoles in a row to further separate compounds of interest from other organic compounds in a sample. The first quadrupole filters the species present by mass with an

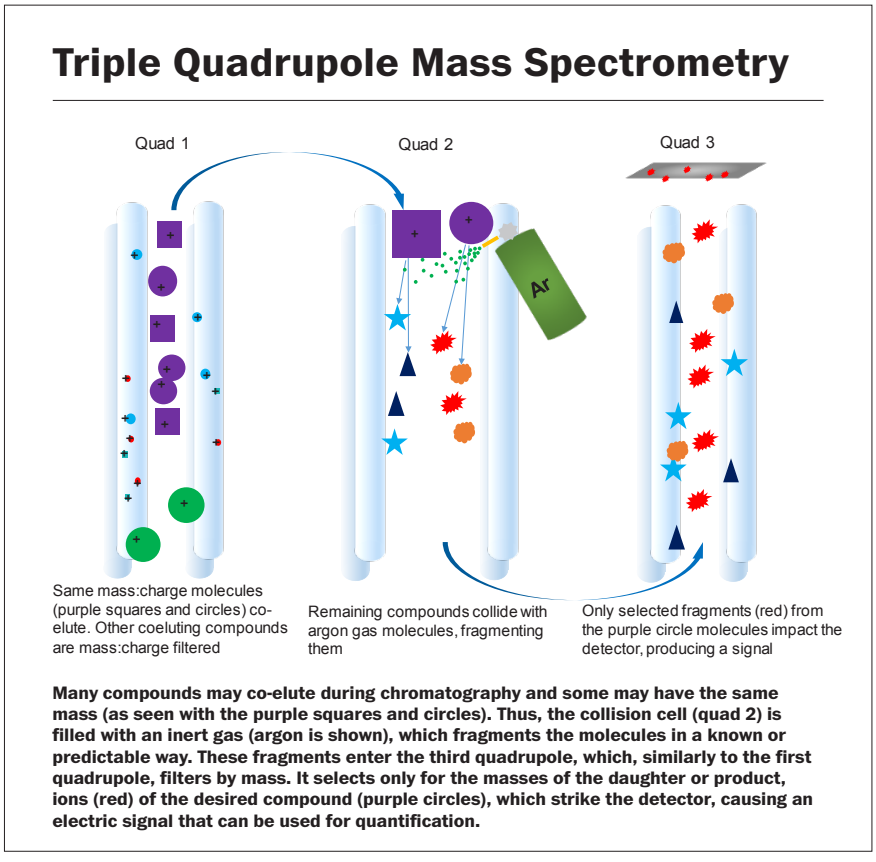


Figure 3

electromagnetic field, as described in the previous section. However, instead of then going into the sensor, the sample passes into a second quadrupole (called a collision cell) that is filled with an inert gas such as argon or nitrogen. The analyte molecules (or the parent ions) collide at high speed with the inert gas molecules, causing them to fragment in a predictable pattern specific to the analyte molecule. The fragments (or product ions) then enter a third quadrupole that filters out the fragments except those that are known to have come from the parent molecule of interest (Figure 3). If the correct fragment is detected at the right time, it can be known with relative certainty that the molecule is the target molecule, despite the thousands of other molecules in the sample.

Let's imagine Waldo finishes the race (chromatography) at a similar time as may other runners. He stands on a scale (first quadrupole), which selects him and a few others that finished the race at the same time and have the same weight to begin a bike race. During the bike race, he rides into a pole, which causes his shoes and hat to fly off (argon gas colliding with the parent ion in the collision cell). Other contestants also ride into poles, but they're wearing different hats and shoes. Race organizers clean up all the hats and shoes (third quadrupole), leaving only the hat and shoes that belong to Waldo. Through these multiple filtering mechanisms—time, mass, and fragment mass—specific molecules can be unequivocally identified and quantified in extremely complex mixtures.

