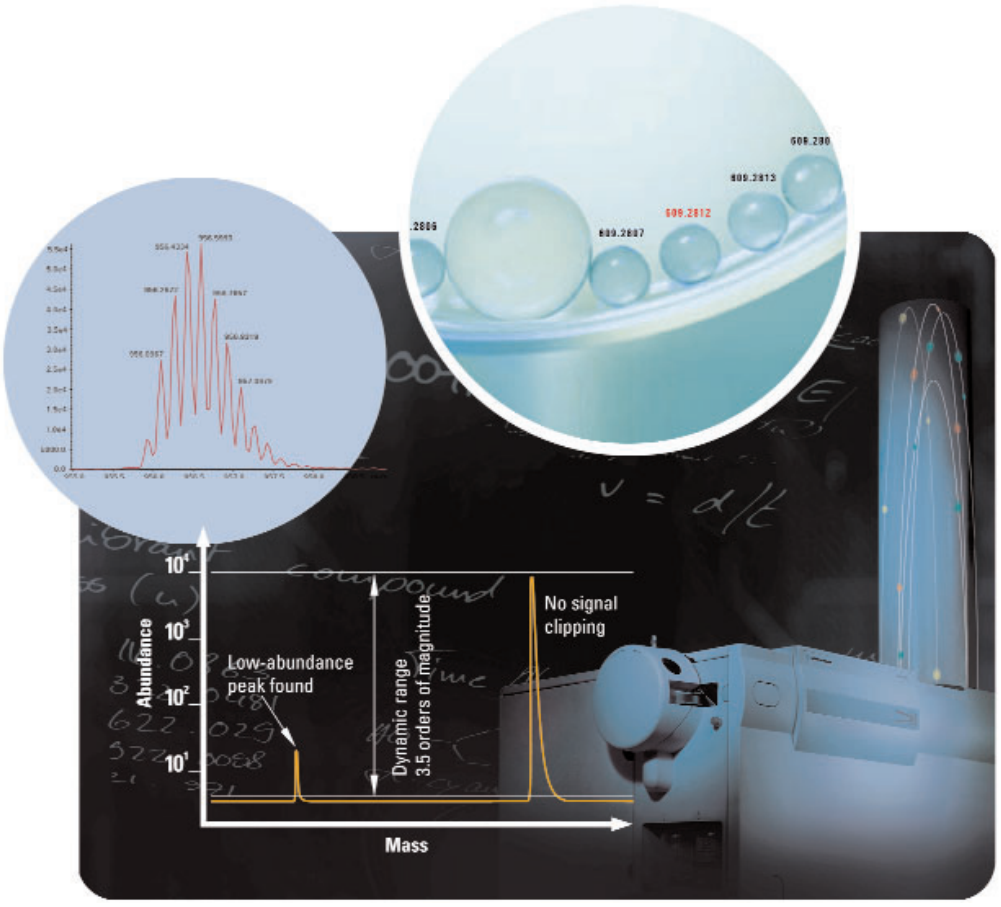
Time-of-flight solutions in pharmaceutical development – the power of accurate mass

Application Compendium



Agilent Technologies

Contents

Technique-related notes

Time-of-flight mass spectrometry 4

Effect of resolution and mass accuracy on empirical formula confirmation and identification of unknowns 14

Advantages of wide dynamic range on an orthogonal acceleration time-of-flight mass spectrometer 16

Accurate mass measurement for analyzing drugs of abuse by LC/time-of-flight mass spectrometry 20

Application-related notes

Using the Agilent LC/MSD TOF to identify unknown compounds 22

Automated empirical formula confirmation using the Agilent LC/MSD TOF 26

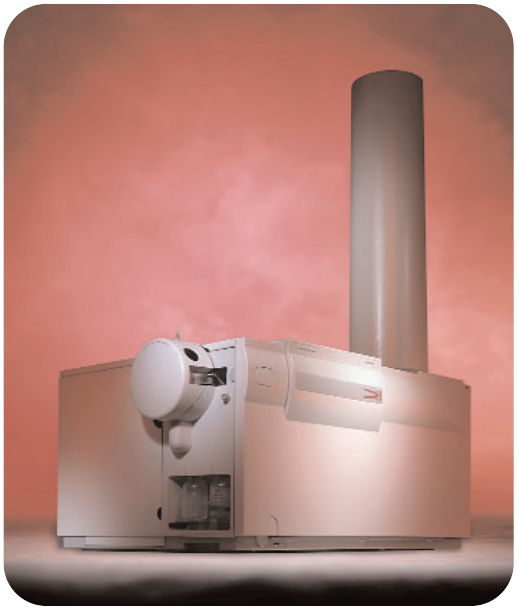
Using accurate mass LC/MS in a walk-up environment 28

Automated empirical formula generation for the identification of unknown compounds 30

Screening for target compounds using the LC/MSD TOF 32

Fast scanning for high throughput screening using the LC/MSD TOF 34

Structure elucidation of degradation products from the antibiotic drug amoxicillin by accurate mass measurement using LC-ESI-oaTOF MS 36



Foreword

This compendium is a collection of examples and applications based on the Agilent LC/MSD time-of-flight mass spectrometer. It focuses on the power of accurate mass in doing both qualitative and quantitative applications. Because this instrument makes obtaining accurate mass measurements routine, the LC/MSD TOF has many uses in the drug development laboratory as well as other areas.

This compendium provides an overview of Agilent's Application Notes and Technical Notes, currently available for using API time-of-flight-mass spectrometry. Most of the notes are presented in a condensed form, for more in-depth reading the full length versions can be downloaded from Agilent's web site at **www.agilent.com/chem/tof**.

Advantages of the LC/MSD TOF

- Routine mass accuracy of 3 ppm or better over a broad dynamic range to allow making accurate mass measurements without requiring special training or careful sample preparation.
- Full scan sensitivity at the low picogram level enabling the identification of unexpected trace contaminants.
- Linear response over three orders of magnitude dynamic range combined with high resolving power which allows for quantification of samples in complex matrices.
- A wide range of sources available including electrospray (ESI), nanoelectrospray, atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), matrix assisted laser desorbtion ionization (MALDI).
- Sample introduction by either HPLC or capillary electrophoresis.

Time-of-flight mass spectrometry

Time-of-flight mass spectrometry (TOF MS) was developed in the late 1940's, but until the 1990's its popularity was limited. Recent improvements in TOF technology, including orthogonal acceleration, ion mirrors (reflectrons), and high-speed electronics, have significantly improved TOF resolution. This improved resolution, combined with powerful and easy-to-use electrospray (ESI) and matrix-assisted laser desorption ionization (MALDI) ion sources, have made TOF MS a core technology for the analysis of both small and large molecules.

This overview describes:

- Basic theory of operation for an orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer
- Flight time and the fundamental equations for TOF mass analysis
- TOF measurement cycle
- Relative advantages of the two most common TOF digitizers – analog-to-digital converter (ADC) and time-to-digital converter (TDC)
- Theoretical and practical limits to mass accuracy
- Dynamic range considerations

Basic oa-TOF MS theory of operation

While an orthogonal acceleration time-of-flight mass spectrometer can be interfaced with many types of ion sources, this discussion will focus on the use of an oa-TOF MS with atmospheric pressure ionization (API) sources. There are several types of API sources that can be used, including:

- Electrospray ionization (ESI) at various flow rates
- Atmospheric pressure chemical ionization (APCI)
- Atmospheric pressure photoionization (APPI)
- Atmospheric pressure matrix-assisted laser desorption ionization (AP-MALDI)

Ions from these sources can be introduced into the mass spectrometer vacuum system via a common atmospheric sampling interface.

Figure 1 depicts the Agilent LC/MSD TOF, an oa-TOF mass spectrometer. Ions produced in the source are electrostatically drawn through heated drying gas and then through a sampling capillary into the first stage of the vacuum system. Near the exit of the capillary is a metal skimmer with a small hole. Heavier ions with greater momentum pass through the skimmer aperture. Most of the lighter drying gas (nitrogen) molecules are pumped away by a vacuum pump.

The ions that pass through the skimmer and enter the second stage of the vacuum system are immediately focused by the first of two octopole ion guides. An octopole ion guide is a set of small parallel metal rods with a common open axis through which the ions can pass. Radio frequency (RF) voltage applied to the rods creates electromagnetic fields that confine ions above a particular mass to the open center of the rod set. The ions are propelled through this first octopole ion guide by the momentum retained from being drawn from atmospheric pressure through the sampling capillary. As the ions transit the first octopole, they also pass into the third stage of the vacuum system, where the pressure is now low enough that there are few collisions between the ions and gas molecules.

Ions exiting the first octopole ion guide immediately enter the second octopole ion guide in the fourth vacuum stage. The second octopole ion guide is similar to the first, but carries a lower direct current (DC) potential. It accelerates the ions. The second ion guide is driven by an RF power amplifier operated at 5 MHz. The high 5 MHz frequency is key to achieving maximum ion transmission over a wide ($> m/z$ 100–m/z 3000) mass range In the fourth vacuum stage, the ion beam leaves the second octopole ion guide and enters the beam-shaping optics. An ion focus lens and DC quadrupole shape the

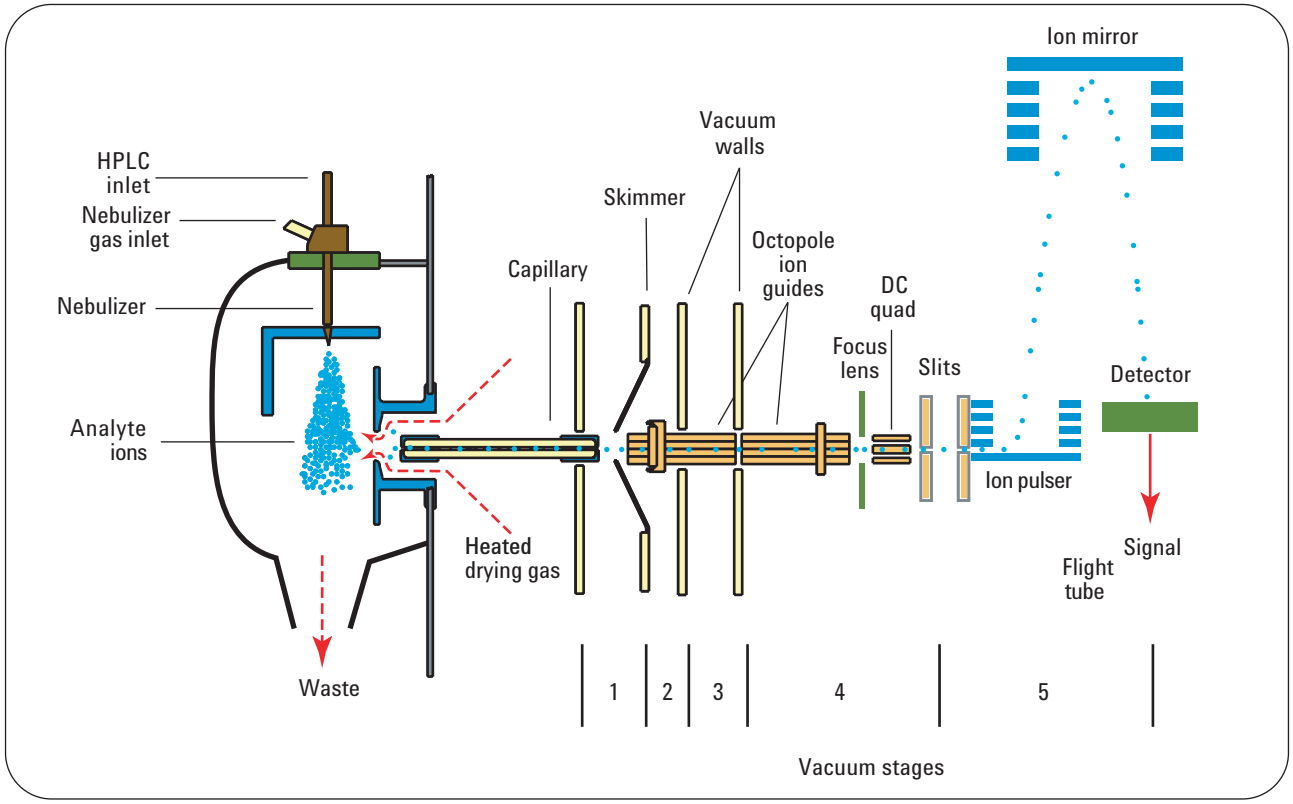


Figure 1
Ion source, ion optics, and mass filter from the Agilent LC/MSD TOF, an API oa-TOF mass spectrometer.

beam to achieve optimal parallelism and size before it enters the time-of-flight mass analyzer. The more parallel the ion beam, the higher the resolving power that can be achieved.

After the ions have been shaped into a parallel beam, they pass through a pair of slits into the fifth and last vacuum stage, where the time-of-flight mass analysis takes place. Because the mass of each ion is assigned based on its flight time, the background gas pressure in this stage must be very low. Any collision of an ion with residual background molecules will alter the flight time of the ion and affect the accuracy of its mass assignment.

In the time-of-flight mass analyzer, the nearly parallel beam of ions first passes into the ion pulser. The pulser is a stack of plates, each (except the back plate) with a center hole. The ions pass into this stack from the side just between the back plate and the first plate.

To start the ion's flight to the detector, a high-voltage (HV) pulse is applied to the back plate. This accelerates the ions through the stack of pulser plates.

The ions leave the ion pulser and travel through the flight tube, which is about one meter in length. At the opposite end of the flight tube is a two-stage, electrostatic ion "mirror" that reverses the direction of the ions back towards the ion pulser. The two-stage mirror has two distinct potential gradients, one in the beginning section and one deeper in the mirror. This improves second-order time focusing of the ions on the detector. Because ions enter the ion pulser with a certain amount of horizontal momentum, they continue to move horizontally as well as vertically during their flight. Thus, they are not reflected directly back to the ion pulsar, but instead arrive at the detector.

Figure 2 shows a schematic of the detector. The first stage of the detector is a microchannel plate (MCP), a thin plate perforated by many precise microscopic tubes (channels). When an ion with sufficient energy hits the MCP, one or more electrons are freed. Each microchannel acts as an electron multiplier. By the time the electrons exit the MCP, there are roughly ten electrons for every incoming ion. The electrons exiting the MCP are accelerated onto a scintillator that, when struck by the electrons, emits photons. The photons from the scintillator are focused through optical lenses onto a photomultiplier tube (PMT), which amplifies the number of photons and then produces a electrical signal proportional to the number of photons. The reason for this conversion of an electrical signal to an optical signal and back to an electrical signal is to electrically isolate the flight tube and the front of the detector, which are at roughly –6,500 volts, from the PMT, whose signal output is at ground potential.

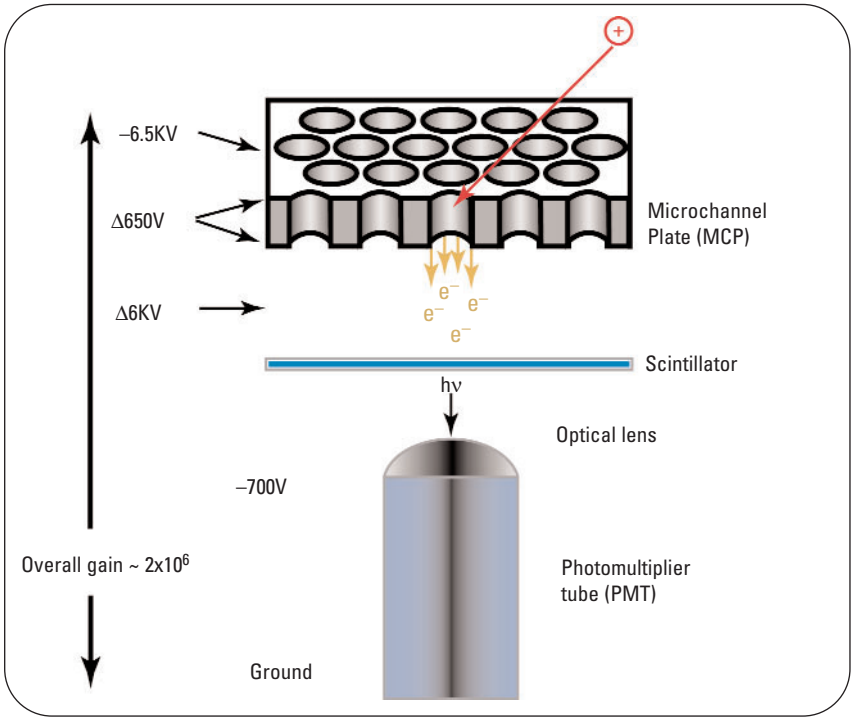


Figure 2
TOF detector with potentials shown for positive ion operation.

