UPLC Protocol – Waters Acquity LC/MS – Research Instrumentation Center

Instrument Overview

LC/MS is useful for analyzing complex mixtures qualitatively or quantitatively and can inform on the mass and polarity of compounds in your sample. The instrument consists of a Waters H-Class UPLC system with a SQD2 single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. Samples are placed in autosampler vials and injected into a flow of mobile phase (usually a combination of water and methanol or acetonitrile) which transports the sample to a reversed phase C18 UPLC column (a nonpolar stationary phase) where analytes are separated primarily by size and polarity. Due to weak interactions with the nonpolar stationary phase, smaller and more polar compounds will tend to elute from the column and be detected by mass spectrometry first, while nonpolar and larger compounds tend to elute later in the LC/MS run. After LC separation the mass spectrometer measures the ionized analytes' *mass-to-charge (m/z)* ratios. Both UV and fluorescence detection are also available.

Appropriate Sample Types & Sample Preparation

- Reversed phase LC/MS is useful for separating and detecting organic molecules (~50 Da to 2,000 Da in mass), peptides, small proteins, and organometallic complexes in complex mixtures
- Samples should ideally be no more concentrated than 0.1 mg/mL. Double the concentration if you do not get any peaks. You should not be getting TIC (total ion counts) larger than e9.
- Please make up samples or dilute by at least 1 order of magnitude in H_2O, methanol or acetonitrile. Highly organic solvents (e.g. toluene, hexanes, anything less polar than acetonitrile) should be avoided.
- Samples should not contain corrosive materials, *e.g.* strong acids and bases, or high concentrations of nonvolatile salts
- Remove all particles from the sample before the analysis using 0.2 µm filters

Column (stationary phase)

- UPLC BEH C18 Column (nonpolar), 130 Å, 1.7 µm, 2.1 mm x 50 mm
- Users may supply their own reversed phase column but should consult with RIC staff prior to making any changes to the instrument

Solvents (mobile phase)

- **Solvent A:** H_2O + 0.1% Formic Acid **Solvent B:** Acetonitrile + 0.1% Formic Acid
  Solvents C, D & Purge: 30% Methanol + 70% H_2O
  Sample Manager Wash: 25% Acetonitrile + 25% Methanol + 25% Isopropanol + 25% H_2O
  Seal Wash Solvent: 70% Acetonitrile + 30% H_2O

Common Mobile Phase Conditions During the LC Run

- *Isocratic*: Mobile phase composition does not vary with time
  - *e.g.* 70% A + 30% B OR 100% C over 10 min.
- *Gradient*: Mobile phase composition varies with time, usually % organic increases linearly
  - *e.g.* (Initial conditions) 95% A + 5% B to (final conditions) 100% B over 10 minutes
- The mobile phase should ALWAYS equilibrate back to the initial conditions at the end of the run

Starting the instrument

1. *Prior to logging into the computer*, turn on the UV detector wait for 1 beep, 3 beeps, and then another 1 beep. Then log in to the computer.
2. Click on *Masslynx* icon to open the instrument control software.
   a. **Make sure to open your own project folder. DO NOT work inside anyone else's folder.**
3. Prime the solvent lines to get rid of any residual bubbles:
   Open *MS Console* and go to solvent manager then click on "Control" → "Prime solvents"
   - Prime A and B, 3 min each.
   - Under "Final Conditions" ensure that the solvent composition and flow rate match the initial mobile phase conditions of your LC run
4. Let the column equilibrate in the initial mobile phase for about 2 min.
   a. While the column and mobile phase are equilibrating, you can set up your method files and sample list.