In the analogy so far, if Waldo hadn't told us his weight or mentioned the color and appearance of his shoes, or if we had never met Waldo, or if we didn't even know Waldo was running the race, the mechanisms described thus far for finding him would be useless. This isn't entirely true for quadrupole mass spectrometry, however, as instruments may be operated in "scan mode," in which the quadrupoles scan through one mass at a time looking for compounds of that mass. This is a full topic on its own, but in short, these methods result in poor sensitivity (high detection limits) and can't unequivocally identify molecules in the mass range a lab might be interested in.

HRMS

The goal of HRMS is to measure the mass of molecules as accurately as possible (i.e., as closely as possible to their known exact mass, equal to the sum of the exact masses of the elements that make up the molecule). If mass is measured accurately enough, it can be used to obtain the elemental composition of a molecule, and from that, a structure can be proposed and confirmed. Determining the elemental composition of a molecule is the first step in identifying the molecule and has been used recently to identify a slew of human-produced chemicals such as pharmaceuticals in wastewater, drinking water, and the environment. This is sometimes called "nontarget" mass spectrometry, where the molecules of interest are not known from the beginning, and instead the scientists or engineers attempt to identify as many molecules in the sample as possible.

To better understand HRMS, let's consider something less abstract. If we have US$6 in bills in our wallet, we would have either six $1 bills, or a $5 and a $1 bill. However, if we have $1,000 in our wallet, the combination of potential bills is much greater, but there is still a finite number of possibilities of bills in our wallet that can be relatively easily determined (10 $100 bills, 20 $50 bills, etc.)

Going back to HRMS, assume the mass of an unknown molecule is measured to be 67.10 amu (it's notable that this is a nominal mass, not an "exact mass," but it is valuable as an example and likely the best measurement that could be achieved from a quadrupole instrument, which is not a high-resolution instrument). We can think of this as the total value of all the bills in a wallet. If it is assumed that only carbon, hydrogen, nitrogen, oxygen, and sulfur are present, which is a reasonable assumption for organic molecules, a system of equations can be used to solve for the summed masses of the variables C, H, N, O, and S (simplified to Eq 1) by using their nominal masses of 12.01, 1.01, 14.01, 16.00, and 32.06 amu, respectively (similar to the sum of the $100s, $50s, $20s, $10s, and $1s). Solving for each of these elements is equivalent to figuring out the number of each type of bill that are in our wallet.

$$12.01\ \mathrm{C} + 1.01\ \mathrm{H} + 14.01\ \mathrm{N} + 16.00\ \mathrm{O} + 32.06\ \mathrm{S} = 67.10\ \mathrm{amu} \quad (1)$$

Since the number of atoms (variables C, H, N, O, and S in Eq 1) must be integers (i.e., no half atoms or half $50 bills), there is only one solution to this equation: four carbons, five hydrogens, one nitrogen, and no oxygen or sulfur (C_4H_5N). From the atomic composition, this compound could be assumed to be pyrrole, although it could also be another compound with the same formula (e.g., allyl cyanide), depending on the structure. For small enough molecules, the atomic composition can be determined using a nominal mass measurement from an instrument such as a quadrupole mass spectrometer, and without highly accurate measurements of mass, although typically one cannot jump from atomic composition to structure.

For larger molecules, the number of possibilities increases exponentially because there are many ways to substitute elements with the same summed nominal

