The use of UHPLC technology in combination with fast Electrospray Mass Spectrometry has advanced the speed and efficiency of research in demanding fields of Organic Synthesis and Catalysis.

In the above diagram, the key features of the Agilent 6140 UHPLC-MS instrument are shown:
1. The **1290 Binary Pump** module delivers water and acetonitrile at pressures up to 1200 bar. It also includes a degasser, an auto-purge and a “Jet Weaver” microfluidic.
2. The **1290 Well-Plate Autosampler** accepts standard HPLC vial trays and 96-well plates.
3. The sample flows through a 2 µm filter to a 2.1 x 50 mm Eclipse Plus 1.8 µm particle size C18 column (part No. 959757-902) at a flow rate of 1 mL/minute and 700-800 bar.
4. The **1290 Diode Array Detector (DAD)** with a 60 mm path length microfluidic flowcell detects the absorbance a 6x improvement in sensitivity over standard 10 mm flowcells.
5. The **6140 MS Single Quad MS detector** with standard ESI source is specifically designed to provide fast data acquisition on a mass range of 50-1350 m/e range.

**Comparison of 1 ng injection on 1100 LCMS vs. 1290 UHPLC-MS:**
- 2-4x Improvement in Resolution, 10x Improvement in Sensitivity
UHPLC-MS System Operation with Agilent Chemstation and Easy-Access

The Agilent UHPLC-MS is operated using an **Online Chemstation** window that runs the instrument and an **Offline Chemstation** window that is used for **data analysis**. In addition, **Agilent Easy-Access** software is used to efficiently handle multiple users in a walk-up format.

I. How to submit samples to the Easy Access queue.

- Hit “Submit New Sample” and enter your login account and password.
- Select the number of samples to submit and enter the **Sample ID(s)**.
- Choose from the set of available Methods. Either **Positive** (PES) or **Negative** (NES) detection can be used in LCMS electrospray. However, positive electrospray ionizes a wider range of compounds. The methods have run times of 4, 7 or 10 minute lengths. The “AX” methods have a **100-800 amu** mass range. For compounds that are above 190 amu, the “BX” has **190-800 amu** (which eliminates low MW impurities from the MS trace) and “CX” runs from **190-1350 amu**. If no other annotations are included, the gradient proceeds from 5% to 95% acetonitrile during the run time and the MS detector is bypassed until t = 0.5 min in order to minimize salts entering the MSD. For polar compounds, the _MSOpen0min can be used to open the MS at t = 0 min. Both the default Inj. Vol. and the UV_A wavelength may also be changed.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PES10BX</td>
<td>Pos. 10 min run, 5-95% ACN, 190-800 amu</td>
</tr>
<tr>
<td>PES4AX_20-95</td>
<td>Pos. 4 min run, 20-95% ACN, 100-800 amu</td>
</tr>
<tr>
<td>NES7CX_MSOpen0min</td>
<td>Neg. 7 min run, 5-95% ACN, 190-1350 amu, MS valve open 0 min</td>
</tr>
</tbody>
</table>
II. Common Questions on Samples:

• How much sample do I need? The LCMS easily detects analytes at the 1 ng level which corresponds to a 1 µL injection from a 0.001 mg/mL sample. However, most samples are about 0.1 mg/mL and give UV peaks ~ 500 mAu. Samples that are at or above 1 mg/mL are unnecessarily concentrated and will give poor data and diminish the life of the instrument.

• What solvents are OK? Acetonitrile is available for making up samples at the instrument. Most other standard organic solvents and water are suitable for sample submission. Chloroform and dichloromethane can affect the peak shapes if used at higher than 1 µL injection volumes.

• How much solution do I need? The WP autosampler needs about 0.5 mL in a hplc vial as it draws from about ¼ inch above the bottom. Vial inserts are available for smaller sample sizes.

• Do I need to filter my sample? No, unless the sample is obviously heterogeneous.

• Can I submit “crude reaction mixtures”? Yes. For reaction monitoring, usually a tip of a pipette diluted to 1 mL will provide a suitable sample.

• What about biological samples? Well, the worst thing for a C18 column to see is biological material including membrane proteins, phospholipids and biopolymers. These materials need to be removed from LCMS samples prior to submission or they will clog the column and degrade the peak shape of analytes.