Setting up an experiment

There are 3 files needed to set up the experiment,

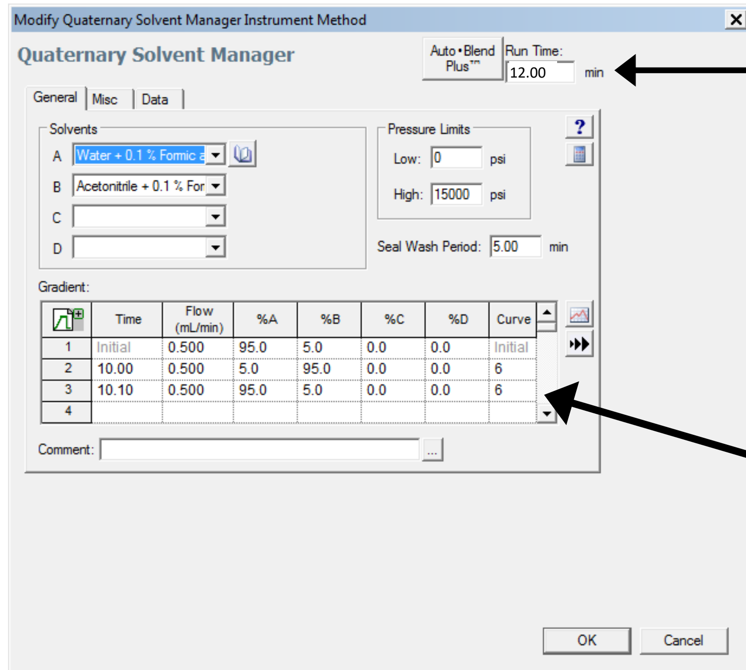
1) *Inlet (LC) method file*: In this file you will specify the overall LCMS runtime (the length of your LC elution program), the mobile phase composition as a function of time (isocratic or gradient), the autosampler temperature control and wash time, and the UV and fluorescence wavelengths to monitor.

2) *MS method file*: In this file you will specify the mass range you want the mass spectrometer to monitor, the scan time (1 / # of scans per second), and the ion polarity (+ or - or both).

3) *MS Tune File*: This file holds details about the best voltages, temperatures, and gas flow rates for optimum sensitivity in the mass spectrometer. Typically you should use the file called "default", "tuning_mix", or "LCMS_tune". These files have been optimized for sensitivity by RIC staff.

A. Setting up the Inlet (LC) method file

1. On the sidebar on the left in *MassLynx* open "Inlet Method". Then click "Inlet". Set up the following conditions under the "Gradient"

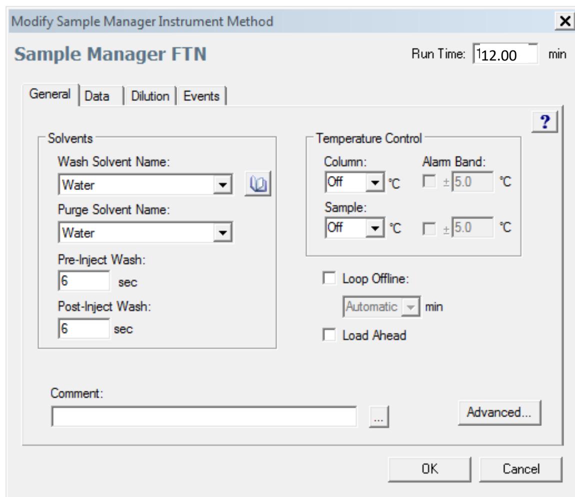


2. Set the total run time of your method. A typical runtime includes 10 min. of LC separation (gradient elution) followed by ~2 mins to equilibrate the column back to the initial mobile phase conditions. A longer runtime will typically increase resolution.

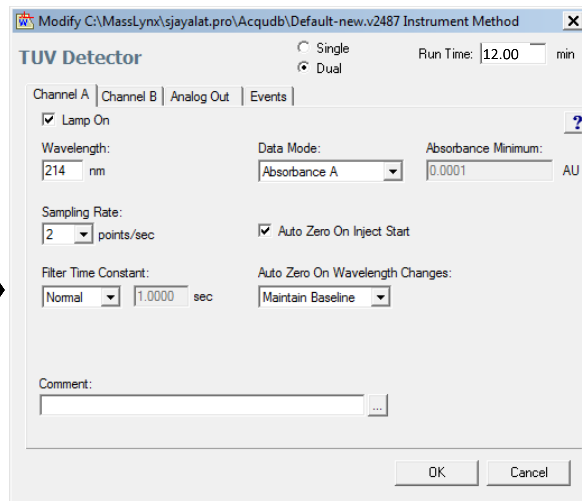
3. Edit your gradient here. For *isocratic elution* you only need to specify the initial conditions. For *gradient elution* you typically vary the solvent composition from 95% water + 5% organic to 100% organic over ~10 mins. You must include:

1. Initial conditions
2. Final conditions
3. Equilibration period to return to initial conditions
4. For “Curve” use “6” (linear).