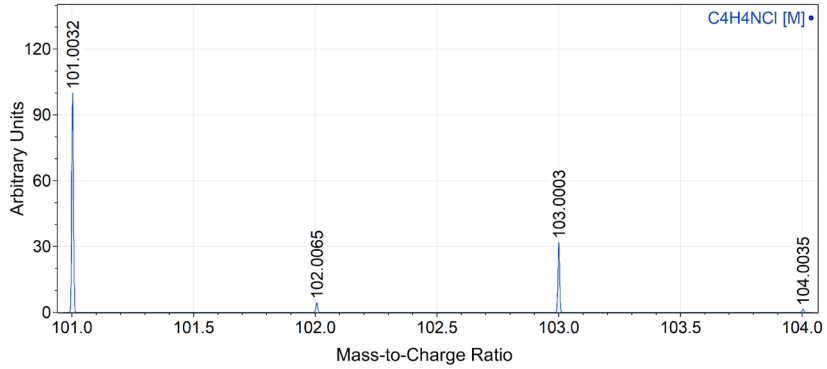
mass, similar to if we have more and more cash in our wallet and we know only the total, the possibilities of bills that are present becomes exponentially greater. To explain how HRMS instruments resolve this issue, we must first explain that isotopes are forms of the same element with the same number of protons but different numbers of neutrons—meaning, they have different atomic mass but the same chemical properties. On the periodic table of the elements, the atomic masses are averages of the atoms' isotopes, weighted by their environmental abundance. For example, chlorine occurs naturally as the principal isotopes of ^{35}Cl and ^{37}Cl, where 75.8% of chlorine occurs as ^{35}Cl and 24.2% as ^{37}Cl. With masses of 34.9689 and 36.9659 amu, respectively, the abundance-weighted average of the two is 35.45 amu, which is found on the periodic table. For molecules, the summed masses listed on the periodic table (i.e., nominal mass) of the individual elements that make up the molecule can be used with a low-resolution instrument (e.g., quadrupole), but this approach isn't very useful because the number of compounds that can be identified using nominal mass is very small and limited to low-molecular-weight compounds. Most molecules of interest in our field are considered low molecular weight by the general field of chemistry, but unfortunately, not low enough for quadrupole instruments to be helpful in identifying them.

For a high-resolution instrument, it's important to consider that a compound will exist with a distribution of its natural isotopes. For example, consider C_4H_4NCl, which has a mass of 101.0032 amu if only the isotopes of highest environmental abundance are present (^{14}N, ^{12}C, ^{1}H, and ^{35}Cl). With a high-resolution instrument, we can expect to detect this compound at this mass, assuming no other atoms become attached during ionization in the instrument source (this is a poor assumption but allows for the most simplistic example). Similar to Eq 1, we may solve for the elemental composition given that the number of each element must be an integer, except with incorporation of the more accurate masses of the highest abundance isotopes rather than nominal masses (Eq 2).

$$12.0000\ C + 1.0078\ H + 14.0031\ N + 15.9949\ O + 34.9689\ Cl = 101.0032\ amu \quad (2)$$

As the measurement of mass accuracy increases, it becomes very difficult to substitute one element for multiple other elements and still arrive at the same mass, making HRMS a powerful tool for determining the elemental composition of a compound. Although these calculations can be conducted by hand, it becomes quite laborious as the number of potential elements increases, and therefore modern HRMS instrument software includes these calculations.

In nature, some molecules will inevitably contain ^{37}Cl rather than ^{35}Cl, so a signal at 103.0003 amu of 0.31 times the intensity of the 101.0032 amu molecule (i.e., ratio of the natural abundance of the isotope, 0.76/0.24) can also be expected. This becomes important when interpreting mass spectra, where observing one peak and a co-eluting peak with mass 1.9970 amu greater and intensity of approximately ⅓ the larger peak may suggest a chlorinated compound is present. Similar principles apply for other isotopes, which can also be used for molecular fingerprinting and provide some information to the true elemental composition, but for brevity, other isotopic fingerprints are not discussed here. Figure 4 shows the expected mass spectrum for chlorinated pyrrole, incorporating other possible substitutions of N, C, and H isotopes.



The first peak, 101.0032 amu, contains the highest abundance isotopes of C, H, N, and Cl. The peak at 103.0003 amu represents the same compound, but with the less abundant ^{37}Cl isotope. Other peaks represent combinations of various lower natural abundance isotopic combinations of ^{13}C, ^{2}H, ^{15}N. Figure generated with nMass v5.5.0 (https://doi.org/10.1021/ac100818g).