Flight time and its relationship to mass

Equations for time-of-flight

The flight time for each mass is unique. It starts when a high voltage pulse is applied to the back plate of the ion pulser and ends when when the ion strikes the detector. The flight time (t) is determined 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:

$E = {}^{1}/_{2}mv^2$

which solved for m looks like:

$m = 2E/v^2$

and solved for v looks like:

$v = -(2E/m)$

The equation says that for a given kinetic energy, E, smaller masses will have larger velocities, and larger masses will have smaller velocities. That is exactly what takes place in the time-of-flight mass spectrometer. Ions with lower masses arrive at the detector earlier, as shown in figure 3. Instead of measuring velocity, it is much easier to measure the time it takes an ion to reach the detector.

The second equation is the familiar velocity (v) equals distance (d) divide by time (t):

$v = d/t$

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 flight time of the ion.

In the design of an oa-TOF mass spectrometer, much effort is devoted to holding the values of the energy (E) applied to the ions and the distance (d) the ion travels constant, so that an accurate measurement of flight time will give an accurate mass value.

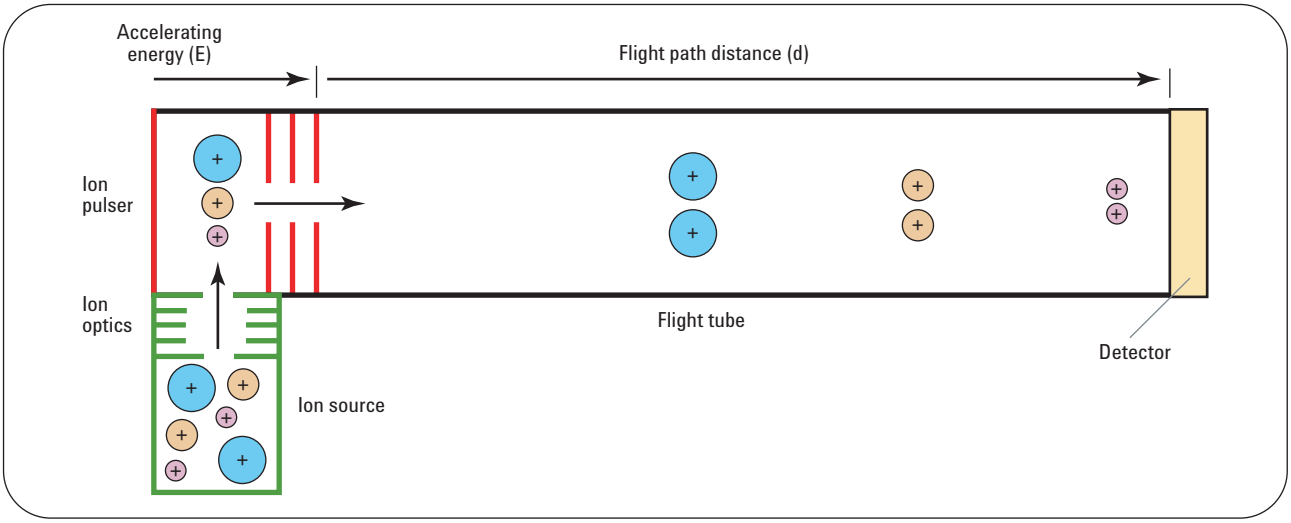


Figure 3
Time-of-flight analysis of ions of various masses, each with a single charge. For clarity and simplicity, this shown in a linear time-of-flight mass spectrometer that does not have an ion mirror.

As these terms are held constant they are often combined into a single variable, A, so:

$m = At^2$

This is the ideal equation that determines the relationship between the flight time of an ion and its mass. Because the relationship is a squared relationship, if the observed flight time of the ion is doubled, the resulting mass is not doubled, but rather it is four times greater.

In practice, there is a delay from the time the control electronics send a start pulse to the time that high voltage is present on the rear ion pulser plate. 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 significant.

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_o.

$t = t_m - t_o$

By substitution, the basic formula that can be applied for actual measurements becomes:

$m = A(t_m - t_o)^2$

Mass calibration

To make the conversion from measured flight time, t_m, to mass, the values of A and t_o must be determined, so a calibration is performed. A solution of compounds whose masses are known with great accuracy is analyzed. Then, a simple table is established of the flight times and corresponding known masses. It looks something like this:

Calibrant compound mass (μ)	Flight time (μsec)
118.0863	20.79841
322.0481	33.53829
622.029	46.12659
922.0098	55.88826
1521.971	71.45158
2121.933	84.14302
2721.895	95.13425

Table 1
TOF mass calibration.

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_o. It employs nonlinear regression to find the values of A and t_o so that the right side of the calibration equation,

$m = A(t_m - t_o)^2$

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_o is highly accurate, it is not accurate enough to give the best possible mass accuracy for time-of-flight analysis. A second calibration step is needed. So after the calibration coefficients A and t_o 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 with an equation that corrects the small deviations across the entire mass range. This correction equation, 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.

Reference mass correction

Achieving an accurate mass calibration is the first step in producing accurate mass measurements. When the goal is to achieve mass accuracies at or below the 3 ppm level, even the most miniscule changes in energy applied to the ions can cause a noticeable mass shift. It is possible, however, to cancel out these factors with the use of reference mass correction. With this technique, one or more compounds of known mass are introduced into the ion source at the same time as the samples. The instrument software constantly corrects the measured masses of the unknowns using the known masses as reference. Reference mass correction is a technique that has been automated on the Agilent LC/MSD TOF mass spectrometer. To introduce reference compounds, a second nebulizer has been integrated into the ESI ion source. This reference nebulizer is connected to the 'A' bottle of the calibrant delivery system (CDS), which is controlled via software. Bottle A contains the reference compounds. The mass spectrometer control software has an editable table that contains the exact masses of these reference compound ions. During the acquisition of each spectrum from the time-of-flight analyzer, these known masses are identified and the A and t_o values are re-optimized. Each stored spectrum has its own A and t_o values so that the software can adjust for even the smallest instrument variation. Each spectrum is then corrected using these values and using the correction equation (the higher-order polynomial function) determined in the second calibration step described previously. The correction equation needs to be determined only once because the small deviations across the mass range are nearly constant over time.

To determine the two unknowns, A and t_o, the reference compounds must contain at least two components of known mass. In order to achieve a good fit for both A and t_o, at least one reference mass needs to be a low mass value and at least one needs to be a higher mass value. For best results, the low *m/z* and high *m/z* reference masses should bracket the masses of analytical interest. The reference mass correction algorithm for the LC/MSD TOF requires that one mass be at or below *m/z* 330 and that a second mass be at least 500 *m/z* above the low mass ion. If these conditions are not satisfied, but at least one reference mass is found, then only the A term is recalculated.

TOF measurement cycle

TOF measurements do not rely on the arrival times of ions coming from just a single pulse applied to the ion pulser, but instead are summations of the signals resulting from many pulses. Each time a high voltage is applied to the plates of the ion pulser, a new spectrum called a single transient is recorded by the data acquisition system. This is added to previous transients until a predetermined number of sums has been made. For analyses requiring a scan speed of one spectrum per second, approximately 10,000 transients can be summed before transferring the data from the instrument back to the host computer to be written to disk. If the target application involves high speed chromatography, then fewer transients are summed, increasing the scan speed. The mass range limits the number of times per second that the ion pulser can be triggered and transients recorded. Once the ion pulser fires, it is necessary to wait until the last mass of interest arrives at the ion detector before the ion pulser is triggered again. Otherwise light ions triggered from the second transient could arrive before the heavier ions of the first transient, resulting in overlapping spectra. Table 2 shows some example masses with their approximate flight times and possible transient rates. These are calculated for a

flight length of two meters and a flight potential of 6,500 volts. Under these conditions, a ion with *m/z* 3200 has a flight time of about 0.1 milliseconds (msec), or 100 microseconds (µsec). Because there is essentially no delay time between transients, this means that 10,000 transients per second correspond to a mass range of 3200 *m/z*. For a smaller mass range, the ion pulser can be triggered at higher rates. For example, a mass of *m/z* 800 (one-fourth of 3200 *m/z*) reduces the flight time to 0.1 msec/-4, or 0.05 milliseconds, allowing for 20,000 transients per second over an 800 *m/z* mass range. Conversely, extending the transient to 0.141 milliseconds doubles the mass range to 6400 *m/z* (mass is a function of the time squared).

m/z	Flight time (µsec)	Transients/ sec
800	50	20,000
3200	100	10,000
6400	141	7,070

Table 1
Flight time and transients/second as a function of mass*.
*Two-meter flight tube, flight potential 6500V. The minimum allowed transient is 50 µsec (50,000 points). The maximum is 160 µsec (160,000 points) or about 8,000 *m/z*.

Because transients are so short, the number of ions of a specific mass from a particular compound in any given transient is generally quite small. For many oa-TOF instruments, this number averages to substantially less than one. This fact plays an important role in the basic design of the data acquisition system of many of today's commercial instruments.

Digitally recording ion arrival

While there is an exact instant when each ion strikes the detector, it is difficult to transfer this perfectly into the digital world. There are two basic approaches used to translate a detector signal into a digital measurement: the analog-to-digital converter (ADC) used in the Agilent LC/MSD TOF and the time-to-digital converter used in many other commercial TOF systems. The next two sections discuss these two approaches.

Analog-to-digital converter systems

The function of an analog-to-digital converter (ADC) is to represent digitally the signal that comes from the ion detector. An ADC does not attempt to determine the exact arrival time of the ions; it is simply a data recorder. As a data recorder, it samples the amplified detector output at a fixed interval. In the case of the LC/MSD TOF, this interval is one nanosecond (10^{-9} seconds). This translates to a frequency of one gigahertz (GHz), or one billion cycles per second. During each cycle the detector output signal intensity is converted into a digital value. The digital value is represented by eight bits, corresponding to a dynamic range of 2^8 counts, or in decimal notation 0 to 255 counts. When the acquisition system signals the pulser to fire, the ADC begins to convert the signal arriving from the detector amplifier. It stores each successive conversion in memory. Each time the pulser fires, the ADC adds the new measurement to those already recorded in memory from the previous transients. When an ADC is used in this way, it is called an integrating transient recorder. With an ADC, some care must be used to bias the detector amplifier (Amp Offset) to a value close to zero so that when no ion signal is present, zero signal is recorded. Otherwise, the signal present in the absence of an ion signal would add to system noise. The gain of the detector and amplifier must be sufficient so that an individual ion registers at least one count. In practice, the gain is normally set so that the average number of counts per ion is greater than one. The LC/MSD TOF autotune routine automatically sets the detector gain and Amp Offset parameters to satisfy these conditions. The advantage of the ADC acquisition system relative to the TDC acquisition system (discussed in the next section) becomes apparent when multiple ions of a given mass arrive at the detector within a single transient. The detector is an analog device and amplifies the combined signal from the several nearly simultaneous ion arrivals. An ADC with its eight bits can translate this rising and falling signal into a digital profile of the mass peak, as shown in figure 4. Each successive transient builds the values in memory. This accurately represents the detector output signal, whether it is from a small or large ion current. The next section will show why the TDC does not have this dynamic range.

Time-to-digital converter systems

The time-to-digital converter (TDC) represents the second approach to digitizing a TOF signal. A TDC acquisition system begins with a discriminator. A discriminator is an electronic device that triggers when a particular signal level is reached. This trigger

signal from the discriminator is registered by a counter, which marks the flight time. After a brief dead time, the discriminator and counter are ready to record the next ion arrival. Since the discriminator triggers on the leading edge of the mass peak, the advantage of a TDC system is its ability to eliminate any broadening of the mass peak originating in the detector and amplifier. One disadvantage is loss of dynamic range. Since the discriminator triggers on the leading edge of the incoming ion signal, it ignores the remainder of the detector signal and gives the same response regardless of whether the signal is the result of one ion or many ions. The TDC simply marks ion arrival, but cannot convey how many ions. Because the repetition rate for transients is high, and the average number of ions for any given mass has been substantially less than one per transient, this has generally been an "acceptable" solution. However, as ion sources and ion optics become more efficient, the number of ions of a given mass in a single transient increases to the point of significance. To illustrate this, consider a hypothetical instrument equivalent to the LC/MSD TOF that uses a TDC acquisition system. Figure 5 shows the number of ions for a single compound that arrive in a single transient, as a function of sample amount. At sample concentrations above 1000 picograms, the hypothetical TDC system no longer gives an increased signal response because the TDC cannot reflect the fact that multiple ions of a given mass are arriving in each transient. A second problem associated with TDC acquisition systems is an observed shift in measured ion arrival time at high ion currents. When less than one ion for any given mass arrives at the detector per transient, the TDC accurately records arrival time to within the limit of the counter's resolution. If ions arrive for a given mass just slightly separated in time (as determined by the instrument's resolving power) then, unless the signal from the detector has returned to below the threshold point, the second ion is unable to trigger the discriminator (see figure 6). This phenomenon and the associated reset time of the discriminator and counter are called TDC dead time. TDC dead time

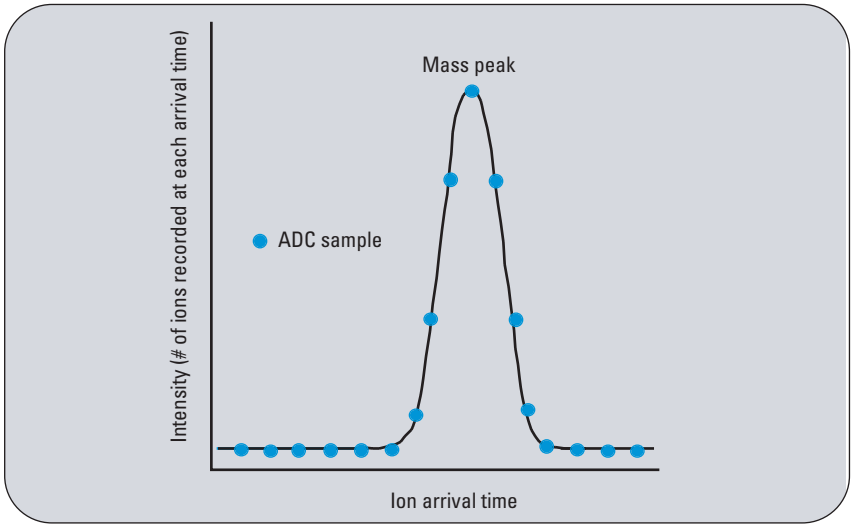


Figure 4
An ADC can record multiple ions per transient, so it accurately tracks ion signal intensity.

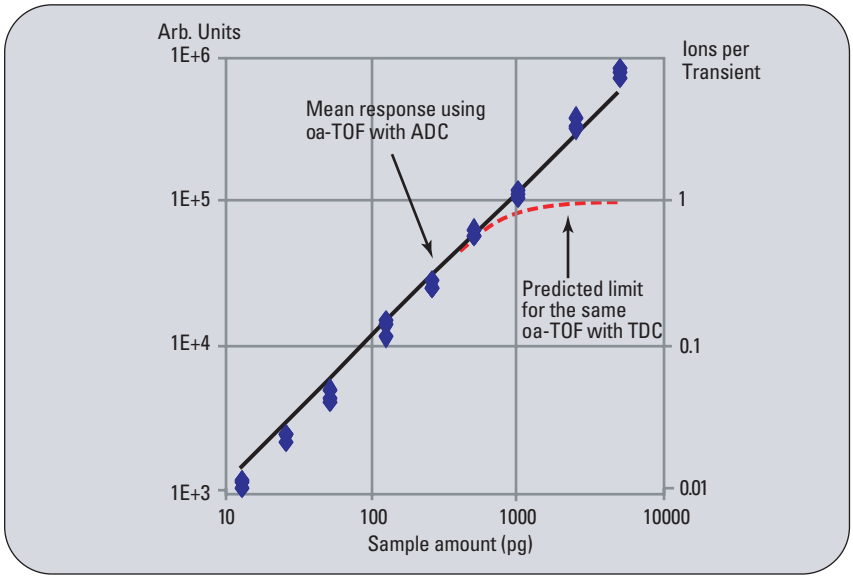


Figure 5
ons per transient as a function of sample amount, showing TDC limitations.

can have a significant effect in attempts to accurately measure average ion arrival times. If a significant number of ions arrive at the detector during the TDC dead time, then a shift in the average of the arrival distribution occurs. The shift in the measured ion arrival time is always to shorter arrival times, because it is always the second ion to arrive in a given transient that is dropped. The shift towards shorter apparent arrival time directly translates to a smaller mass value. When attempting to measure mass values to the part-per-million accuracy, even a few ions missed can have a substantial effect. The discriminator used on TDC systems also introduces a third problem. The arrival of each ion produces a peak with measurable width. With an ADC system, the peak is profiled with multiple points within a single transient. These points can be subjected to mathematical centroiding to calculate the arrival time with high accuracy. Centroiding allows calculation of the ion arrival time to a resolution beyond that given by the original data points. With a TDC system, the arrival of the ion is captured by a single value. This means the time between data acquisitions must be shorter to achieve the same time resolution. Because of the loss of the arrival profile information, TDC systems must operate at higher sampling rates to achieve equivalent mass accuracy even when saturation effects are not present.