4. Click “OK”
5. Click the Autosampler icon in the Inlet window. window



10. Click TUV Detector icon in the Inlet



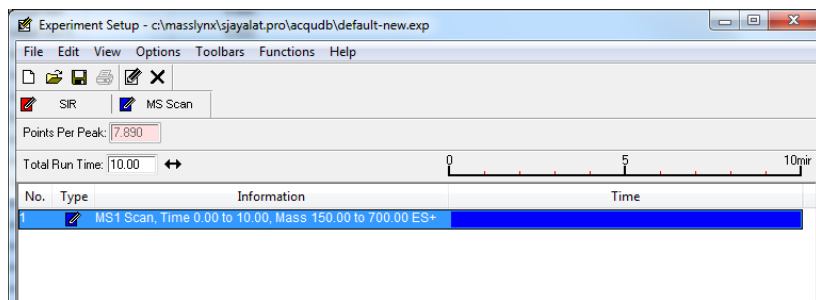
6. Input the same total Run Time as in the previous window.
7. Set the column temperature (range 20 to 90 °C)
8. Set the autosampler (“Sample”) temperature (range 4 to 40 °C)
9. Click OK

11. Input the same total Run Time.
12. Select the wavelength mode (single or dual) Input the wavelength(s) in Channel A (and B) tab(s). Typical wavelengths are 214 nm and 254 nm.
13. Click OK

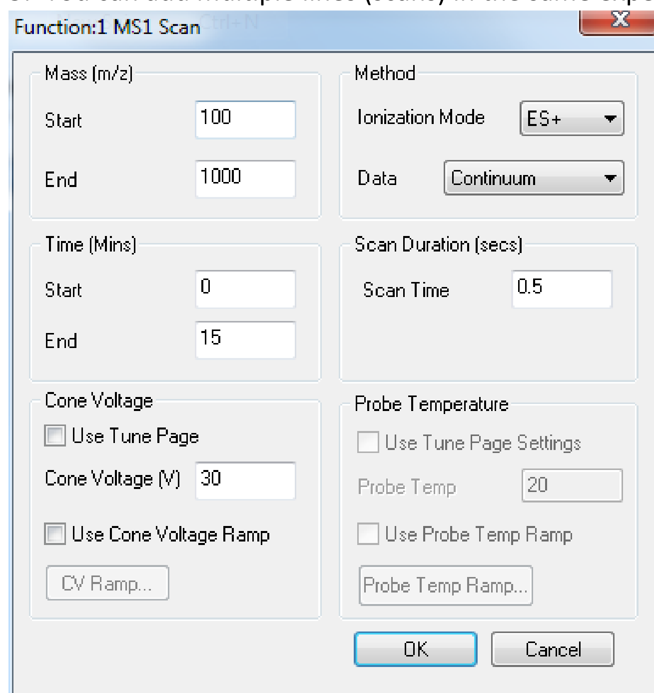
14. Save the Inlet (LC) method file in Aqcudb folder inside your project folder.

B. Setting up the MS method file

1. Click the “MS Method” Icon in the *Masslynx* main window



2. Click on the type of the scan for No. 1. in the list (SIR = selected ion recording or MS Scan)
   a. SIR will monitor the intensity of selected peaks. MS Scan will return the entire mass spectrum as a function of time.
3. Double click the 1st line in the list to open the “Function” window.
4. Fill out the parameters (see below) in the Function window of your choice.
5. You can add multiple lines (scans) in the same experiment.



Set the “Ionization Mode” (polarity) to ES+ or ES-

Set the type of data to collect:

Continuum: Full ion distribution profile.

Centroid: Processed continuum data showing a centered line for each continuum peak.