Further notes on sample submission:

• When submitting more than 3 samples at a time, select the “Night Queue” to allow other samples to run first.

• For samples that are highly lipophilic, 20-95, 40-95 or 60-95 gradients can be used to improve separation and allow shorter run times.

III. Data Analysis

1. Auto-Print From Easy Access – check the box “Print Report” when submitting samples.

2. Snapshot – while a sample is running, Select “Snapshot” in the Offline Chemstation.

3. Load Signal to access previously run Data – then select file on C:/ or D:/ (old data) drive. If only one signal chromatogram is desired, it may be selected individually when loading.

IV. Tools for Data Analysis

When a data file is loaded, it will usually consist of three DAD chromatograms plus one MSD trace. They can be viewed individually or all together by selecting “All Loaded Signals”. In addition, five buttons above the data provide different tools for Integration, Signal selection, etc.

The “Spectrum” button allows one to view the Mass Spectrum or UV spectrum of a selected peak.
Shown below, the dragging button delivers a spectrum for the region that the user drags the mouse over. The UV spectrum is created by subtracting the red and blue UV absorbances from the dragged region in order to compensate for solvent absorbance (the acetic acid used in the aqueous mobile phase absorbs in the 210 – 230 nm range).

**Extract Ions** – used to search for a mass or to evaluate the MS purity of a peak:
Modifying Data Analysis Preferences – The first thing to know is how to get back to the default “LCMS ANALYSIS” Method since previous users often change settings.

Methods for Integration:
2. Using “Integration Events”, the integration settings may be adjusted:
   - To remove a peak from the integration use this tool.
   - Select trace to be modified then "Integration Events".
   - Hit Green Check Mark when done.
   - Select "Integrate" to re-integrate.
UHPLC-MS Data Analysis (continued)

**Printing Report:** The “Specify Report” Window is used to select the format and destination of the printout. When re-loading the LCMSANALYSIS method, it is set to the printer. One can preview by selecting “Screen” or print to file formats such as pdf. When satisfied, Select Report:Print Report.
The Agilent 6230 TOF LC-MS system allows high-resolution mass measurement of analyte ions generated by electrospray or other methods with 1-5 ppm accuracy (0.0005-0.0025 amu for a 500 MW compound) suitable for publication or elemental composition determination. Several design improvements including the 4 GHz detector sampling rate and the Agilent Jet Stream Source (AJS) have increased the sensitivity and stability of the 6230 detector over previous models. Due to the simultaneous sampling of all masses, the Agilent 6230 TOF achieves sensitivity of 1000-10,000 fold over that of quadrupole LC-MS detectors. For these reasons, TOF LC-MS can greatly accelerate the pace of discovery in organic chemistry research.

This primer is organized to assist in the use of the LC-TOF instrument including:

1. Understanding the basic features of the TOF detection method.
2. Sample Preparation Guidelines
3. Data Analysis
4. Standard troubleshooting and evaluation of results

**Training on the TOF Instrument = Respecting the incredible sensitivity of this instrument.**

- The TOF must never see sample concentrations at the 0.1-1 mg level that are customarily submitted to other instruments. All samples need to be diluted properly to avoid contamination and damage to the TOF detector.
A Time-of-Flight MS detector offers a dramatic advancement over the quadrupole MS detector in sensitivity and accuracy. A quadrupole LCMS operating as a “tunable mass filter” is limited by almost nominal mass resolution and requires cycling of the voltage and RF through a range for each scan. For TOF instruments, packets of ions accelerated into the flight tube are separated temporally based on mass. MALDI-TOF instruments using nanosecond laser pulses can achieve high mass resolution in simple reflected geometries. LCMS-TOF instruments require an orthogonal geometry that allows extraction of ion packets by a pulser.

**Quadrupole:** ~200-300 ppm (2-3 scans per second)

**Simple Reflectron TOF:** (not suitable for LCMS design)

Orthogonal-Acceleration Time-of-Flight: 1-3 ppm 10,000 scans per second!