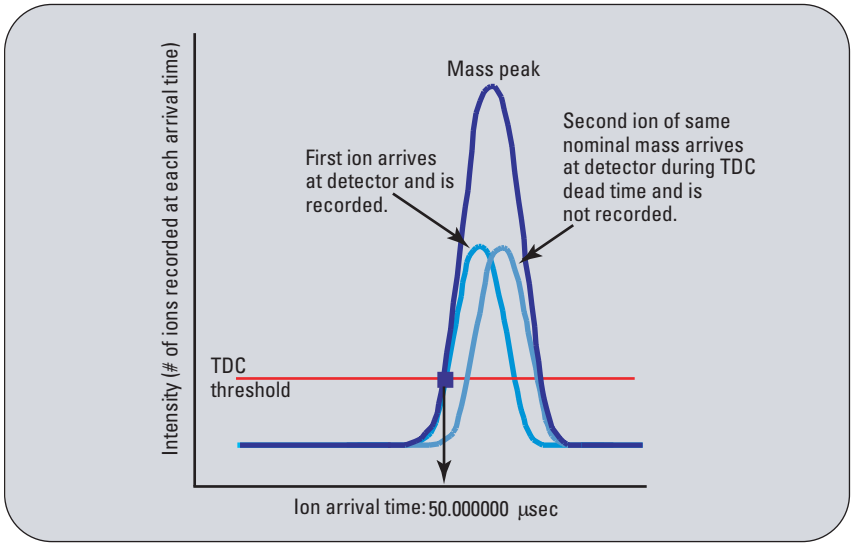


Figure 6
TDC dead time causes shift to shorter arrival times for higher signal levels.

Theoretical and practical limits to mass accuracy

Whether the acquisition system is a TDC or an ADC, the arrival time for the accumulated signal in memory is determined by centroiding the mass measurements from the individual transients. Even though the focus of the design of the TDC was to specifically measure the arrival time of each ion, the nominal arrival time must be the average (centroid) of the population for the summed transients. There are limits to how precisely this centroid can be determined.

Ion statistics

The first theoretical limit is set by the number of ions measured and their time distribution. If the distribution is narrow and well populated, resulting in a quiet and stable signal, then the centroid or average can be precisely determined. The expression is:

$\sigma = 10^6/(2.4 * R * -n)$

where σ is the standard deviation of the resulting measurement, R is the resolving power (often called resolution) of the mass spectrometer and n is the number of ions that are detected in the mass peak. Suppose one desires 95 % confidence 2σ mass accuracy at 3 ppm. Then with a resolving power of 10,000 (and 1σ =1.5 ppm) it is necessary to have approximately 1000 ions. To increase the number of ions in a centroided spectrum, it is general practice to use the data analysis software to average spectra across the width of the eluting chromatographic peak. It should be noted that while oa-TOF has the potential for fast scan cycles, reducing the scan time reduces the number of transients, which reduces the integration of ions required to achieve accurate mass measurements. Fast scanning and accurate mass are opposing performance goals. The most accurate mass measurements are achieved under slower scanning conditions.

Chemical background

The second significant factor that limits mass accuracy is chemical background. The high resolving power of a TOF system helps to reduce the chances of having the peak of interest merged with background, yet even a small unresolved impurity can shift the centroid of the expected mass. The magnitude of this effect can be estimated by using a simple weighted average calculation.

$$\Delta_{obs} = \Delta_{contaminant} \frac{1}{2}Abd_{contaminant} (Abd_{contaminant} + Abd_{sample})$$

where

Δ_{obs} is the observed shift in mass in ppm

$\Delta_{contaminant}$ is the mass difference between the sample and contaminant in ppm

$Abd_{contaminant}$ and Abd_{sample} are the mass peak heights or areas of the contaminant and sample

By way of example, for a resolving power of 10,000, a mass difference between the sample and contaminant of 50 ppm, and relative mass peak heights of 10:1 (sample vs background) the observed mass shift would be $50 \infty 1/(1 + 10)$ or about 5 ppm. There are a number of ways to minimize chemical background. First, the Agilent LC/MSD TOF has a sealed ion source design that minimizes contamination from the laboratory air. Second, very high purity HPLC solvents should always be used. Third, a regular, systematic cleaning program for the HPLC and the MS ion source should be followed. These precautions help ensure the highest quality mass measurements.

Dynamic range

Dynamic range can be measured in various ways. Probably the most exacting definition for mass spectrometry is the “in-scan” condition. This is the dynamic range within a single spectrum, defined as the ratio in signal abundance of the largest and smallest useful mass peaks.

Even when restricted to the in-scan definition of dynamic range, the upper and lower limits must be defined. There are both theoretical and practical limits to consider. Theoretically, it is possible to detect a single ion, but practically, chemical background would, under most conditions, obscure such a low level. Practical limitations depend on the application. For example, when the instrument is used for accurate mass measurement, then the lower limit is set by the minimum sample amount for which accurate mass measurements can be obtained. To determine the minimum sample amount, the limitations based on ion statistics must be considered. Assuming a goal of 5 ppm mass accuracy, achieved with 67 % confidence (1σ) based on a single unaveraged spectrum and allowing for 1 ppm of calibration error, then $1\sigma = 4$ ppm. Staying with the assumption of 10,000 resolving power, then about 200 ions are required for the measurement. This calculation is based on ion statistics and resolving power, and is independent of acquisition technology. This calculation does assume that there is significant sensitivity (signal-to-noise) so that the measurement is unaffected by background contamination. To determine the highest level under which accurate mass measurements can be obtained, the type of acquisition system must be considered. With a TDC system, there is a theoretical limit at one ion per transient at a given mass. With an ADC system, depending on the detector gain, many ions can be accurately measured for a given mass in a single transient. The LC/MSD TOF autotune software targets the detector gain for a mean ion response of five counts. In a single transient, the ADC with 8 bits or 255 counts can therefore measure up to 50 ions for a given mass. Practical considerations limit both TDC and ADC systems with regards to the upper limit for which accurate mass measurements can be achieved. For a TDC system, long before the level of one ion for a given mass per transient is reached, substantial mass shifts are observed. Deadtime correction algorithms compensate for this, but these corrections are effective only up to some fraction of this theoretical limit, typically 0.2 to 0.5 ions/transient. Both ADC and TDC systems, when used to make measurements on rising and falling chromatographic peaks, need to allow for a safety buffer of a factor of two. This is because the chromatographic peak may be rising into saturation, even while the average of the 10,000 transients used to make the final mass measurement is at only the 50 %

level. Table 3 summarizes both theoretical and practical dynamic range limits for ADC- and TDC-based oa-TOF mass spectrometers, based on single- spectrum, in-scan dynamic range. Depending on the application, it is sometimes possible to extend the practical dynamic range. One approach is to sum (average) multiple spectra together. This improves ion statistics and allows for increased mass accuracy at lower sample levels. To extend the dynamic range on the high end, the opposite approach is taken and spectra from the apex of a chromatographic peak are excluded from the average. Intelligent spectral averaging is an important function of the automated accurate mass report generation software of the LC/MSD TOF. Together these techniques can extend the practical limit of dynamic range (~10^3) by a factor of 100, achieving effective dynamic ranges of 10^5 for ADC-based systems in accurate mass applications.

Conclusion

Over the past few years, there has been substantial progress in technologies that take the oa-TOF to new performance levels. High-efficiency ion optics and vacuum system designs have given rise to greater sensitivities. High-speed ADC-based acquisition systems have made greater mass accuracy and wider dynamic range possible. The addition of sophisticated data systems and data processing algorithms has enabled outstanding mass accuracies under routine analysis conditions. By understanding the concepts of oa-TOF mass spectrometry, it is possible to achieve the ultimate in performance with the Agilent LC/MSD TOF system.

	LC/MSD TOF	Hypothetical TDC system
Theoretical limit		
Minimum detectable per spectrum (ions/spectrum)	1	1
Maximum detectable per transient (ions/transient)	50	1
Maximum detectable per spectrum (∞ 10,000 transients)	500,000	10,000
Dynamic range	500,000	10,000
Practical limit (while achieving accurate mass)		
Lower limit per spectrum (ions/spectrum)	200	200
Upper limit per transient (ions/transient)	25	0.1–0.25
Upper limit per spectrum (∞ 10,000 transients)	250,000	1000–2500
Dynamic range	1,250	10–25

Table 3
Single spectrum in-scan dynamic range.

Effect of resolution and mass accuracy on empirical formula confirmation and identification of unknowns

Mass resolution and mass accuracy are both critical aspects of MS performance. With sufficient mass resolution and mass accuracy, a mass spectrometer can positively confirm elemental composition or identify unknowns.

The Agilent LC/MSD TOF design includes unique design features that enhance both its mass resolution and mass accuracy.

Design elements that enhance resolving power include:

- A "beam shaper" and related ion optics that reduce variations in ion position and energy before they enter the mass analyzer.
- One-dimensional "harp" grids oriented in the direction of ion travel in both the pulser and ion mirror (reflectron).
- A mechanical design that automatically creates proper alignment (parallelism).

Design elements for mass accuracy include:

- An analog-to digital (ADC) acquisition system that provides several orders of magnitude of dynamic range.

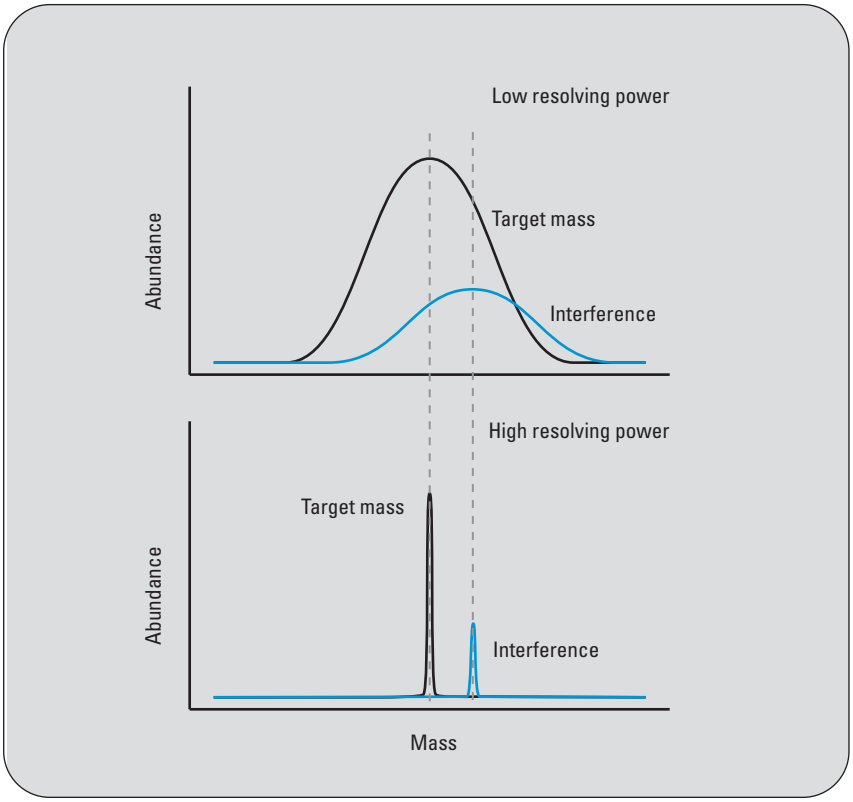


Figure 1
The high resolving power of a TOF mass analyzer helps to reduce the chances of having the mass peak of interest merged with an interfering ion from the sample or the background.

- An automated calibrant delivery system and a second nebulizer that allow the continuous introduction of a reference mass compound at a concentration low enough that it is unlikely to interfere with analyses.
- A flight tube made from a special, ultralow-thermal-expansion alloy that minimizes flight path changes due to temperature changes.
- Mechanical and electronic temperature compensation in the flight tube and electronics.

Even if mass resolution and mass accuracy are not sufficient for positive identification, accurate mass measurements can reduce the number of likely candidates enough that positive identification can be made based on a combination of accurate mass measurements and other information. Information that may help to limit some of the elemental composition possibilities to those that make chemical sense can include starting materials, the isotope distribution, the number of possible nitrogens and the number of unsaturated bonds in a compound. In the case of a target compound, the expected empirical formula is known and can be compared against the measured accurate mass data to confirm identity.

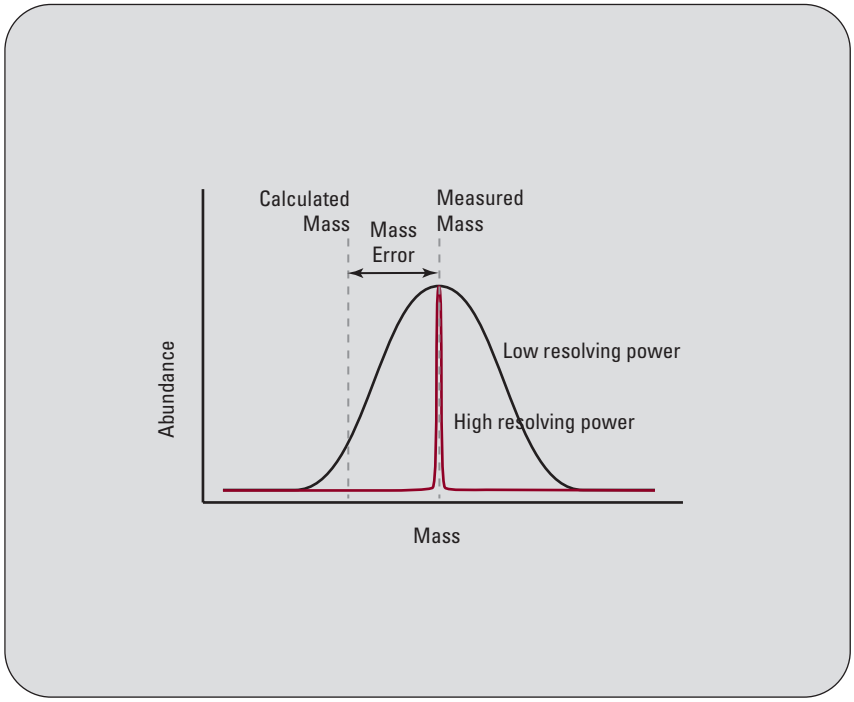


Figure 2
Mass accuracy is independent of resolving power, but the LC/MSD TOF exhibits outstanding resolving power and mass accuracy.

Detailed note: "Effect of resolution and mass accuracy on empirical formula confirmation and identification of unknowns", *Agilent Technologies Technical Overview*, publication number 5989-1052EN **(2004)**.

Visit our website at **www.agilent.com/chem/tof** to download the detailed note.

Advantages of wide dynamic range on an orthogonal acceleration time-of-flight mass spectrometer

Time-of-flight mass spectrometry A wide dynamic range is a desirable characteristic in mass spectrometers. It allows compounds of differing abundances to be analyzed at the same time. This can simplify sample preparation and facilitates “walk-up” access for researchers who are not experts in mass spectrometry. In the case of time-of-flight mass spectrometers, it also facilitates the introduction of a reference mass compound, which enhances the accuracy of mass measurements.

This technical overview reviews the concept of dynamic range. It then discusses how dynamic range is achieved in a time-of-flight mass spectrometer and how a detector using analog-to-digital converter technology can dramatically improve dynamic range compared to older detector technologies.

Dynamic range

The ratio of the maximum signal, which is the upper limit, to the minimum signal, which is the lower limit, to the magnitude distinguished from background, is the mass spectrometer's dynamic range.