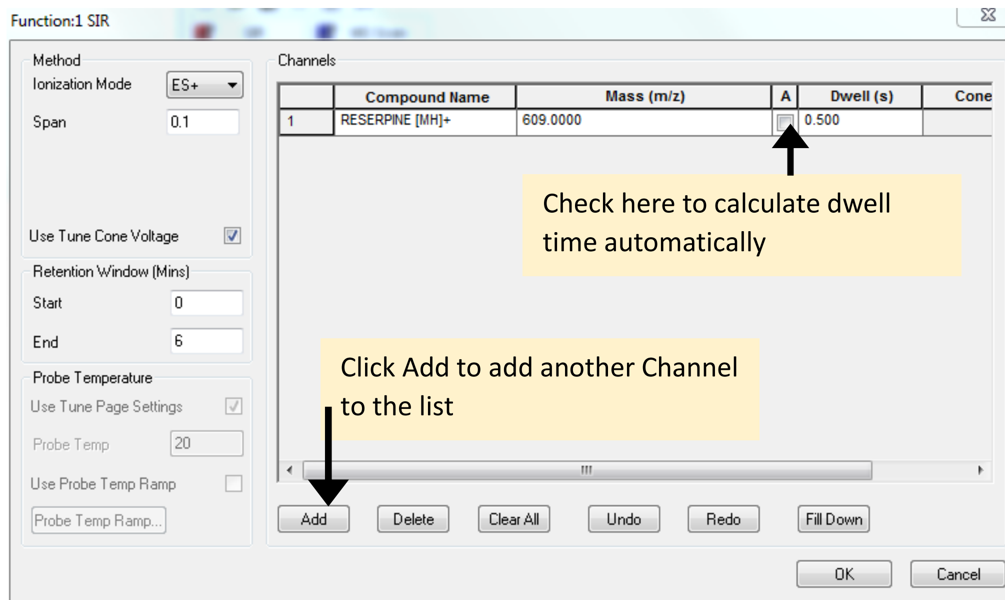
Set the mass range (start and end) and Scan Duration (1 / # of scans per second)

Set the length of time to record mass spectra (should match the length of your LC run)

Click “Use Tune Page” under “Cone Voltage”

6. Click OK.

7. Parameters for the SIR function: Enter in your compound name and its expected *m/z* to monitor. You can monitor multiple ions at a time. In this mode the entire mass spectrum WILL NOT be obtained.



8. Click OK.
9. Once done adding scans and editing parameters, Click "File->Save As" to save the MS method.

Turning on the Mass Spectrometer

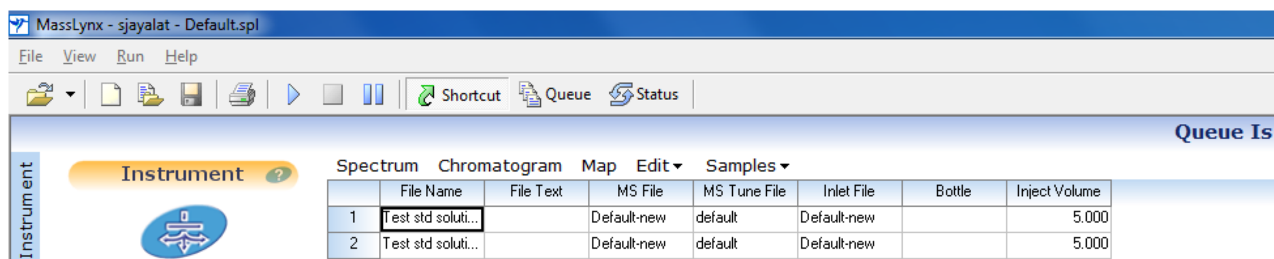
1. Double click "MS Tune" Icon in the Main screen to open the Tune window.
2. Turn on the gas flow by clicking the cylinder icon (API gas) in the toolbar in the Tune window.
3. Click "I" icon in the (bottom right in the Tune window) to initialize the mass spectrometer, turning on the voltages and gas flows.
    a. The mass spectrometer and API gas can also be initialized from the "MS Console" window

Loading the samples and setting up the runs

1. Prior to running your LC method **YOU MUST ENSURE THAT THERE IS ENOUGH MOBILE PHASE (SOLVENT) IN THE SOLVENT BOTTLES TO COMPLETE YOUR RUN**. Failure to do this can result in damage to the column.