Figure 4

Again, modern HRMS instrument software incorporates these molecular fingerprinting techniques.

Thus, for high-accuracy instruments (HRMS instruments), measurement of mass can be used to find the elemental composition of a molecule, and from that a structure can be proposed. This informed guess is then typically confirmed using high-purity analytical standards based on chromatographic retention time and isotopic fingerprinting. The following sections describe three types of instruments (time-of-flight, Fourier-transform ion cyclotron resonance mass spectrometry, and Orbitrap) that can take measurements of mass accurately enough to perform calculations similar to Eq 2.

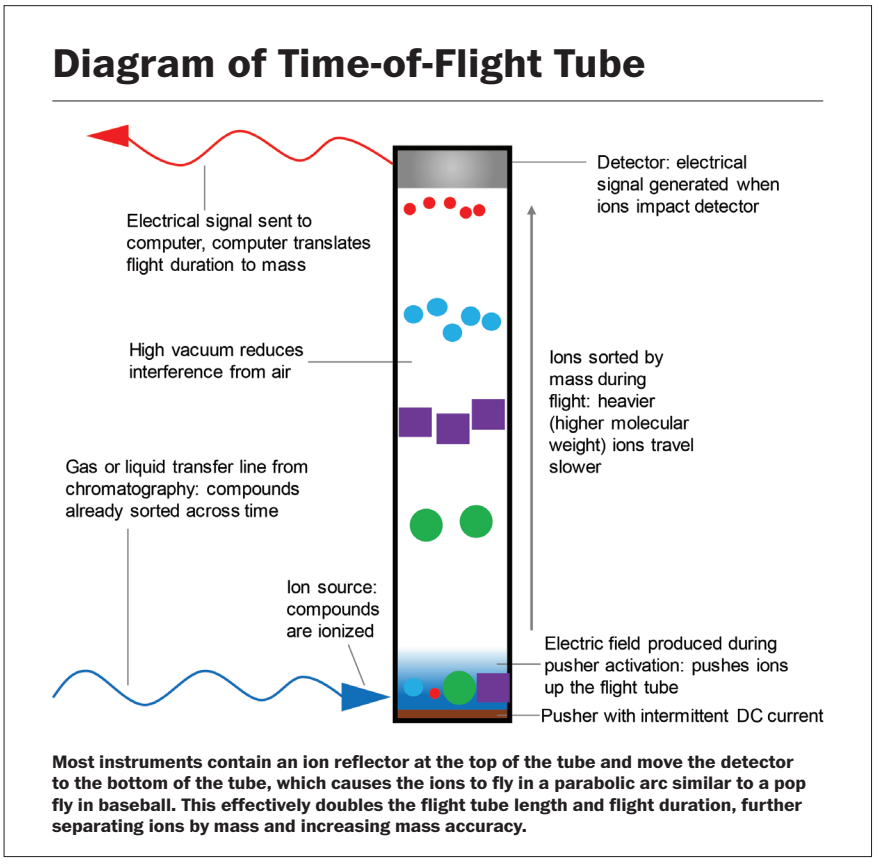


Figure 5

Time of Flight

Time-of-flight (TOF) instruments are similar to their quadrupole counterparts in that they operate under high vacuum to remove interfering molecules and an indiscriminate electrical signal is produced by any ion that impacts the detector. The source, or the part of the instrument that turns molecules into ions, is also similar, as is the chromatograph, either liquid or gas. Different, however, is that while quadrupole instruments function on the principle of using electromagnetic fields to remove ions that are of no interest, TOF instruments send all ions to the detector. Ions are pushed up a flight tube by an intermittent electromagnetic pulse (on, off, on, off) at a rate of thousands of times per second. Ions enter the vacuum space above the "pusher," and upon activation of the pusher (i.e., generation of an electrical field), are repelled up the flight tube. Larger (heavier, or greater mass) ions travel more slowly and thus impact the detector later than lighter ions. TOF instruments measure the time from pusher activation to ion impact extremely accurately, and then translate, through a calibrant of known mass, flight duration into mass.