Effect of dynamic range on MS

Dynamic range has a significant effect on a mass spectrometer’s usability. If an instrument’s dynamic range is very narrow it is easy to introduce the wrong amount of sample and either get a clipped signal (too much sample) or no signal (too little sample). If mixtures of chemicals are being analyzed, a narrow dynamic range means that all of the compounds in the mixture must have similar abundances. If they do not, either the signals from high-abundance compounds are clipped, affecting response linearity and making quantitation difficult, or signals from low-abundance compounds are not detected and those compounds are not identified (figure 1).

In contrast, a wide dynamic range makes it much easier to use a mass spectrometer (figure 2). Sample preparation and abundance becomes less critical. If mixtures are being analyzed, both high- and low abundance compounds can be identified. Linearity is improved, making quantitation easier and more reliable.

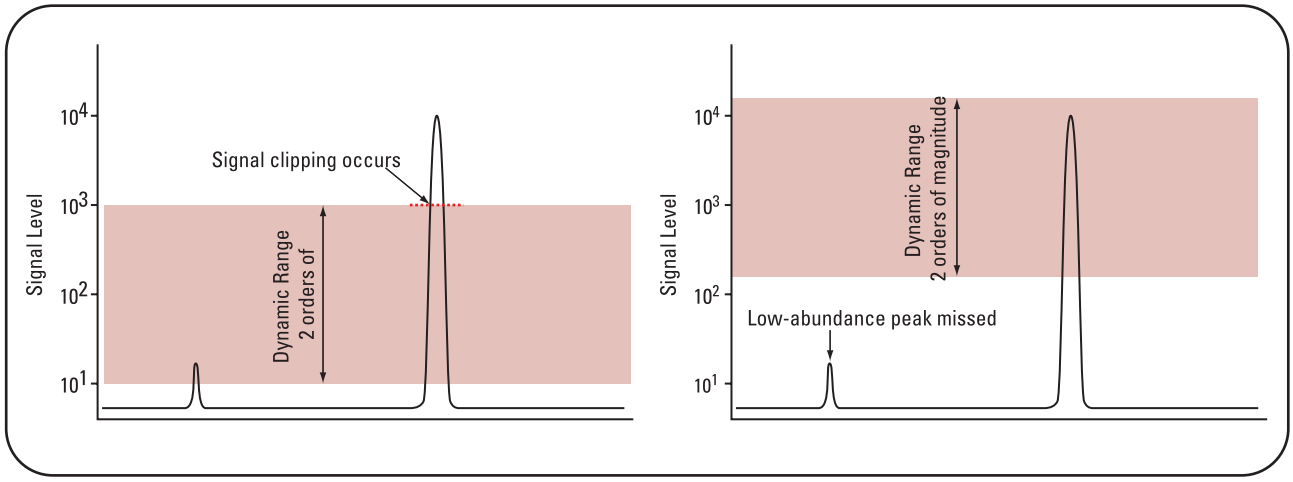


Figure 1
Consequences of narrow dynamic range.

Implications of dynamic range for TOF MS

For time-of-flight (TOF) mass spectrometers, a wide dynamic range has an additional benefit. To maximize mass accuracy, a reference mass compound whose mass is known to a very accurate degree is often introduced into a TOF MS for purposes of calibration. If a TOF MS has a narrow dynamic range, the reference mass compound must be introduced at an abundance nearly the same as the abundance of samples being analyzed. This can create significant chemical interferences and affect analytical results.

If, on the other hand, a TOF MS has a wide dynamic range, a reference mass compound can be introduced at an abundance much lower than the abundances of typical samples. This eliminates, or at least minimizes, interferences. Thus, a reference mass compound can be introduced continuously and the mass accuracy of the TOF MS never has a chance to drift, ensuring maximum mass accuracy (figure 3).

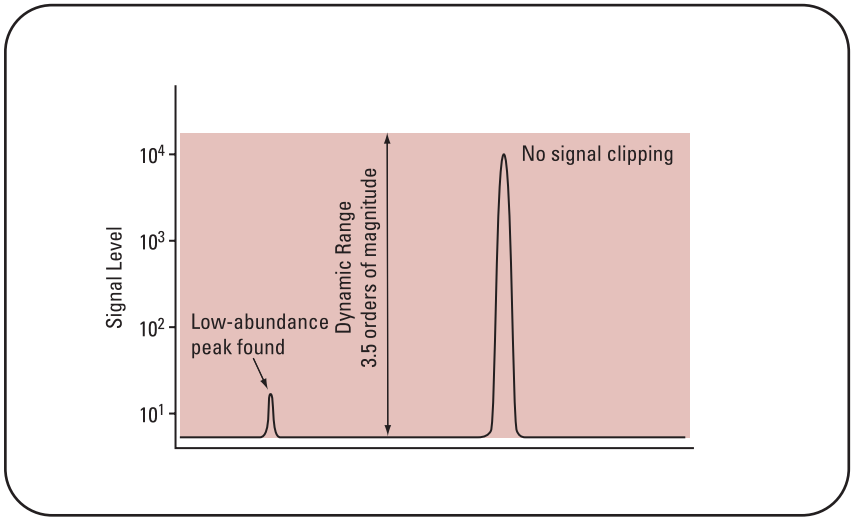


Figure 2
Advantages of wide dynamic range.

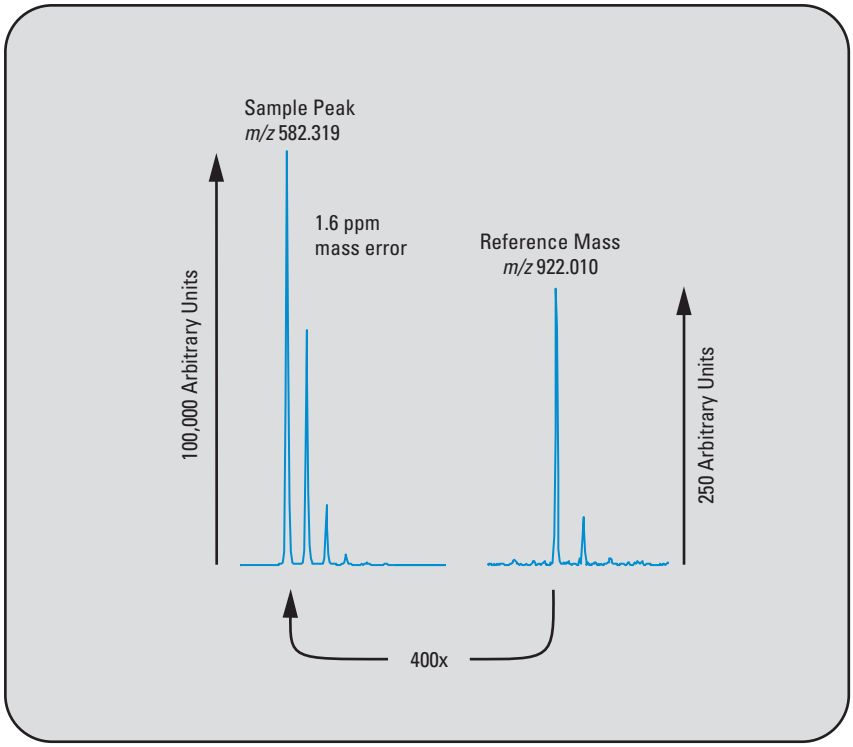


Figure 3
Wide dynamic range enables continuous introduction of reference mass compound at a low level, providing maximum mass accuracy with minimum interference.

TOF MS design and dynamic range

The dynamic range of a TOF MS is determined by many aspects of the instrument design. One of the most influential is the detector and its electronics. The detector senses ions as they impact it and generates a signal based on those impacts. The signal is converted to digital form so that it can be processed. A time-of-flight mass

spectrometer determines ion masses based on the time it takes them to "fly" from a starting point to an ending point. TOF is a pulsed technique, with each electrical pulse starting a group of ions on their flight to the detector. For each pulse, the detector records a corresponding spectrum, called a transient. Many transients are summed to create a mass spectrum.

Time-to-digital signal conversion

Traditionally, TOF mass spectrometers have used a detector with a time-to-digital converter (TDC). A TDC records a precise arrival time for each mass within a transient. Because a TDC records the arrival time based on the arrival of the first ion of a given mass, it cannot tell the difference between one ion at a given mass arriving in a transient and several ions with the same mass arriving in a transient (see figure 4). This was acceptable in previous generations of TOF mass spectrometers, when inefficient ion sources and ion optics made it unlikely that more than one ion of a given mass would be present in a single transient. However, with the improved efficiency of modern ion sources and ion optics, there is a good chance that a transient will include multiple ions at a given mass. The TDC's inability to determine how many ions are arriving severely limits the instrument's dynamic range. TOF mass spectrometers using TDC detectors often have dynamic ranges of only one or two orders of magnitude. Sample abundance must be adjusted to match the instrument's range. Often this entails a time-consuming trial-and-error, dilute-and-reshoot process. One partial solution to TDC dynamic range limitations is beam-splitting or defocusing. While this helps prevent detector saturation, it also results in a large portion of the ions being discarded. This can reduce sensitivity and cause low-abundance components of a sample to be lost entirely.

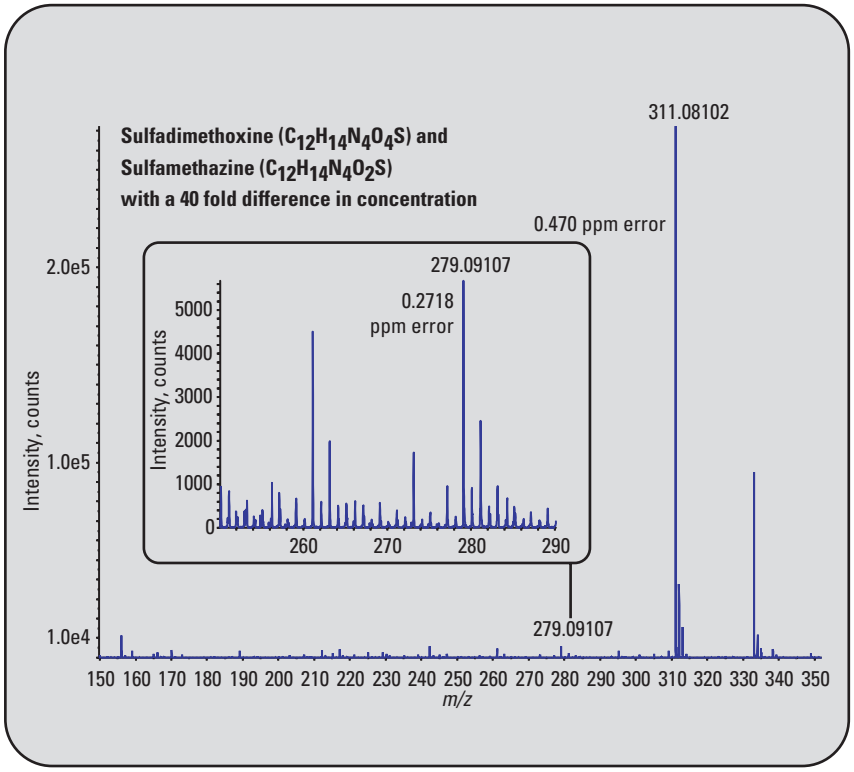


Figure 4
Wide dynamic range makes it easier to detect lower-abundance compounds in the presence of higher-abundance compounds.

Analog-to-digital signal conversion

In contrast, the Agilent LC/MSD TOF mass spectrometer uses a different approach, a detector with an analog-to-digital converter (ADC). The ADC does not try to record arrival times for individual ions. Instead, it records the total signal strength the detector is outputting at fixed, and very frequent, intervals – as often as a billion times per second. The ADC records differing signal levels, so it can tell the difference between one ion of a given mass or many ions of a given mass arriving in a

single transient. The result of this is that the LC/MSD TOF, with its ADC detector, has a dynamic range of three to four orders of magnitude. A resulting advantage compared to older TOF MS designs is that the LC/MSD TOF features continuous introduction of reference mass compound at a very low abundance. This greatly enhances the mass accuracy of the LC/MSD TOF (better than 3 ppm) while minimizing interference with actual samples. The wide dynamic range also enables the LC/MSD to detect less-abundant compounds in the presence of significantly more abundant compounds, even within a single scan (see figure 4). This can be of great benefit when trying to identify minor impurities in the products of synthetic chemistry and purification, or when looking for post-translationally modified proteins in the presence of native proteins.

Conclusion

A wide dynamic range provides significant benefits for mass spectrometers, facilitating the detection of lower-abundance compounds in the presence of higher-abundance compounds. For time-of-flight mass spectrometers, this allows the continuous introduction of reference mass compounds that greatly enhance mass accuracy. The Agilent LC/MSD TOF features a detector with analog-to-digital signal conversion that greatly enhances its dynamic ranges compared to older TOF designs that rely on time-to-digital signal conversion.

Detailed note: "Advantages of wide dynamic range on an orthogonal acceleration time-of-flight mass spectrometer", *Agilent Technologies Technical Overview*, publication number 5989-1728EN **(2004)**.

Visit our website at **www.agilent.com/chem/tof** to download the detailed note.

Accurate mass measurement for analyzing drugs of abuse by LC/time-of-flight mass spectrometry

The Agilent LC/MSD TOF provides routine and seamless accurate mass measurement for unambiguous identification of chemical substances of forensic relevance like drugs of abuse.

14 basic drugs of abuse were separated using the LC/MSD TOF. Although in some cases chromatographic separation is not complete (figure 1), examination of extracted ion profiles for each of the [M+H]+ ions shows that each compound can be identified without interference as in the case of the isomers, hydrocodone and codeine (figure 2).

Table 1 shows the mass accuracy achieved from 50 pg of these compounds to 50-ng injected on-column. Accuracy of better than 5 ppm is achieved for amphetamine and better than 2 ppm for oxycodone. Note that for low mass measurements, the number of possible empirical formulas is far less and a 5-ppm range is more than sufficient. At a higher mass, the possibilities increase and a lower

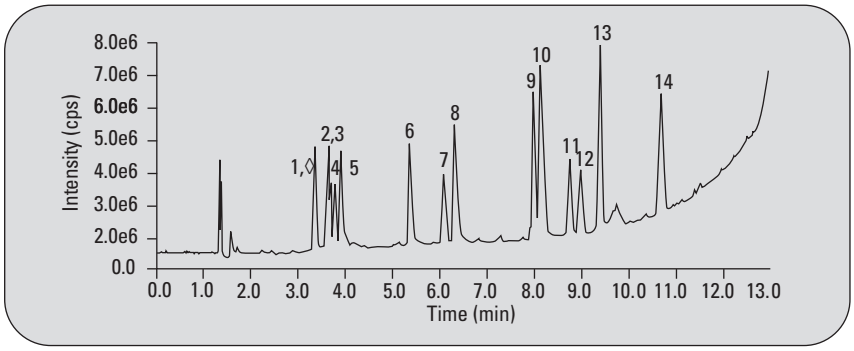


Figure 1
TIC of basic DA. LC/ES-MS TOF separation of 14 basic DA used as targeting compounds in toxicological sample screening.

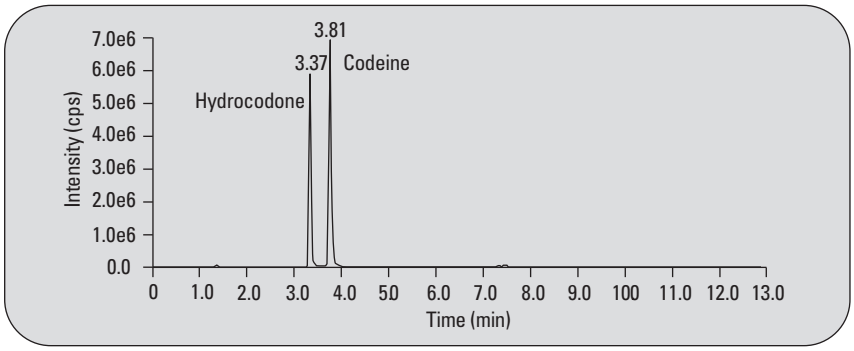


Figure 2
Extracted ion chromatogram. Ion chromatogram of m/z 300.0-300.2 extracted from the data shown in figure 1. Isomers are chromatographically separated, facilitating their identification.