2. Place sample vials in the Sample Manager tray and note the placement (i.e. 1:41 = Tray 1, position 41) of each vial.

3. Go to the main *Masslynx* window and edit the sample list (see below).



4. Fill out the table with Sample Name in the “File Name” column and the vial location (i.e. 1:41) in the “Bottle” column for each sample.
5. Then for each sample,
   I. Use 5.000-10.000 µL in the “Inject Volume” column
   II. Double click inside the “Inlet File” cell and select the correct Inlet method file and from the list.
   III. Double click inside the “MS file” cell and select the MS method file from the list.
   IV. Double click in the “MS Tune File” cell and select “default” tune file or another optimized tune file that has been supplied for you.
6. Select all the rows containing samples you need to run by dragging down on the numbers in the sample list.
7. Click the play icon in the toolbar.
8. Click Yes on the prompt to update sample list file
9. Check that samples you want to run are listed and only “Acquire Sample Data” box is checked

Shutting down the instrument

Follow these steps in sequence to properly shutdown the instrument. If you are analyzing your data, skip step number 4 and close the software and logoff once you are done with data analysis.

1. Put the MS into Standby mode (‘O” icon in the left bottom corner in the Tune window) then turn off the gas flow by clicking the cylinder icon (“API gas”). This can also be done in MS Console.

2. Turn off the TUV detector lamp from the software (“Bulb” icon in the Inlet window or “lamp” icon in the TUV detector tab in MS Console)

3. Turn off the flow (“Faucet” icon in the Inlet window)

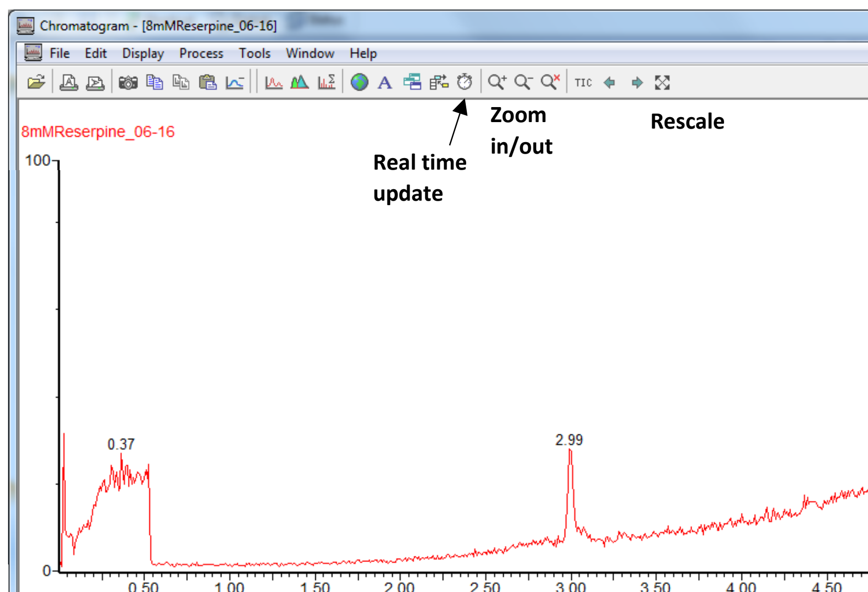
4. Close the software and logoff

5. Turn off the TUV detector (Power button on the Detector) and fluorescence detector (if used). Leave the other components on.