Because multiple ions of varying mass are pushed up the flight tube, multiple impacts are recorded with various flight durations, which are then translated into mass. In other words, no ions are intentionally filtered out, and the mass spectra of all ions entering the mass spectrometer are captured with high sensitivity. This comes with the additional benefit that flight duration is simple to measure very accurately, and thus measurement of mass is very accurate (good approximation of the fourth decimal place for compounds in the 50–1,000 amu range). A diagram of a TOF mass spectrometer flight tube is shown in Figure 5.

In very simple samples with few molecular solutes present, it may be possible to completely skip chromatography and directly inject the sample into the TOF mass spectrometer. All ions are detected, and so all compounds are likely to be shown in the mass spectrum, given that they ionize well (i.e., if Waldo tells us his weight accurately enough, there's no need to have anyone run the race; we can simply make everyone stand on very accurate scales). Of course, if the sample contains two compounds with the same elemental composition (e.g., pyrrole and allyl cyanide are both C_4H_5N), this method isn't appropriate and requires other fingerprinting techniques or chromatography to deconvolute the separate molecules.

With the accurate masses of many compounds, structures may be proposed and again confirmed using analytical standards. Some TOF instruments also incorporate a quadrupole in front of the flight tube to intentionally fragment an unidentified compound, and the fragments can be used to confirm the structure of the proposed compound or verified against an external analytical standard.

TOF instruments are popular because they are relatively inexpensive compared with other high-resolution instruments; an LC/qTOF mass spectrometer that can identify environmental unknowns generally costs around $350,000. However, there are limitations; for example, if we want to know how much of a specific molecule is present but are not interested in other dissolved compounds, TOF instruments are less sensitive for targeted quantification than quadrupole instruments because the intermittent electromagnetic pusher field results in some ions being lost during the flight of other ions. Also, like many mass spectrometers, TOF instruments are quite large and cumbersome, with flight tubes extending approximately 1 meter above the instrument itself. This requires adequate bench space and ceiling clearance, in addition to gas requirements and vacuum pump exhaust.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

Another limitation of TOF-based mass spectrometry for identification of unknowns is that mass resolutions are typically on the order of 25,000–50,000, which may not be high enough to unambiguously assign a molecular formula to a peak (particularly for higher-mass compounds) or to resolve two very closely spaced peaks. For instance, two peaks at m/z of 1,000.01 and 1,000.02 would require mass resolution of 100,000 to discern the two. After initial rapid gains in mass resolution by TOF instrument technology (e.g., extending TOF path length by adding reflectors to the top of the flight tube to increase effective length), improvements to mass resolution have been incremental.

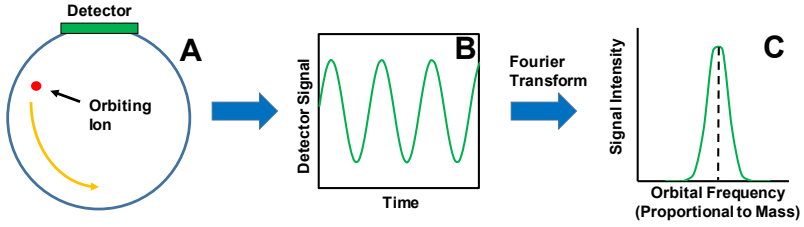
To dramatically increase mass resolution, an entirely different approach is used for mass separation: ions are electromagnetically trapped in a circular chamber under a high magnetic field (typically >5 tesla) in an instrument called a Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer. In this situation, ions orbit in an electromagnetic field at a frequency proportional to the field strength and inversely proportional to their mass (Eq 3).

$$f = qB/2\pi m \quad (3)$$

where q is the charge of the ion (typically 1 elementary charge = 1.6×10^{-19} C), B is the magnetic field strength in tesla ($T = kg/C \times s$), and m is the ion mass (in kilograms).