Design of the LCMS-TOF ion optics and ion path achieves entropic cooling of the ion stream leading up to the pulser. The pulser, operating at ~6000V, pushes each ion packet into the 1 meter long flight tube with the same kinetic energy in the z-direction. The spread of velocities from K.E. = ½ mv² leads to a series of arrivals at the detector in the 20-100 microsecond range. Mass differences of 1-2 ppm in the 100-2000 amu range require the time measurements to be accurate to fractions of a nanosecond – an early challenge to the TOF technology.

\[
\Delta t = \sqrt{\frac{\text{KE}}{eV}} = \sqrt{\frac{\text{KE}}{1.602 \times 10^{-19} \text{J}}}
\]

With a linear time-of-flight geometry, the spread of initial ion velocities (due to their depth in the pulser at the time of extraction) would be too great to achieve ppm mass resolution. Use of an ion mirror and placement of the detector at the time-of-flight focus position allows correction for the velocity spread of all ion masses simultaneously.

The raw time data obtained for a series of ions of known exact mass (the tuning mix) agrees with square relationship above to ~ 2-6 ppm using \(a\) and \(t_0\) adjustments. A residual mass error polynomial correction is performed by calibration to the tuning mix and achieves a ≤ 1 ppm fit.
1. **Reference Ions** The TOF instrument needs to deliver data with ≤ 1 ppm accuracy in an environment which has inherent instability. If the temperature of the room changes by 1 °C, will the 1.000000 meter flight tube lengthen by 1 micron? How about the electronics of the medusa (power distribution board) that have to convert the voltage from the wall to 6500V for the pulser, are there 1 ppm fluctuations in that process? Any high-resolution experiment must include reference ions so that the data may be adjusted to the correct high-resolution value. In positive ESI, the TOF uses ions 121.0509 and 922.0098 as reference ions to adjust the raw data with $a$ and $t_0$ coefficients.

2. **Transient Integration** The process would be easier if each pulse event contained both reference ions and the ion of interest racing each other through the flight tube. But only about 1% of individual spectra contain a given ion (calculate 0.1% of $10^{11}$ ions being chopped into 0.50 ns packets) and the events are arriving so fast at the detector that they can’t be sent to the computer individually. So the TOF detector (multichannel plate and photomultiplier tube) accumulates the spectra in packets of $10^4$ transients that are sent to the computer each second.

3. **Centroiding** When the TOF is operating in “2 GHz mode” the time axis of the spectra contains data points separated by 0.50 ns. But we said that 2 ppm accuracy for a 450 MW compound corresponds to a $\Delta t$ accuracy of 0.04 ns. *How does the TOF achieve this leap of resolution?* The TOF uses the intensity of each data point near the top of the peak to discern where the “top of the peak” lies between the two data points separated by ~ 0.0050 – 0.0100 amu. If the peak is saturated (sample too concentrated), this process fails. (The math details of centroiding are known as “ion statistics”.)

4. **Spectral Averaging** In order to determine an accurate mass, we need several of these 1 second spectra (not just one!). Using multiple spectra in the chromatogram increases the signal so that the maximum effect of centroiding can be achieved. We can see as we watch the individual spectra in real time that the 922 mass moves around each second. Sometimes 922.0063, sometimes 922.0120... In order to add spectra across the chromatogram, we can’t apply individual mass corrections to each spectrum or they won’t line up.

So a single $a$ and $t_0$ correction is used for the entire run. When we select a portion of the acquired spectra, we may need to recalibrate the spectra to get the most accurate results.

I. Sample preparation:
For the TOF, you will need your sample within a concentration range of 0.1 to 1 \( \mu \text{g/mL} \) so that a 1 \( \mu \text{L} \) injection will send, ideally, ~0.1-1.0 ng into the system. (You can get good results if you have even as little as 0.01 \( \mu \text{g/mL} \)). The easiest way to achieve this is to take one small drop from a “good” low-res LCMS sample (0.1 mg/mL) and dilute it into 1-2 mL of clean ACN in an HPLC vial. Once you prepare your sample at TOF dilution, be careful not to post-contaminate it by leaving it in contact with a dirty cap, etc. (Please don’t try to make a TOF sample directly from the solid.)

II. Click “On” in the Acq window
• When you start the TOF, it will prepare for the run by the 3 steps shown (~ 6 min for the TOF pane to turn green (Ready).
• When you start your run in IIA or IIB below, the TOF will proceed with steps 4 & 5. You want to see the Reference Ions appear in Step 5 (if not bottle A may be empty). They don’t need to be exactly 121.0519 and 922.0098, but if they are very far off, then the TOF needs to be recalibrated.
• This figure also shows what good samples and bad samples look like (you don’t want TIC >1E8).