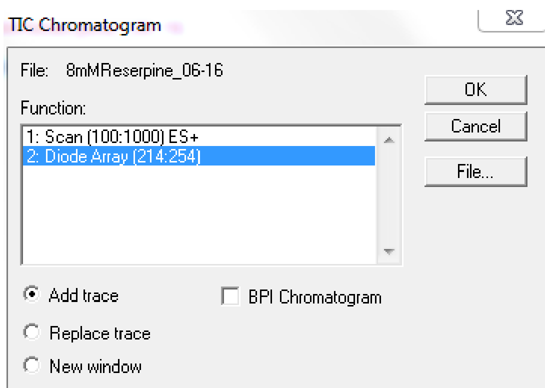
6. Make sure there are no flashing red lights on any of the LCMS components. There should be a single steady green light on QSM, SM and MS and no lights on the TUV detector.

Viewing and processing data

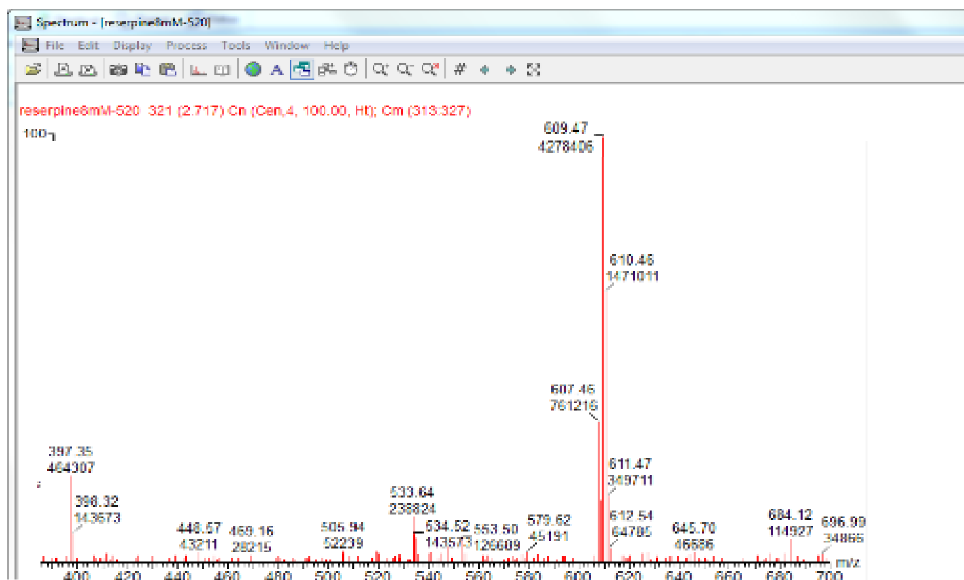
1. Highlight a sample row in the sample list and click on the Chromatogram button in Masslynx main window to view the chromatograms.
2. If you want to view the data in real time for the sample that is currently being analyzed, click Display → Real-Time Update or click the small stopwatch icon in the toolbar.



3. You will only see a single trace even if you collect data in multiple modes (UV and MS).
4. Click View >> TIC then select the traces you want from the list in the TIC window and click OK.



5. Click and drag from left to right to zoom horizontally or up and down to zoom vertically.
6. Right click on a point in the Chomatogram (i.e. Peak maximum) to view the corresponding Mass Spectrum for that point.



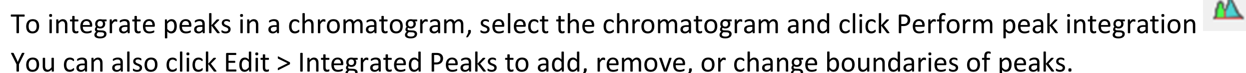
7. Right click and drag over a peak (left to right) in TIC chromatogram to view the combined Mass Spectrum of the peak.

UV Chromatogram processing

If both channels are selected in UV detector the initial DAD chromatogram will produce a combined chromatogram from both channels. Single channel chromatograms can be obtained by following method:

Right click and drag on a peak in DAD combined chromatogram. This will produce a UV spectrum with two lines, one at each channel. Right click and drag over a line to produce a UV chromatogram specific to the channel.

To perform baseline correction, select the chromatogram and click Perform background subtract

To integrate peaks in a chromatogram, select the chromatogram and click Perform peak integration 
You can also click Edit > Integrated Peaks to add, remove, or change boundaries of peaks.