The movement of the orbiting ion produces a small electromagnetic field, which is measured at a location in the orbit such that each pass of the ion is detected as oscillating electrical current. This oscillating electrical signal is then mathematically transformed via a Fourier transform into a series of peaks corresponding to different orbital frequencies, which each corresponds to a unique mass. Calibration with mass standards allows an assignment of the mass corresponding to each orbital frequency (Figure 6).

Because the frequency of ion orbit can be measured very accurately, FT-ICR instruments can achieve extremely high mass resolution (>1,000,000 or the fifth and even approximations of the sixth decimal place in compounds with mass between 50 and 1,000 amu), allowing unambiguous determinations of atomic composition for each peak and avoiding peak overlap issues common with TOF-based instruments. However, unlike TOF instruments, FT-ICR instruments are prohibitively



Part A shows a schematic of an ion orbiting in an FT-ICR instrument, part B shows the output signal from FT-ICR instrument, and part C shows the Fourier-transformed output signal with peak corresponding to a unique mass.

Figure 6

expensive to own and operate for single investigator labs or even most universities, so they're typically housed at national laboratories or specialized research institutes (e.g., the National High Magnetic Field Laboratory in Tallahassee, Fla.).

Orbitrap

As a compromise between the accessibility of TOF instruments and the mass resolution of FT-ICR instruments, Orbitrap instruments have recently become popular. Orbitrap instruments are miniaturized FT-ICR mass spectrometers that fit on a benchtop and can be obtained for between $500,000 and $1,000,000, opening up accessibility for academic departments or even certain individual investigators. The first Orbitrap instrument, the LTQ Orbitrap, was introduced by Thermo Scientific in 2005, and successive models have refined the technology. Typical commercial Orbitraps can achieve mass resolutions of 240,000 (approximately the fifth decimal place for environmental applications), which is usually high enough to resolve all mass peaks for small molecules, and usually feature a quadrupole in front of the Orbitrap detector to allow intentional fragmentation.

However, there are tradeoffs for high mass resolution, and the Orbitrap has not completely dominated the HRMS market. Most obviously, because Thermo Scientific holds the exclusive patent on parts of the Orbitrap technology, the prices remain significantly higher than for TOF instruments, which are produced by several vendors. Technologically, the Orbitrap is not without disadvantages. The highest mass resolutions are only achievable with relatively long scan times, extending the run time per sample, so Orbitraps are typically operated at lower than maximum resolution (e.g., 70,000—only slightly better than a typical TOF instrument) to increase throughput. Resolution may then be adjusted in a second injection of the sample if peaks overlapped in the first trial. Additionally, all ion trap instruments can suffer from the space charge effect, in which the trap becomes too full and the ions begin to repel one another (as a result of having the same polarity charge), resulting in reduced mass accuracy. For these reasons, TOF instruments remain popular despite their lower resolution.

Hopefully by now, the notion of television sci-fi chemical analysis has met reality, and Waldo is seeking treatment for his bicycle accident. Though this article is simply an introduction for people interested in learning more about how mass spectrometry works, it should help individuals working in the water industry understand the complexity involved in chemical analysis and what is involved to determine a low-concentration pollutant's presence and structure in a water sample. It should help water professionals understand what's going on "under the hood" in their own analytical instruments, allowing them to better troubleshoot instrumentation issues and understand the full capabilities of the tools in their labs. Finally, understanding these analytical techniques should help water professionals better evaluate the work of others and think more critically about water research.

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About the Authors

Elizabeth McKenna is a master's student at the University of Nevada, Reno, Nev.

Priyamvada Sharma is a PhD student at the University of Nevada.

Daniel L. McCurry is an assistant professor at the University of Southern California, Los Angeles, Calif.

David Hanigan is an assistant professor at the University of Nevada, Reno, Nev.; dhanigan@unr.edu.

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