IIIA. Submit SINGLE SAMPLE.
• Click “Method Editor” on the bottom pane of the TOF Acq Software. Open a Method.

The TOF has Positive and Negative ESI Methods, DI. Usually, the PosDI_50ACN will be the best run to start with. If a compound is especially lipophilic, 85% ACN may help keep it in solution on a DI run.

→ You can Open the method that you plan to use while the TOF is getting ready in Step II.
• Click “Sample Run”.
• Enter Vial Position, Inj Vol, FileName.d and choose your Data Folder from “Path”.

• Hit Start. If your run proceeded with the reference ions found, the single sample vial icon will be green. You will have to click the “Off” button on the screen to put TOF in Standby when you are done.
IIIB. Submit WORKLIST (Sequence)

- Click “Worklist Pane” then Click “Open Worklist” NEW_WORKLIST.w.
- Then add you runs either one at a time or “Add Multiple Samples”.
  You can choose Inj volume from 0.1-3 uL or just leave it as “As Method” to inject 1uL .
- Set your data folder in the “Data File Settings” tab in “Worklist Run Parameters”. With the settings shown, your files will be saved in a datestamped subfolder inside your personal folder with the Data File name of each line.
- Under “Run Parameters”, the “Acq Clean-up: SCP-Standby” will automatically put TOF in Standby when your worklist is done. • Hit Start.

III. Open and Examine your file in the TOF Qualitative Analysis Program.

The key idea is that a sample that is too strong cannot deliver suitable Hi-Res data. Of course, some compounds ionize more readily than others, but with practice, you will become adept at submitting samples within the correct concentration range for the TOF.

Step 1-3. Find (or Open from the desktop) the Qualitative Analysis Program, CloseAll unwanted previous data and Open your data file from D:\Projects\New\Data. The data will open with your TIC Chromatogram as shown. Then left-click-drag to select a portion of your chromatogram.

Step 4. Extract MS Spectrum.

In “Range Select” mode, you can manually select the portion of the peak to extract your MS spectrum from.

Left click and drag to select the region of interest, then right click within the region and select “Extract MS Spectrum”.

Step 5. Examine Your MS peak.

If it is saturated (with a star) or just scary looking, you need to run it again at lower concentration.

Step 6. Examine the Reference Ions

For Pos mode, the two reference ions should be 121.0509 (purine) and 922.0098. For Neg mode, 119.0363 and 1033.9881.
Step 6. Examine the Reference Ion Masses. If the reference masses are exactly correct, then you are done. But if they significantly differ from the correct values, then you can recalibrate the spectrum to obtain the calibrated accurate mass. The reason that they can slightly off is described in the section “Spectral Averaging”.

Step 7. Recalibrate the Spectrum. After highlighting the MS spectrum of choice, click in the MS field and select “Recalibrate”. Click on “Calculate manual recalibration and apply” button.

Printing Your Results:
Short Report: Select File: Print>Workflow Report (Ctrl+P) and hit OK to get a printout of the DI run, the MS spectrum and the peak table.

Report with Formulas:
Open the Method Editor and select “Identification:Generate Formulas” Then hit the Play button (>) to apply a formula search to the peak table (see page 8). When you print this report (Ctrl+P), the printout will include a table with the proposed molecular formulas.

➔ In many cases, working up a DI run can take about 15 seconds: Open data file, drag to select the peak, extract MS spectrum, ref ions OK √, print.
The new accurate mass value (240.1384) is not much different than the old one (240.1386). But it is the correct result to report. In most cases, the recalibration will lead to only a minor correction and either number would be within 5 ppm accuracy. Now that we have the calibrated accurate mass, we will use the MassHunter software to provide a Molecular Formula.

But first, consider what our accurate mass value represents.

1. **Ionization** – LCMS generally produces "even electron ions", that is (M+H⁺), (M+Na⁺) and the like. Only extremely electron rich compounds (like methoxyanthracene) will produce "odd electron ions" like M•⁺ under LCMS conditions. If you use Chemdraw to calculate the exact mass that you are looking for, you need to account for the fact that it is a cation or you will be off by the electron mass.