<u>Saving the data</u>

In the spectrum or chromatogram window click edit > copy picture to copy as a picture. Paste in paint or in Microsoft Word or Powerpoint and save. Make sure to take your data with you on a flash drive or vial email.

In the spectrum or chromatogram window click edit > copy chromatogram/spectrum list to copy the data as XY format numbers. Paste in Microsoft Excel or Notepad and save.

In the chromatogram window click edit > copy detected peaks to copy the data as XY format numbers. Paste in excel and save.

Copy Raw data files from C > Masslynx > project folder > data and paste on a flash drive or on a shared drive space.

Common Issues and Solutions

1. **Mass calibration is out of date (measured masses are not correct)**
   Solution: The Waters LC/MS mass calibration is updated several times a year and should be the default calibration file used by the instrument **UNLESS** your MS Tune file is linked to a particular older calibration file. To resolve this issue, do the following:
   a. Open MassLynx
   b. Open your own project folder.
   c. Click "MS Tune" to open the mass spectrometer control software
   d. Open the tune file you will be using for your experiments ("File->Open"). Make sure you select the tune file in your own folder.
   e. In MS Tune, select "Set Linked Calibration" under the "Calibration" menu.
   f. If the text field is blank, then your tune file is using the latest calibration file automatically.
   g. If the text field is NOT blank, then select "Clear" and then click "Link" so that your tune file uses the most recent calibration automatically.
   h. Now save your tune file ("File->Save").
   i. Do this for all tune files you plan to use for your experiments.
2. **LC/MS component is in an error state**
   Solution 1: In *MS Console*, click "Reset" under the "Control" menu in the component showing the error.
   Solution 2: Restore instrument communication by logging out and logging back in. Turn off the UV lamp, the mass spectrometer (and API gas), and the LC flow, close *MassLynx*, and log out of the computer. Then turn on the UV detector, wait for a series of beeps, log in, and open *MassLynx*. At this point communication with the instrument should be restored.
3. **LC/MS used a previous version of a method file after a change was made**
   Solution: If a method file is changed, you must re-load the file in the sample list grid before starting a new run.
4. **Peaks not showing up**
   Solution 1: Your molecules may not have ionized by ESI. The mass spectrometer detects *ions*, not neutral molecules. Most molecules will ionize by proton addition or loss to create a singly charged positive or negative ion, respectively. Ensure your molecule has heteroatoms that can protonate. Amines and molecules with carboxyl functionality will tend to form positive ions and carboxylic acids and phenols will tend to form negative ions. Molecules without sufficient acidity or basicity may not be detected by MS (but might be detected by UV).
   Solution 2: Your molecule may be in an ionic state in solution, particularly if it has extremely basic or acidic functional groups. If your molecule is ionized, it will not have any interactions with the C18 column and may elute in the "void" at the very beginning of the run. Changing the pH of the sample or mobile phase may resolve this issue.
   Solution 3: The concentration of your sample might be inadequate, but this should not be your first assumption. Mass spectrometers are extraordinarily sensitive instruments; the SQ2 detector should be able to detect organic molecules in the low ppm concentration range if they can be protonated or deprotonated. If you inject too high of a concentration, you may contaminate the tubing, the column, and the mass spectrometer.
5. **Can't identify a peak or unexpected peaks**