Peak Number	3	5	1	4	2
Compound	Amphetamine	Methanphetamine	Hydrocodone	Codeine	Oxycodone
Nominal *(m/z)*	136.10	150.10	300.15	300.15	316.15
Conc. (ng-injected)	Measured error (ppm)	Measured error (ppm)	Measured error (ppm)	Measured error (ppm)	Measured error (ppm)
50.00	–4.97	–2.41	0.94	0.94	1.32
25.00	3.53	–2.49	1.17	1.60	0.27
5.00	–4.71	–3.01	0.37	0.37	0.04
5.00	–4.53	–3.03	0.47	0.37	–0.16
5.00	4.53	–3.05	0.30	0.30	–0.75
2.50	5.00	–2.78	1.60	1.60	–0.42
0.50	–4.23	–2.20	1.34	1.90	0.04
0.50	–5.01	–2.48	1.11	1.27	–0.33
0.25	–5.70	–2.69	0.95	1.27	–1.16
0.05	5.00	–5.42	3.60	2.26	0.53

Table 1
Accurate mass measurements vs. concentration of some DA in reference material using targeted automatic search of empirical formula.

range for error is needed to provide confirmation or suggest a reasonable empirical formula to aid the identification of an unknown.

Figure 3 shows a response vs. concentration plot for codeine. The highly-linear response indicates this instrument can also be used for quantitative analysis. Note that the 50-ng injection was excluded, with this compound and others, because of detector saturation. For these compounds at saturated concentrations, accurate mass measurement was made at the edges of the chromatographic peak with an automated script. Also, a detection limit was not set and the 50-pg injection was made as an arbitrary low standard. The LC/MSD TOF specification for reserpine is 10 pg at a signal to noise ratio of 10:1. With the instrument's high mass resolution and seamless auto-calibration of every spectrum collected, selectivity of the extracted ion is increased.

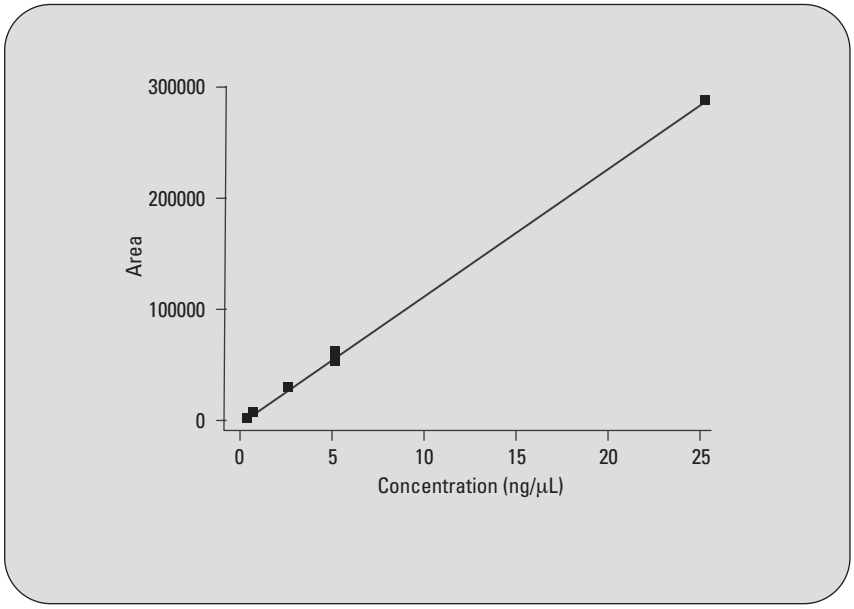


Figure 3
Codeine linearity. Plot of codeine extracted ion (ms/300.0-300.2) chromatographic peaks measured from 50-pg injected on-column to 25-ng on-column. TOF detector saturated at 50 ng.

Conclusion

Very high sensitivity is achieved and, with the TOF detection, all data are full scan, allowing compounds that are not targeted to be detected. The system offers a wide dynamic range capable of providing accurate mass measurements across that range without having to match lock-mass signal intensity with analyte intensity. Finally, a linear response is achieved within a concentration range below detector and electrospray saturation.

Detailed note: "Accurate mass measurement for analyzing drugs of abuse by LC/time-of-flight mass spectrometry", *Agilent Technologies Technical Overview*, publication number 5989-0667EN **(2004)**.

Visit our website at **www.agilent.com/chem/tof** to download the detailed note.

Using the Agilent LC/MSD TOF to identify unknown compounds

In the pharmaceutical industry, it is not unusual to generate small quantities of unknown compounds in addition to the intended products when synthesizing lead molecules, when doing larger scale synthesis of promising candidates, or during manufacturing. While HPLC with a UV detector can detect the presence of unknowns, mass spectrometry is usually required to positively identify them. Since the synthesis or manufacturing process generally provides some clues to the composition of unknowns, it is frequently possible to propose and/or confirm a logical structure. One approach to identifying these unknowns is to interpret the combination of precursor ion spectra and product ion spectra produced by MS/MS analysis. An alternate approach is to use the accurate mass capabilities of a time-of-flight (TOF) mass spectrometer. Accurate mass TOF systems are used to confirm target compounds,1 but can also be used to identify unknowns.

While a quadrupole mass spectrometer is typically operated with unit mass resolution and can assign the mass to the nearest 0.1 *m/z*, modern TOF systems produce spectra with a resolution of 4000 to 10,000, depending on the mass of the ion, and can assign a mass to better than 5 ppm accuracy. This has significant implications when trying to propose possible empirical formulas. Table 1 shows what happens when you consider a molecule such as reserpine (MW 608.2734) and restrict the elemental composition to combinations of C, H, O, and N.

Mass Accuracy (ppm)	Possible Formulas
165 (quadrupole)	209
10	13
5	7
3	4
2	2

Table 1
Number of theoretical formulas for a compound composed of C, H, O, and N with a molecular weight 608.2734.

Note that even with 2 ppm mass measurement accuracy, there are still two possible molecular formulas. Furthermore, a unique formula does not translate to a unique structure. Typically, other information such as knowledge of the synthesis, other spectral data, or product ion spectra from collision-induced dissociation (CID) is needed for unambiguous identification.

Experimental

The system used was an Agilent LC/MSD TOF coupled to an Agilent 110 Series HPLC.
For more information about the applied experimental conditions see the original Application Note.

BPCs and EICs covering a mass range narrower than the complete scan range often gave signals that more clearly showed the presence of trace compounds. Figure 1 shows an example of a TIC (a) and an EIC (b) from the same data. The vertical scale on the EIC is also expanded 10 times. Spectra from the appropriate chromatogram were averaged and suspected molecular ions were selected. Using the elemental composition calculator built into the data analysis software with a tolerance accuracy set to 3 ppm, and applying constraints on the elemental composition, possible formulas were determined for simulated unknowns, where the actual compound was known, and for true unknowns that were trace components in synthetic mixtures.

Results and discussion

The first sample analyzed was the antibiotic, chloramphenicol, which was treated as an unknown in order to demonstrate the general technique. The elements were restricted to C, H, O, N, S and Cl

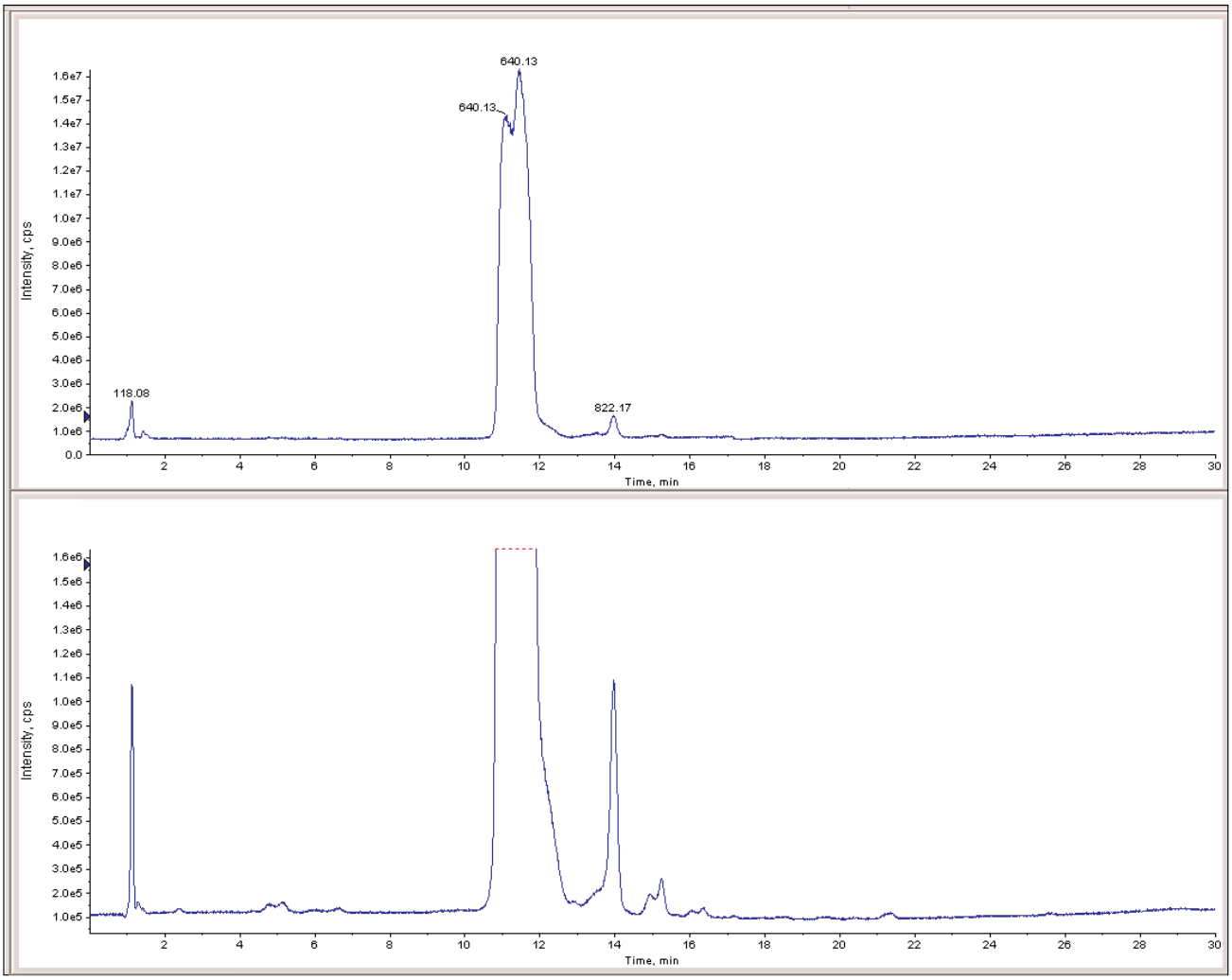


Figure 1
TIC and EIC of a mixture with lower-level unknown components.

and the number of charges was –1 because the compound was analyzed in negative ion mode.

The elemental composition calculator produced four possible formulas based on the ion at m/z 321.0046 as shown in figure 2. In order to narrow the choices, the isotope ion at m/z 322.0081 should be consistent with the number of carbons in the molecule and the naturally occurring abundance of the ^{13}C isotope. In addition, the ion at m/z 323.0017 should reflect the naturally occurring abundances of ^{34}S and ^{37}Cl. Selecting "Show Isotopic" from the calculator will actually overlay the theoretical isotope abundances on the spectrum. Taking these criteria into account, the first formula, $C_{11}H_{11}N_2O_5Cl_2$ turned out to be the correct formula.

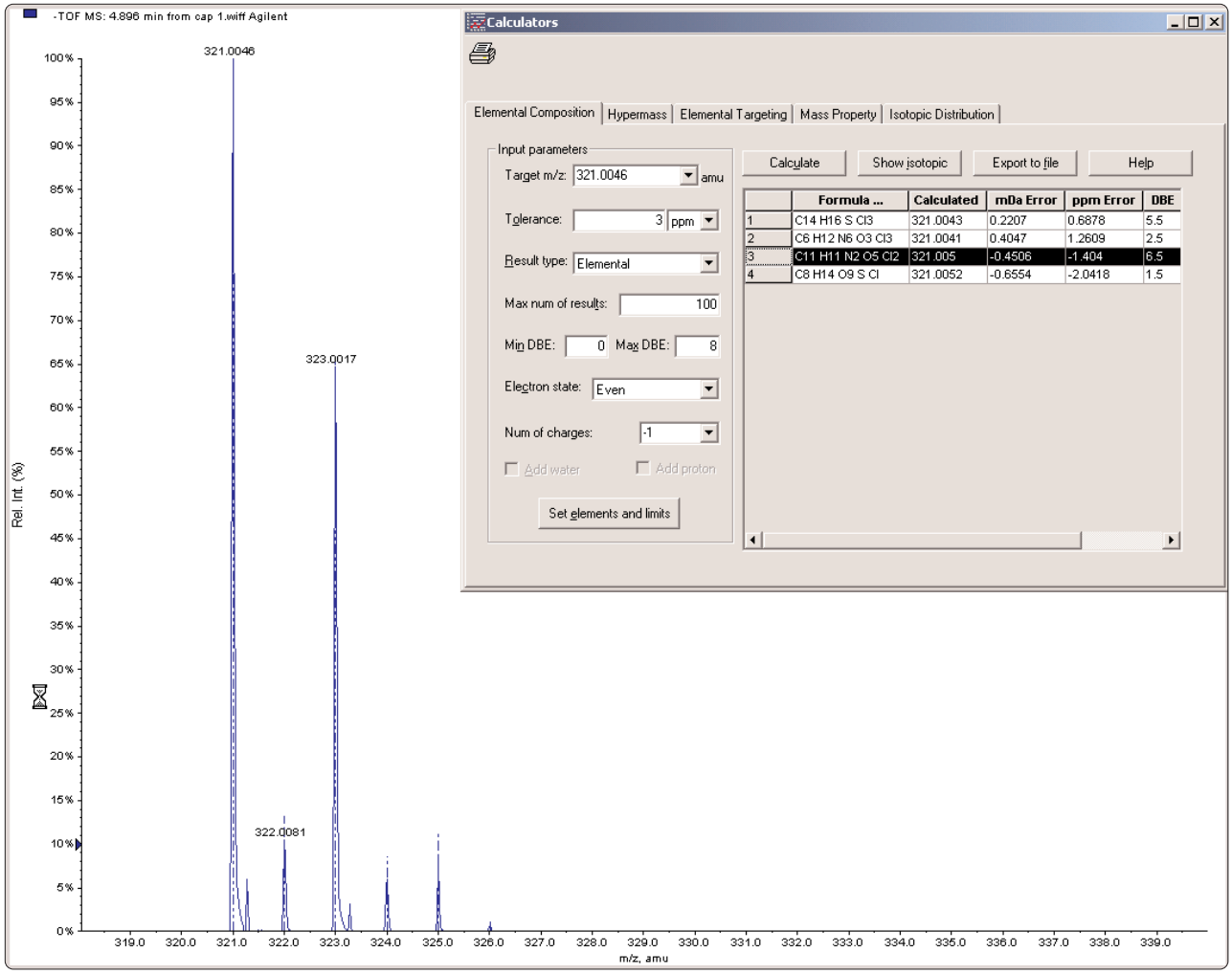


Figure 2
Possible formulas generated from chloramphenicol data by the elemental composition calculator.

In addition to isotope information, adduct information can be used to reduce the number of possible formulas. For example, a mixture of four sulfa drugs: sulfamethizole, sulfamethazine, sulfachloropyridazine, and sulfadimethoxine, gave a chromatographic peak that produced a mass spectrum with ions at *m/z* 311.0814 and 333.0624 as shown in Table 2. Using the elemental composition calculator the following possible formulas were proposed, assuming the *m/z* 333.0624 ion could contain sodium.

Ion at *m/z* 311.0814		Ion at *m/z* 333.0624	
A	$C_5H_{15}N_{10}O_2S_2$	D	$C_{10}H_9N_{10}O_2S$
B	$C_{12}H_{15}N_4O_4S$	E	$C_{12}H_{14}N_4O_4SNa$
C	$C_{13}H_{11}N_8S$		

Table 2
Possible ion formulas consistent with observed ions.

Choice A has too few carbons to match the isotope data. Choices B and E are the only formulas that are consistent with a protonated and sodiated adduct.

Even in instruments that are not capable of MS/MS, in-source CID can yield structural information that can be used to aid in the identification of unknowns. An analysis of the sulfonamide antibiotic, sulfachloropyridazine, demonstrates the usefulness of this approach. The LC/MSD TOF can acquire alternating scans at different fragmentor voltages. By using values of 150 and 215 volts, spectra with and without fragmentation were obtained across the chromatographic peak representing sul-

fachloropyridazine. Figure 3 shows a resulting mass spectrum. In addition to the protonated and sodiated ions at *m/z* 285.0207 and 307.0027, there is a fragment ion at *m/z* 156.0016. By observing the isotope ratios, it is possible to narrow down the number of proposed formulas from seven to one. Since the ion at *m/z* 156.0016 must come from the parent ion, its list of possible elements is based on the five elements (C, H, N, O, S) that make up sulfachloropyridazine. With this constraint, the calculator only comes up with a single formula, $[C_6H_6NO_2S]^+$ to match the data.