2. **The Electron mass** – If you use Chemdraw to calculate the exact mass that you are looking for, you need to account for the fact that it is a cation or you will be off by the electron mass.

3. **The Nitrogen Rule** – Because Nitrogen is the only element that has an odd valence and an even mass of its most abundant isotope (¹⁴N), a compound that has an odd number of nitrogens will have an odd mass and therefore an even (M+H⁺) or (M+Na⁺) ion. Since our mass is even, it has an odd # of nitrogens.

4. **Isotopic Series** – For any compound, we will see an isotopic series for the ion ascending to higher mass in units of 1 amu with decreasing intensity of each peak. If we see the peaks separated by ½ integral mass units, then the ion is a +2 charge. If we see an isotopic peak (M-1)+H⁺ of any significant intensity (like >10%) it could indicate the presence of a boron for which ¹⁰B/¹¹B ≈ 0.2. Alternatively, a highly electron rich compound could produce some M⁺ ion which would be 1amu less than M+H⁺.

4. **The Mass Defect** – When we ask the Formula Calculator to calculate our molecular formula, it will try to combine elements to produce our accurate mass number. The four digits after the decimal represent the sum of the combined mass defects of all the elements contained in the ion minus the electron mass. The spectrum above with several contaminants in the 200-240 range would be a pretty embarrassing TOF run, but it serves to demonstrate a collection of ions with different mass defects together. Our ion, 240.1383, has a mass defect of 0.1383. We also see 208.1901 (lots of hydrogens) and 197.0001 (heavy atom contributes negative mass defect). We can also appreciate that if we ask the formula calculator to provide a formula for 240.3383, there is no way it can come up with a mass defect of 0.3383 for a 240 MW compound. For compounds containing only C,H,N,O our four-digit mass defect will be approximately the # of ¹H’s/80 because most of the mass defect will be coming from the hydrogens in our compound.
Step 7: Using the Formula Calculator. Under Tools, select the Formula or Mass calculator. Plug in the mass from your spectrum and select charge (+1 or -1) and the ion type (M+H⁺), (M+Na⁺) usually. You may occasionally find (M+NH₄⁺) arising from the small amount of ammonium trifluoroacetate present in the tuning or reference mix. In addition, you may notice (2M+H⁺) or (2M+Na⁺) ions for some compound types.

IV. What constitutes a “publishable” HRMS result.

Finally, appreciate that journals that allow HRMS data as evidence of a compound’s molecular formula (in lieu of Elemental Analysis, for example) are placing a great degree of trust in the mass spectroscopist to provide acceptable data. Most journals do not require the raw MS data, only “C₁₆H₁₇NO calcd for (M+H⁺): 240.1383, found: 240.1380”, so you should be extra diligent in producing data that is solid. Here are four guidelines:

• Don’t use data from runs that have too much compound and saturated peaks.
• A Hi-Res hit for publication should come from a DI run (not one peak of many in an LC run).
• Use data from the entire mass peak – not just the part that gives you your number.
• Always recalibrate the data to produce a spectrum that has correct reference masses.
• Don’t get creative with compound ions and fragmentation ion assignments. Keep it simple.

When you use the TOF to determine the molecular formula of an actual unknown, you will come to realize that the 5 ppm publication range isn’t much use in identifying a compound over 300 MW. For this reason, you should expect the TOF to regularly deliver High-Res hits within 1-2 ppm.
If you choose an LC run of 4, 7 or 10 minutes, your peaks will come out a little later than the similar runs on the UHPLC-MS instrument because the TOF uses a similar 2.1x50 mm C18 column (Agilent #727700-902 600bar) at 0.5 mL/min (instead of 1 mL/min).

Since the TOF is so sensitive, a normal sample will be \( \leq 1 \) nanogram and will often barely register on the G1315 Agilent DAD detector (0.5 – 2.0 mAu usually) for most compounds.

You can select the MS peaks individually or integrate the MS chromatogram using Actions>>Integrate and Extract Peak Spectra, the unwanted peak can be deleted as shown and each chromatogram peak can be recalibrated if necessary to give accurate mass values for identification. The threshold can be raised to limit the size of the MS PeakList or reduced to deliver the masses of smaller peaks. In order to print the results, select “File: Print>Workflow Report” as shown.