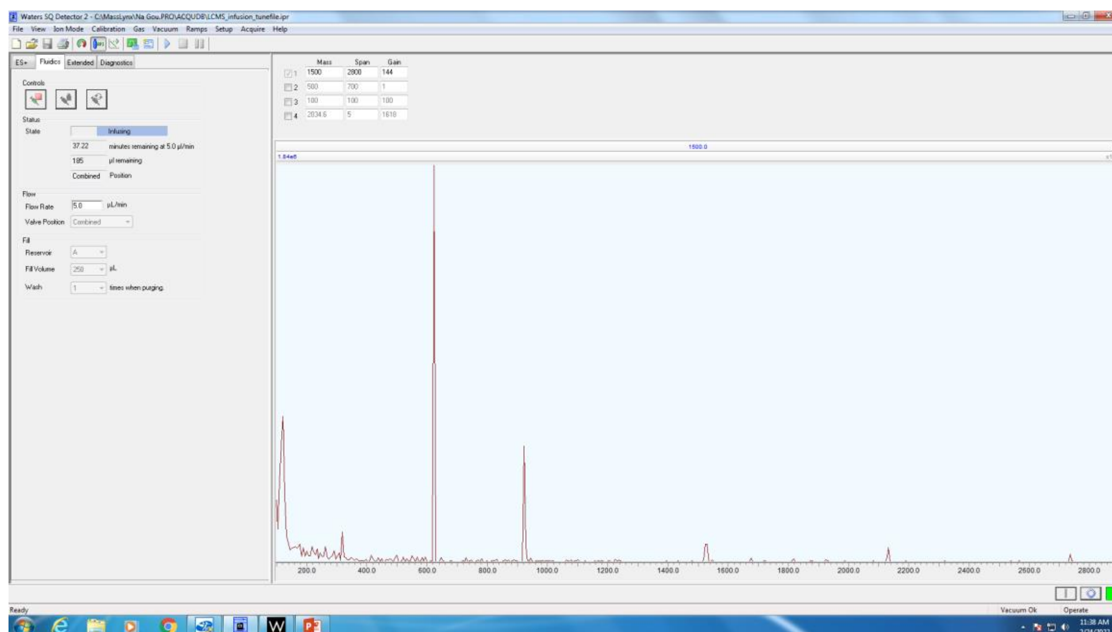
Solution: It is strongly recommended to run a solvent blank at the beginning and end of your run to identify contaminant peaks in your solvent and peaks that have carried over from prior users. If the unexpected peak shows up only in your sample and not in your blanks, then there is an unexpected molecule in your sample.

6. **Mass spectrometer error, API gas pressure too low.**
   Solution: Check that nitrogen is being supplied to the instrument (*i.e.* that the nitrogen tanks are not empty and that the valves are not closed at the gas manifold) and is being supplied at a high enough pressure. The regulator at the nitrogen tank should read 80 psi minimum to supply enough pressure.

Mass Calibration

Mass calibration need to done at least once per month. The previous mass calibration files are stored in "Masslynx->Intellistart->Result->Default Res". To perform a Mass calibration, API calibration solution is needed. Current the API calibration solution is in vial B.

1. Purge the infusion line
   Open MassLynx, then open "Mass Tune", in the "fluidics" panel, set flow rate to 5-10ul/min, then select the solvent reservoir that is filled with calibration solution, which is "B" now.
   The instrument will start to purge the infusion line automatically.



2. Mass calibration
   a. Open the API gas by click "gas"
   b. Set fill volume, which is 250ul now
   c. Set MS to ON, start infusion and wait until peaks coming out
   d. Open the window "MS console", choose "waters SQ detector 2", then click "intelli Start", expand the resolution and calibration option by click "open instrument set-up options", select "Instruments resolution" (select both positive and negative) and

“Instruments calibration”, then click Start on the right side, wait until mass calibration work automatically.

e. Wash calibration mix when you are done.

Mass Tune

Mass tune need to done once per three month. To perform a Mass tune, ESI tuning mix is needed. Current the ESI tuning mix is in vial A.

1. Purge the infusion line
   Open MassLynx, then open “Mass Tune”, in the “fluidics” panel, set flow rate to 5-10ul/min, then select the solvent reservoir that is filled with tuning mix, which is “A” now.
   The instrument will start to purge the infusion line automatically.
2. Mass calibration
   a. Open the API gas by click “gas”
   b. Set fill volume, which is 250ul now
   f. Set MS to ON, start infusion and wait until peaks coming out
   c. The parameter can be tuned are in the ES+ window, the capilliary voltage, cone voltage, desolvation temperature.
   d. To tune in LC/MS mode, switch valve position to “combined” and turn on LC flow. To record a spectrum, “acquire”->”start”, the real time windows will stop to update after start acquiring.
   e. LC/MS requires higher temperature and gas flow then infusion, capilliary voltage also might be difference.
   f. Cone voltage, higher ,better high m/z, wash tune mix when you are done.