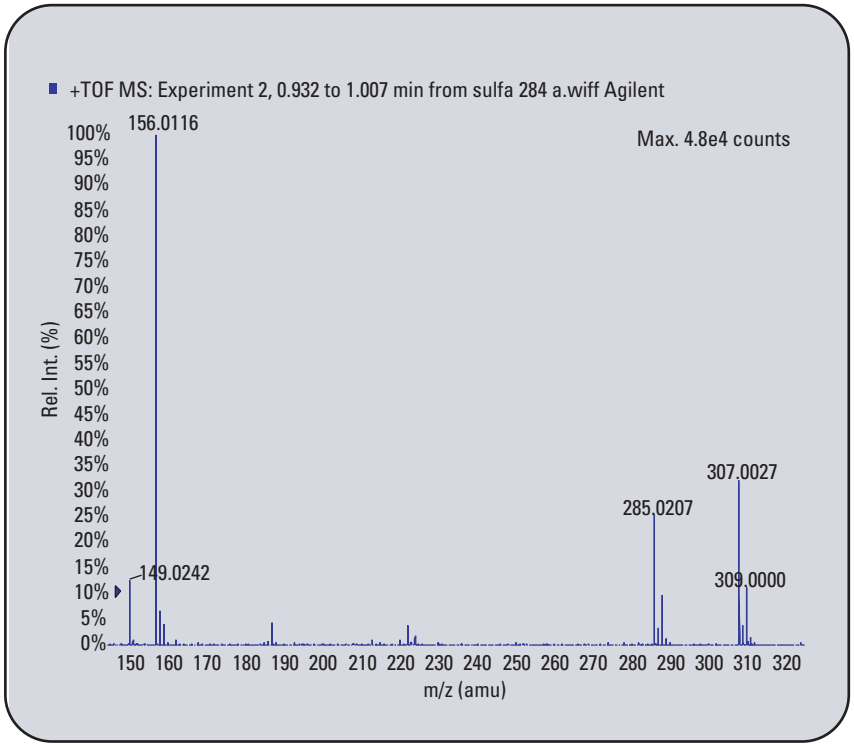


Figure 3
Sulfachloropyridazine spectrum with adduct and fragment ions.

Conclusion

The identification of unknown components in synthetic mixtures is made much easier by the Agilent LC/MSD TOF. Mass accuracy of better than 3 ppm results in a fewer potential formulas for most compounds. When other information such as isotope ratios, fragment ions, and adduct ion are considered, it is often possible to propose a unique empirical formula.

Detailed note: "Using the Agilent LC/MSD TOF to identify unknown compounds", *Agilent Technologies Application Note*, publication number 5989-0626EN **(2004)**.

Visit our website at **www.agilent.com/chem/tof** to download the detailed note.

Automated empirical formula confirmation using the Agilent LC/MSD TOF

In the process of drug discovery, a major objective is the generation of lead molecules. Laboratories may synthesize anywhere from a few new molecules per week to thousands of molecules per week. Typically, before these lead molecules are screened for suitability, mass spectrometry is employed to confirm that the intended molecules were synthesized, because screening an incorrect compound can waste time and money. Most of this confirmation has been done by LC/MS, using single quadrupole instruments that have only unit mass resolution. The problem with this approach is that while negative results prove the compound was not synthesized, positive results at this resolution only indicate a high probability that the correct compound was synthesized. If, instead, a mass spectrometer capable of mass accuracy better than 5 ppm is used, the chemist will have a much higher confidence that the correct compound was made when a positive result is obtained.

Quadrupole mass spectrometers are generally accepted as routine tools, and have been refined to work over a broad range of sample conditions with little user intervention. Historically, the same has not been true of high-resolution mass spectrometers such as time-of-flight (TOF) mass spectrometers. The Agilent LC/MSD TOF mass spectrometer has features and attributes that make it much closer to quadrupole mass spectrometers in its ability to work more reliably over a wide range of sample conditions. This note describes the use of the Agilent LC/MSD TOF for automated empirical formula confirmation in a high-throughput environment.

Experimental

A detailed technical overview of the LC/MSD TOF is available in this compendium on pages 4-13. A high-throughput HPLC system with alternating column regeneration was used. The sample used for this experiment contained sulfamethizole, sulfamethazine, sulfachloropridazine and sulfadimethoxine in a 96-well plate at the 10 ng/µg level. The experimental conditions are described in detail in the full length Application Note.

Results and discussion

Empirical formula confirmation

The data processing method automatically calculated the target molecular weight from the formula. An extracted ion chromatogram (EIC) was produced to cover the mass range of all chosen adducts. An average mass spectrum was taken across the largest peak in the EIC and the masses for each target were compared against the theoretical mass. A one-page report was produced for each target compound showing the EIC, the average mass spectrum, an expanded mass spectrum showing the adduct ions, and the results for each adduct in both absolute mass error and ppm error. An example of this report is shown in figure 1. Early results showed the system typically provided accurate mass to within 3 ppm for sample amounts in the low picogram range to low nanogram range. At the high end, the detector eventually saturated resulting in distorted mass peaks that were difficult to assign mass and yielded poor isotope ratios. The data processing method was modified to evaluate the spectra across the EIC peak for saturation. The user can set a threshold (50% was used in these experiments) and only spectra below this threshold are considered in producing the average spectrum used for the subsequent calculations. This significantly increased the dynamic range over which samples could be run without diluting and reinjecting. The data from the four 96-well plates were processed in Excel. The mass error was plotted against signal abundance and molecular weight. The average error was less than 3 ppm RMS for each plate. Some plates gave less than 2 ppm

error. For one plate, the error on samples below molecular weight 250 was between 4 and 10 ppm. However, when the possible formulas were calculated for the measured mass, each sample gave a unique formula within the calculated mass error.

High-throughput results

The injection volume of the sample described was 0.5 µl. This produced four separate peaks, each approximately three seconds wide, eluting in less than 0.8 minutes (see figure 2). The system acquired 15 scans across each peak. Based on 12 runs, the mass accuracy was 0.8 ppm RMS.
By using automatic column regeneration, as well as the overlapped injection and automatic delay-volume reduction features of the well plate sampler, the cycle time per run was one minute and 23 seconds including the time required to process and produce the empirical formula confirmation report.

Conclusion

The Agilent LC/MSD TOF is capable of reliably performing automated empirical formula confirmation over a broad range of sample concentrations and molecular weights. Mass accuracy averaged better than 3 ppm. Using short, small-particle-size columns at high flow with automatic column regeneration, the system had sufficient scan speed to confirm the empirical formula of a 96-well plate library in just over 2 hours.

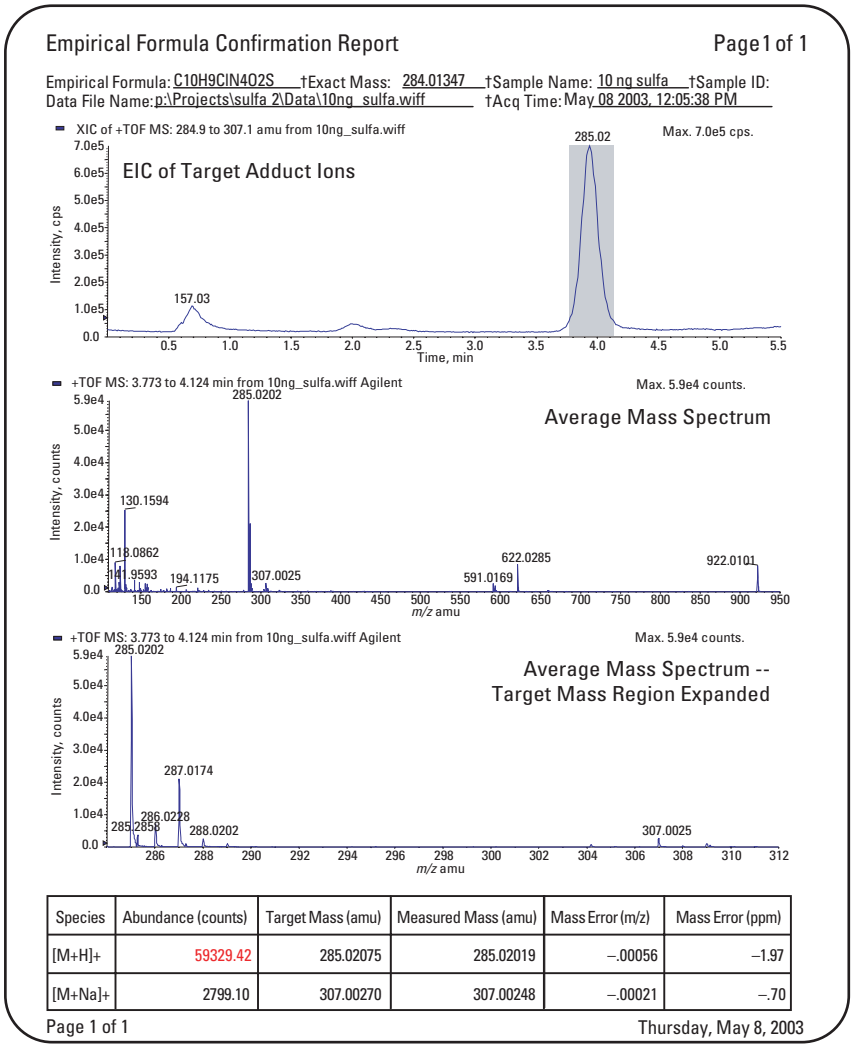


Figure 1
Automated empirical formula confirmation report for sulfachloropyridazine.

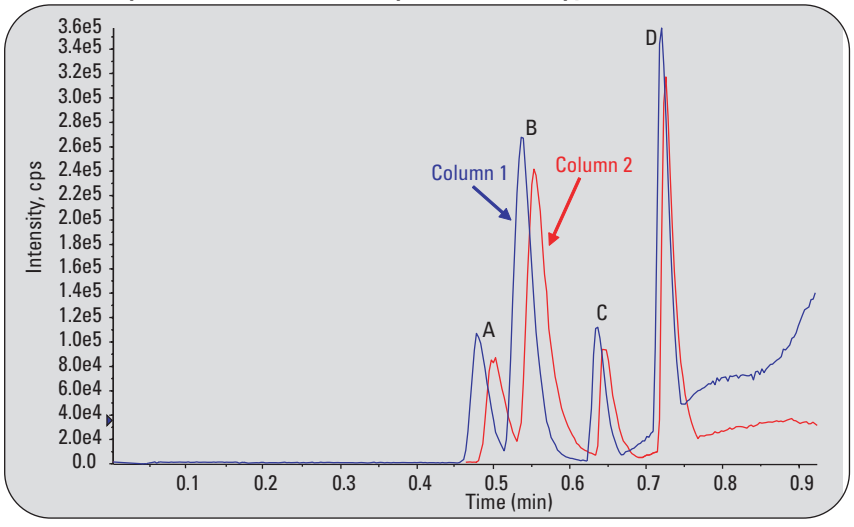


Figure 2
Chromatograms showing separation sulfa drugs on each of the alternating columns. A) sulfamethizole, B) sulfamethazine, C) sulfachloropyridazine, D) sulfadimethoxine

Detailed note: "Automated empirical formula confirmation using the Agilent LC/MSD TOF", *Agilent Technologies Application Note*, publication number 5989-0625EN **(2004)**.

Visit our website at **www.agilent.com/chem/tof** to download the detailed note.

Using accurate mass LC/MS in a walk-up environment

Instruments providing accurate mass have been used in the development of pharmaceuticals both for confirming the identity of synthesized compounds and as a powerful tool in the identification of unknowns. However, obtaining the needed mass accuracy has required the samples to be run by highly skilled mass spectrometrists. In today's pharmaceutical development laboratories there is a desire for samples to be run by the chemists and not by dedicated operators. The Agilent LC/MSD TOF is capable of providing accurate mass routinely. By having a calibrant delivery system to automatically dispense calibrant and a solution for doing automated internal reference mass correction, any operator skilled enough to run a single quadrupole instrument can obtain accurate mass spectra.

To make the operation even simpler, the Agilent Easy-Access Software used on the LC/MSD Quadrupole was adapted for use on the LC/MSD TOF. To the sample submitter, the user interface is virtually indistinguishable from the quadrupole version. The submitter merely logs in some sample information, selects from some methods previously set up on the system by a system administrator and if empirical formula confirmation is desired, the target formula is input. The submitter is prompted where to place the samples in the autosampler and the system indicates when they will be completed. The samples are logged into a queue and when analyzed, a report is printed out or e-mailed to the submitter. The user interface is shown in figure 1. If a specified time has passed since the last sample was run, the system will automatically introduce the calibrant mixture and recalculate the time-to-mass coefficients before running the next samples.

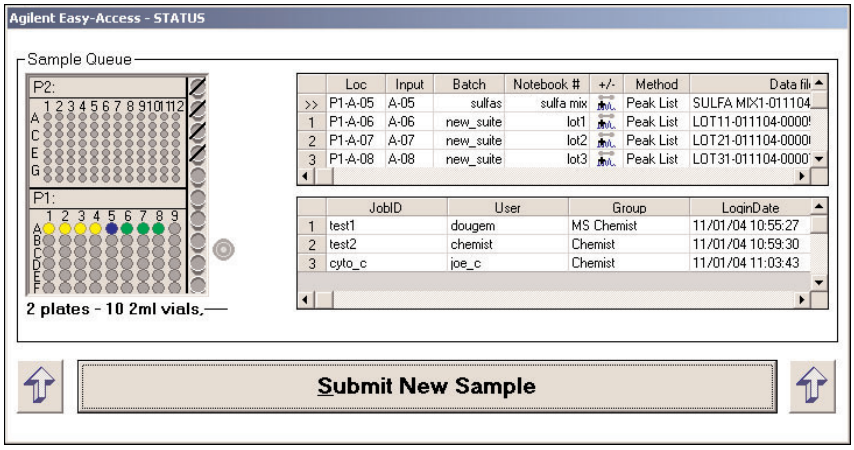


Figure 1
The Easy-Access user interface showing status and for submitting samples.

A variety of automated reports can be produced by the system for either confirming the synthesis of lead compounds or aid in identifying unknowns. The user can choose from methods that print out the major ions for each peak in a chromatographic signal or propose empirical formulas for each of those ions. If a compound database is available, the system will identify if any of the compounds in the database are present. For the confirmation of correct synthesis of recombinant proteins, the submitter inputs a molecular weight or sequence and the system outputs a protein confirmation report. An example of e-mailed reporting is shown in figure 2.

The system can be set up by a system administrator to match most laboratory protocols in areas such as security, terminology, methodology, etc. Changing the setup is under password control. The administrator can even choose to receive e-mail notification of system errors or low solvent state. The system can be set up to work with submitters with a range of skill levels. For example, some users may be able to specify injection volume, move samples in the queue and have a wider range of methods while others may have much more limited capability. In laboratories where tracking samples for accounting purposes is needed, the system will report the usage to a file or database.

The Easy-Access software allows laboratories to obtain results requiring accurate mass measurement without requiring highly skilled instrument operators.

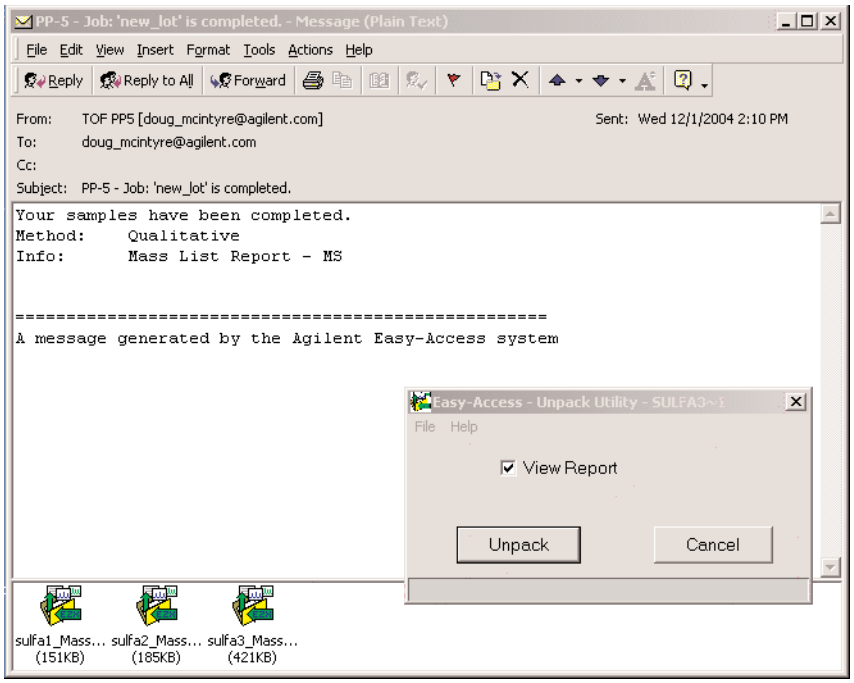


Figure 2
Example of an email notification of sample completion with atttached report.

Detailed note: "Using accurate mass LC/MS in a walk-up environment", *Agilent Technologies Application Note*, publication number 5989-2548EN **(2005).**

Visit our website at **www.agilent.com/chem/tof** to download the detailed note.

Automated empirical formula generation for the identification of unknown compounds

Mass spectrometers that provide accurate mass measurement are a powerful tool in the identification of unknown compounds. Modern LC/MS instruments with Time of Flight analyzers can often provide mass measurements with errors under 3 ppm. While this cannot usually provide a unique empirical formula for a complete unknown, in many cases such as the analysis of impurities in the chemical synthesis of lead molecules in pharmaceutical development, this mass accuracy can be used with other information to provide a single logical empirical formula. This note describes how the Agilent LC/MSD TOF can be used to provide automated reporting of empirical formulas for the peaks in a HPLC chromatographic signal.

Experimental

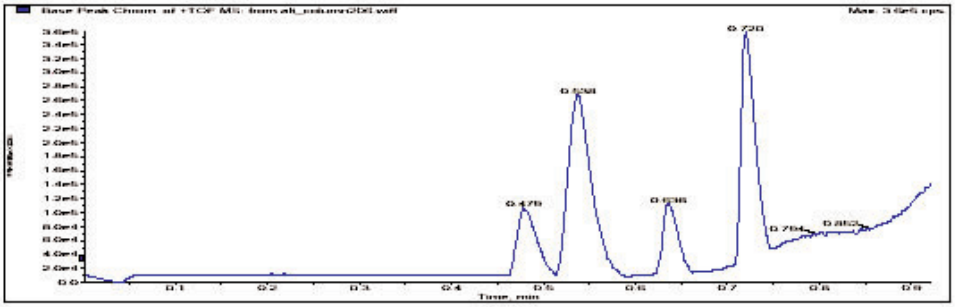
The Agilent LC/MSD TOF is capable of providing routine accurate mass measurements with minimal user interaction. A calibrant delivery system (CDS) automatically provides both a calibrant mix for the tuning and calibration of the instrument but also a reference mix through a second nebulizer for doing real time internal reference calibration[1]. Earlier versions of the software provided an automated Mass List Report that provided the mass spectra from each peak in a Total Ion Chromatogram (TIC). This method was modified to expand the allowed signals to include a Base Peak Chromatogram (BPC), UV signal or the analog signal of any other detector such as a light scattering detector. Additionally, the user can select empirical formula generation and specify a list of elements to consider with minimum and maximum values for each. The electron state and charge state are also specified. The user can select a mass error range to consider and a maximum number of hits. The results reported can be sorted by mass error or by considering the isotope values. In this case, the isotopes of the adduct ion cluster are also considered. For example, they can be compared to the theoretical isotope ratio and a score out of 1000 reported[2]. In most cases, the correct formula will have a score of 990 or greater. Figure 1 contains an example of the first page of a report based on the BPC of a mixture of 4 sulfa drugs. In this example, the top formula on the list, sorted by mass error, is the correct one.

Conclusion

Using the automated generation of empirical formula, the Agilent LC/MSD TOF is a powerful tool in the identification of unknowns while still being easy to use.

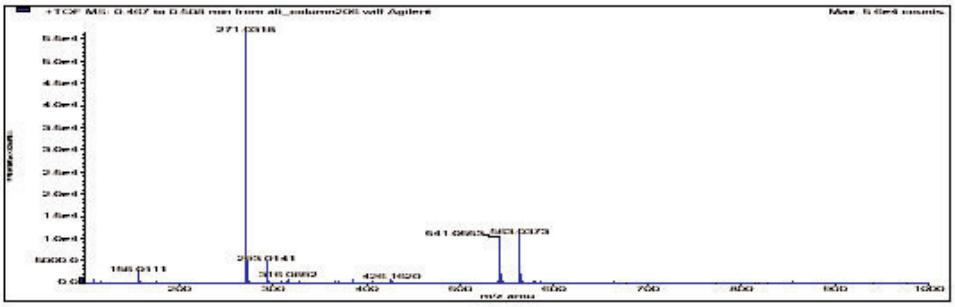
Mass List Report Page 1 of 3

Sample#: sulfa mix Sample Location: Sample Id: Operator:
Data File Name: d:\pe sciex data\Projects\report app note\Data\alt_column206.wiff Acq Time: November 19 2003, 04:44:23 PM
Method: D:\TOF_Data\damethods\EZX_EFG.anm\mass_list.xml



Period# : 1 Experiment# : 1

Peak#	Experiment#	Time	Area	Most Abundant Masses/scan
1	1	0.48	1.41327 E5	271.03181
2	1	0.54	4.38872 E5	279.09140
3	1	0.64	1.12099 E5	265.02057
4	1	0.72	3.52979 E5	311.08153



Peak# : 1 Experiment# : 1 Retention Time : 0.48 min

Mass Value = 271.03181

#	Formula (+1)	Calculated m/z	Error (mDa)	* Error (ppm)	DBE	Isotope Match	Intensity Match
1	C9H11N4O2S2	271.03180	0.01	0.1	6.5	997	66290
2	C10H17O2SCl2	271.03208	-0.27	-1.0	1.5	732	66290
3	C9H3N8O3	271.03226	-0.45	-1.7	12.5	998	62049
4	C17H7N2S	271.03245	-0.64	-2.3	15.5	0	0
5	C4H12N8S2Cl	271.03094	0.87	3.2	2.5	846	56655
6	C8H7N4O7	271.03093	0.88	3.3	7.5	999	62049
7	C19H8Cl	271.03090	0.91	3.3	15.5	937	66290
8	C7H12N2O7Cl	271.03276	-0.95	-3.5	2.5	0	0

Figure 1
First page of an Empirical Formula Generation report.

Detailed note: "Automated empirical formula generation for the identification of unknown compounds", *Agilent Technologies Application Note*, publication number 5989-2779EN **(Summer 2005)**.

Visit our website at **www.agilent.com/chem/tof** to download the detailed note.

Screening for target compounds using the LC/MSD TOF

Mass spectrometry has long been used to identify compounds using database searching. In GC/MS, databases of hundreds of thousands of compounds exist. These databases contain the electron ionization spectra of (EI) each compound in the database, typically reduced to 10 to 25 nominal mass values. The unknown spectrum is compared to the entries in the database and the hits are ranked using one of a variety of algorithms. In LC/MS a compound typically produces far fewer ions and the ones obtained vary greatly depending on the HPLC conditions used. The MS/MS spectrum can be used as it typically contains ions other than those indicating molecular weight. However, rather than identifying a compound based on the presence of many ions at nominal mass, another approach is to use simply an isotope cluster indicative of the molecular weight but using accurate mass measurement. In this case, the database need contain no spectral information at all, merely an empirical formula and the calculated monoisotopic weight. This note describes how this can be done with the Agilent LC/MSD TOF, an instrument capable of routinely providing mass measurements with better than 3 ppm mass accuracy.

Two approaches are used for doing this form of target screening or compound confirmation. The first is to extend the standard mass list report that takes each peak in the chromatographic signal and obtains a mass spectrum. This signal can be the Total ion chromatogram (TIC), base peak chromatogram (BPC), UV trace or the analog signal from another detector. The user selects possible adducts, charge states, etc to consider and how many masses in each spectrum to consider. For each ion, based on the adduct list, possible molecular weights are calculated. These molecular weights are compared against the entries in the database. The database is a comma separated value (CSV) file containing the following information for each compound:

Formula, Retention Time, Molecular Weight, Compound Name, Description

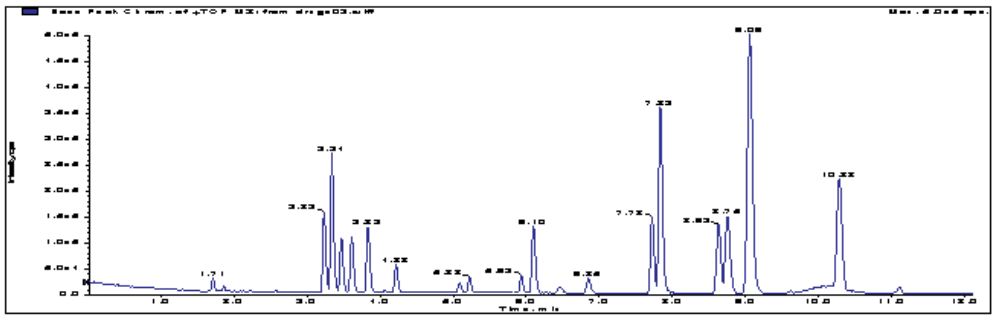
The retention time is an optional value that allows for excluding entries that do not fall within a specified window.

The second approach is to do a reverse search and is an extension of the empirical formula confirmation (EFC) report described in a previous note[1]. Instead of specifying a specific empirical formula to confirm, the user specifies a database and for each formula in the database, considers the selected adduct ions and generates an extracted ion chromatogram (EIC). An average spectrum is obtained if the EIC is of sufficient abundance and the ions in this spectrum are compared to the calculated *m/z* values. This type of search is very useful when looking for targets in complex matrices and the compounds do not show up as peaks in any of the possible signals.

As an example of this type of screen, a mixture of 14 street drugs[2] were searched against a database of 50 pharmaceutical and drug of abuse compounds. The search was based on the BPC from *m/z* 125 to 600. The first page of the report can be seen in figure 1. By considering retention time, the isomers hydrocodone and codeine are correctly identified.

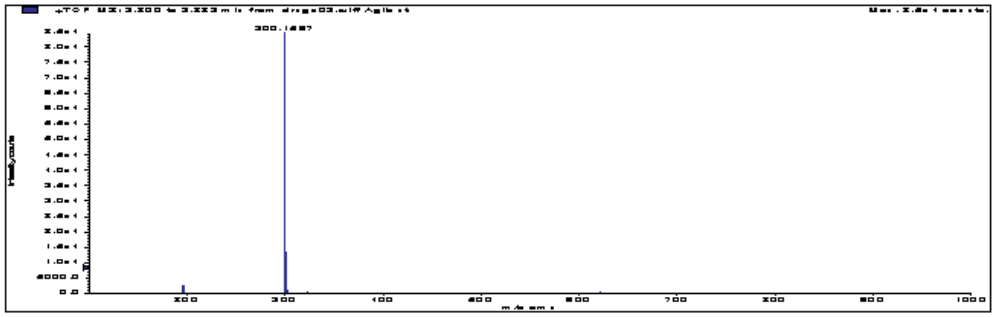
By using a database of known empirical formulas, the accurate mass capabilities of the LC/MSD TOF allow for confident identification of target compounds in unknown samples.

Sample#: street drugs Sample Location: Sample Id: Operator:
Data File Name: C:\PE Sciex Data\Projects\drugs of abuse\Data\drugs03.wiff Acq Time: February 06 2005, 03:53:47 PM
Method: C:\Program Files\Agilent\TOF Software\damethods\conf screen.anm\mass_list.xml



Period# : 1 Experiment# : 1

Peak#	Experiment#	Time	Area	Most Abundant Masses/scan
1	1	3.23	4.56218 E5	300.15969
2	1	3.34	7.95911 E5	148.11261
3	1	3.47	3.22841 E5	316.15462
4	1	3.61	3.41856 E5	300.15953
5	1	3.83	4.00653 E5	150.12826
6	1	4.22	1.64976 E5	290.13880
7	1	5.93	1.02273 E5	318.17038
8	1	6.10	4.58278 E5	244.20622
9	1	7.72	5.05485 E5	340.22748
10	1	7.83	1.30421 E6	310.21701
11	1	8.63	5.53416 E5	309.09064
12	1	8.75	7.03625 E5	271.06381
13	1	9.06	2.43672 E6	251.11872
14	1	10.28	1.04656 E6	285.07932



Peak# : 1 Experiment# : 1 Retention Time : 3.23 min

Mass Value = 300.15969								
#	Formula	Compound	Adduct	Mass	Error (mDa)	* Error (ppm)	Ret. Time Error	Description
1	C18H21NO3	Hydrocodone	[M+H]+	300.15942	0.27	0.9	-0.14	
2	C18H21NO3	Codeine	[M+H]+	300.15942	0.27	0.9	-0.58	

Figure 1
First page of an Empirical Formula Generation report.

Detailed note: "Screening for target compounds using the LC/MSD TOF", *Agilent Technologies Application Note*, publication number 5989-2780EN, **(Summer 2005)**.

Visit our website at **www.agilent.com/chem/tof** to download the detailed note.

Fast scanning for high throughput screening using the LC/MSD TOF

In pharmaceutical drug discovery, the large number of lead molecules being analyzed has put pressure on investigators to run increasing number of samples in less time than in the past. The use of short, small particle size columns run at relatively high flow rates now results in chromatographic run times of less than a minute. The resulting peaks elute with baseline peak widths of less than 2 seconds. This high-speed chromatography places demands on the total HPLC system. In particular, it is important that the detector acquisition rate produces enough data points across the peak without sacrificing the quality of the information. For mass spectrometer detectors, the traditional 1-2 spectra per second rate is no longer suitable.

The time an ion takes to pass through a quadrupole mass analyzer limits the possible scan speed unless resolution is sacrificed. For a time-of-flight analyzer, a single transient or scan takes place in 70 to 100 microseconds. At normal scan speeds, 5,000 – 10,000 transients are summed to get a single spectrum. If faster scan speeds are desired, one merely sums up fewer transients. Of course, at faster acquisition rates, the data processing requirements are much more demanding. The LC/MSD TOF is capable of acquiring spectra over a range of m/z 100 – 1000 at the rate of 20 per second or better, with no loss in resolution or mass accuracy. Because fewer transients are summed, the data will be noisier.

The LC/MSD TOF had the HPLC modified for high throughput as described in a previous note. 4.6 x 50 mm x 1.8 micron SB C-18 columns were used at 2 ml/min. Because of the high flow, a simple splitter using 2 5 cm pieces of 0.005" ID PEEK tubing and a tee reduced the flow to MSD to about 1 mL/min. This yielded peaks approximately 2 seconds wide at baseline. In order to obtain a strong ion indicative of molecular weight as well as fragment ions for structural information, a dual experiment analysis was performed. The fragmentor was toggled from 175 volts to 250 volts 10 times per second. This gave a total of 20 spectra per second or 10 per second at each voltage. Mass accuracy is still maintained and sufficient data points are obtained across even a 2 second wide peak.

The chromatogram obtained for a four-component mixture of sulfa drugs is shown in figure 1. Representative spectra are shown for both the low and high fragmentor voltages.

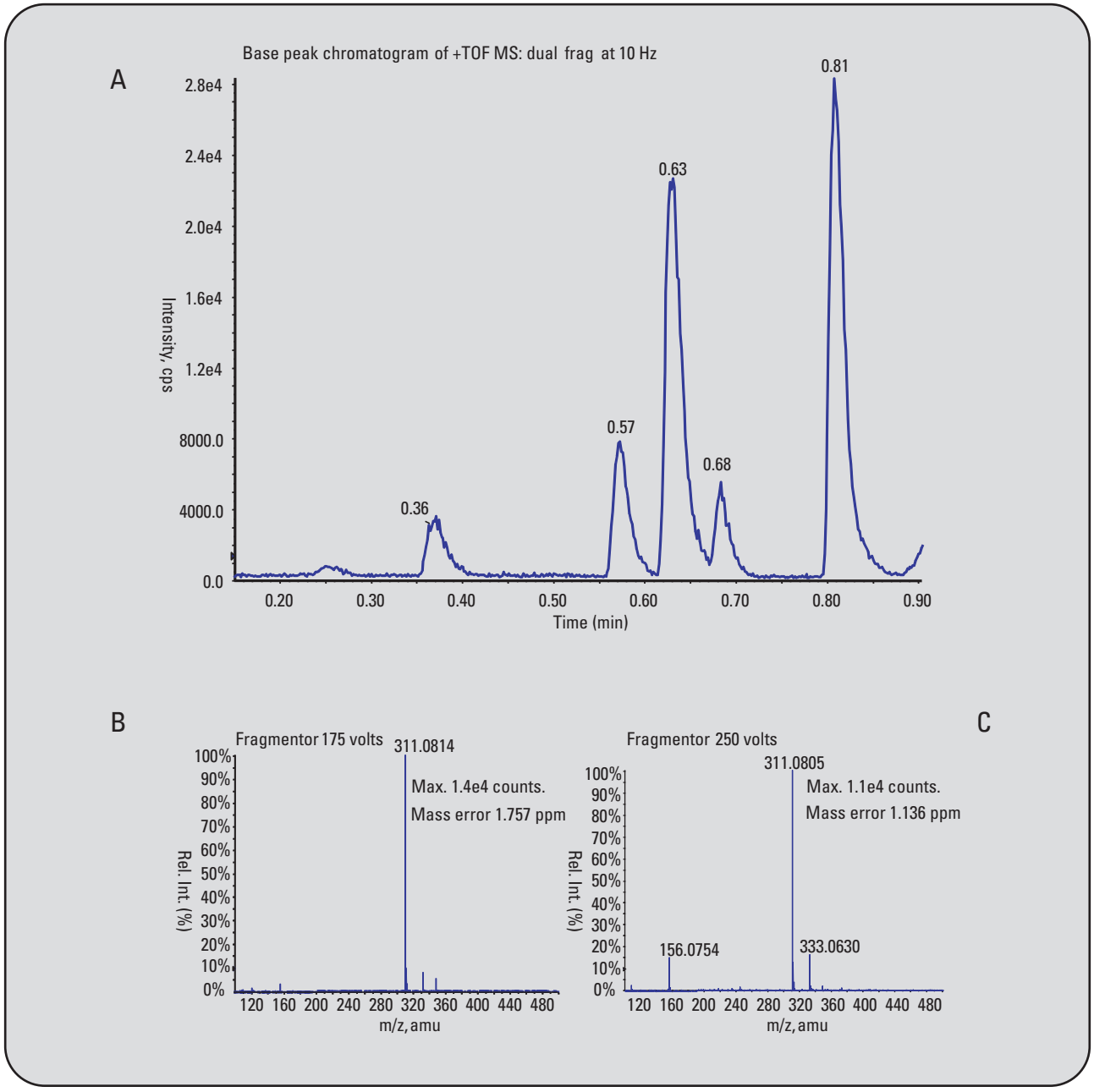


Figure 1
A) Base peak chromatogram of 4 sulfa drugs.
B) Spectrum of sulfadimethoxine at 175 volts.
C) Spectrum of sulfadimethoxine at 250 volts.

Detailed Note: "Fast scanning for high throughput screening using the LC/MSD TOF", *Agilent Technologies Application Note*, publication number 5989-2558EN, **(2005)**.

Visit our website at **www.agilent.com/chem/tof** to download the detailed note.

Structure elucidation of degradation products from the antibiotic drug amoxicillin by accurate mass measurement using LC-ESI-oaTOF MS

A final dosage from the antibiotic drug amoxicillin was degraded under induced stress conditions. Herein we describe the identification and confirmation of degradation products by structure elucidation with accurate mass determination of the molecular ions and of the CID fragments by LC-ESI-oa TOF MS.

In modern pharmaceutical drug discovery and development it is of crucial importance to identify an unknown compound with the highest possible degree of confidence because of its potential toxic effects on humans. The compound could be, for instance the pharmaceutical active substance itself, a minor byproduct of the production process, a secondary substance in a drug isolated from a natural source, a metabolite created in the human body or a degradation product of the pharmaceutical agent created under harsh storage conditions. In addition to the repertoire of analytical methods for structure elucidation a common method for the identification and identity confirmation of an unknown compound is the mass spectrometric determination of accurate molecular mass and consequently the calculation of the empirical formula. Several years ago only operation intensive magnetic sector field and FT mass spectrometers were able to perform these measurements with sufficient accuracy. Nowadays, with the advent of new TOF technologies comparably easy to use and inexpensive ESI orthogonal acceleration TOF instruments are also capable of handling this task. Recently published examples of an LC-ESI-TOF instrument used in structure elucidation are the identification of a photo oxygenation product of a broad-spectrum antibiotic for livestock as well as the identification of highly complex polyene macrolides isolated from Streptomyces noursei.
In this work the identification by structure elucidation of degradation products from the antibiotic drug amoxicillin obtained under stress conditions with accurate mass determination of the molecular ions and of the CID fragments with LC/ESI-oaTOF for the confirmation of the molecular formula and the fragment formulas is described.

Experimental

ESI-oaTOF instrument: Agilent LC/MSD TOF equipped with a dual sprayer source (positive mode) for the simultaneous infusion of the lock mass reference solution.

Dry gas:	7.0 L/min
Dry temp.:	300 °C
Nebulizer:	15 psi
Scan:	50-1000 *m/z*
Fragmentor:	150 V or 300 V for CID
Skimmer:	60 V
Capillary:	5000 V

LC system: Agilent 1100 Series capillary LC system containing a capillary pump with micro vacuum degasser, a micro well-plate autosampler with thermostat and a column compartment.

Solvent A:	Water, 10 mM ammonium formate, pH 4.1
Solvent B:	AcN
Column flow:	8 µL/min
Primary flow:	500-800 µL/min
Gradient:	0 min 0 % B, 1 min 0 % B, 13 min 25 % B, 23 min 25 % B
Stop time:	23 min
Post time:	15 min
Column:	ZORBAX SB Aq 0.3 mm x 150 mm, 3.5 µm

Sample preparation: To treat the antibiotic amoxicillin with acidic stress conditions a solution of amoxicillin (25 mg/µL) in 0.1 M HCl was stirred for one hour at room temperature (RT = 25 °C).

Results and discussion

The degradation of Amoxicillin (1) was induced by subjecting the pure drug substance to harsh conditions as described in the experimental section. Dilutions of this solution were collected at various time frames and subjected to capillary chromatography to separate the accumulated degradation prod-

ucts. The obtained total ion chromatogram (TIC) clearly shows the degradation of amoxicillin into various products (figure 1). The outlined chromatogram also assigns also the degradation products, which were identified by structure elucidation with accurate mass determination using ESI-TOF MS for the confirmation of the molecular ions and the CID fragments. The measurement was performed twice using different fragmentor voltages of 150 V and 300 V in the TOF MS. When the fragmentor voltage is set at 150 V there is no collision induced dissociation (CID) observed whereas a good fragmentation for the molecular ion from the separated degradation products is observed at a voltage of 300 V. The first degradation product of amoxicillin (1) obtained after braking the four membered beta lactame ring amoxicillin penicilloic acid (2) was confirmed by accurate mass determination with the ESI-TOF MS with *m/z* 384.1212, with 4.50 ppm mass accuracy and formula calculation (insert in figure 1). The structure of this degradation product was elucidated by the appearance of specific fragments in the ESI-TOF measurement applying a fragmentor voltage of 300 V with *m/z* 323.1063 with 0.78 ppm mass accuracy, which is the product of a deamination followed by a decarboxylation reaction (figure 2). The complete fragmentation pattern of amoxicillin penicilloic acid (2) also shows the fragments with *m/z* 189.0697 and 122.0605 with a respective high mass accuracy of 0.39 ppm and 0.70 ppm, which are important for the structure confirmation (insert in figure 2).

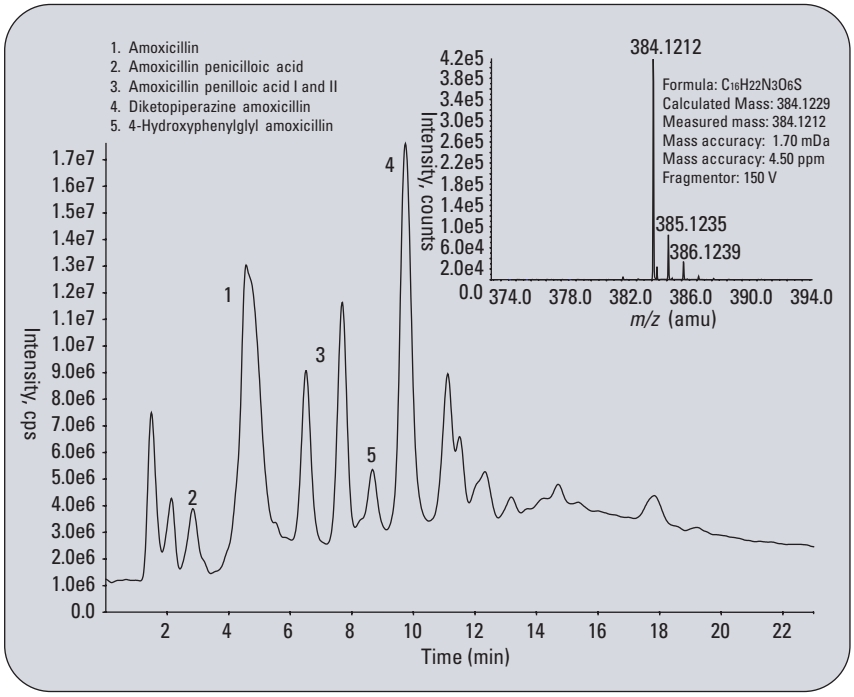


Figure 1
Total ion chromatogram of TOF from amoxicillin (1) and its degradation products. Insert: Exact mass determination of amoxicillin penicilloic acid (2) ($C_{16}H_{21}N_3O_6S$), [M+H]=384.122 *m/z*.

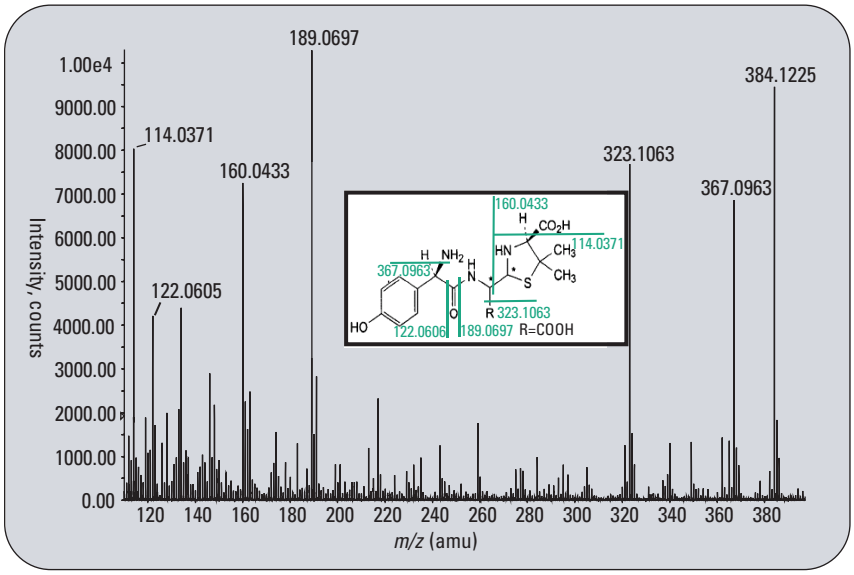


Figure 2
Accurate mass determination of the CID fragment ions of amoxicillin penicilloic acid.

The complete information obtained from the CID fragments of amoxicillin penicilloic acid (2) is summarized in table 1 with their molecular formula and the measured mass accuracies. In the degradation pathway of amoxicillin (1) several other compounds were identified. For instance, there is the product of a decarboxylation of (2) the stereo isomeric amoxicillin penilloic acids I and II (3), the product built by the formation of a six membered ring in (2) the diketopiperazine amoxicillin (4) and the product of a reaction of amoxicillin (1) by itself the 4-Hydroxyphenylglyl amoxicillin (5). The elucidated degradation pathway is shown in figure 3.

Measured mass	Calculated mass	Formula	Mass accuracy [mDa]	Mass accuracy [ppm]
384.1225	384.1229	$C_{16}H_{22}N_3O_6S$	-0.40	1.12
367.0963	367.0964	$C_{16}H_{19}N_2O_6S$	-0.10	0.22
323.1063	323.1066	$C_{15}H_{19}N_2O_4S$	-0.30	0.78
189.0697	189.0698	$C_7H_{13}N_2O_2S$	-0.10	0.39
160.0433	160.0432	$C_6H_{10}NO_2S$	0.10	-0.46
122.0605	122.0606	C_7H_8NO	-0.10	0.70
114.0371	114.0377	C_5H_8NS	-0.60	5.60

Table 1
Mass accuracy of all CID fragments of amoxicillin penicilloic acid.

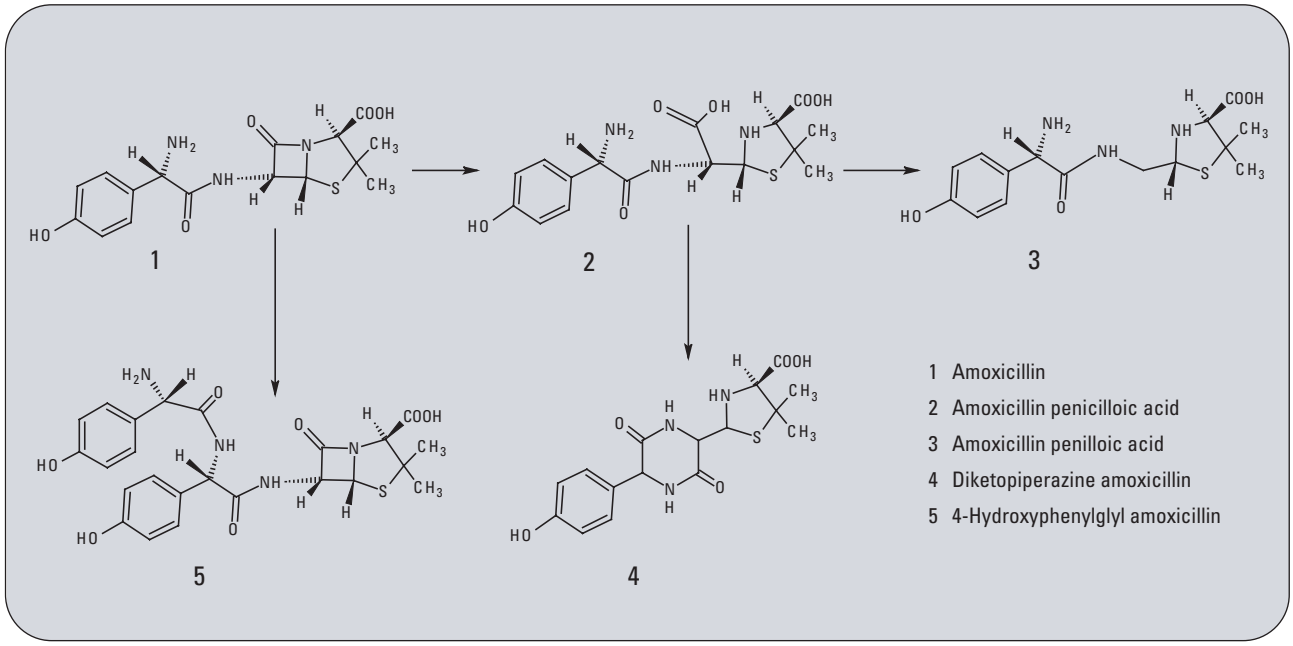


Figure 3
Degradation pathway of amoxicillin.

Conclusion

The degradation products synthetically created from amoxicillin, which may also be formed under harsh storage conditions, were separated by capillary LC and analyzed by LC-ESI-TOF MS. The molecular mass obtained for the molecular ions of the degradation products and the molecular masses of their CID fragments, which were measured with highest mass accuracy of less than 1 ppm by ESI TOF support the suggested amoxicillin degradation pathway. The identity of the proposed products was confirmed by accurate mass measurement with ESI TOF and empirical formula calculation for the molecular ions of the degradation products.

Detailed note: "Structure elucidation of degradation products from the antibiotic drug amoxicillin by accurate mass measurement using LC-ESI-oaTOF MS", *Agilent Technologies Application Note*, publication number 5989-2348EN **(2005)**.

Visit our website at **www.agilent.com/chem/tof** to download the detailed note.

wwww.agilent.com/chem/tof

Printed April 1, 2005
Publication Number 5989-2549EN

Agilent Technologies