Standard Operating Procedure

Liquid Chromatography with Orbitrap Mass Spectroscopy (LC-Orbitrap-MS)

Written by: Levi Zanders

University of Tulsa Department of Chemistry and Biochemistry

Introduction:

Orbitrap Mass Spectroscopy is a type of ion trap mass spectroscopy, which uses an integrating sensor in an ion trap which is generating resonant cyclotron frequencies, allowing for extremely high resolution. Orbitrap sensing employs a chamber surrounding an electrode, allowing for an orbital suspension of ionized particles. A cross-section of this design is shown below.

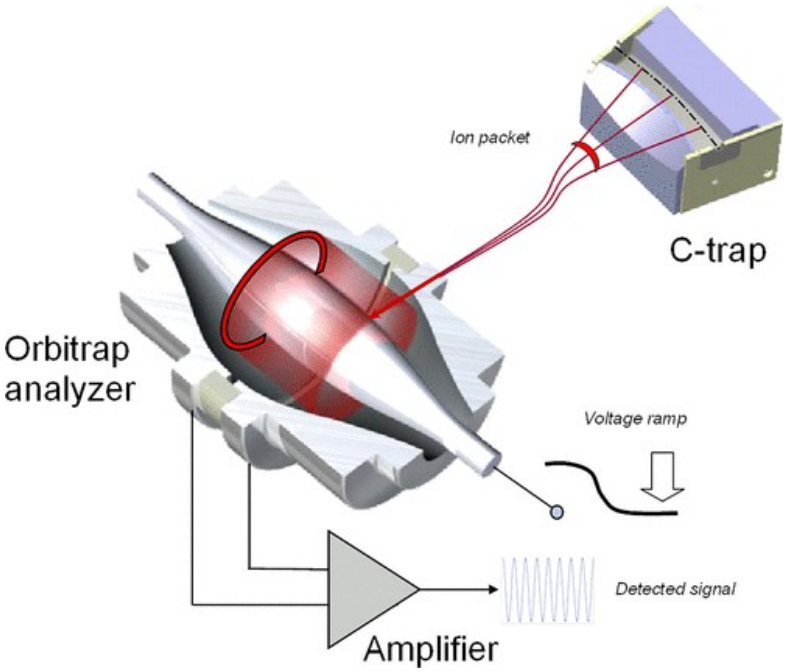


Figure 1: A standard orbitrap analyzer – figure obtained from: *https://pubs.acs.org/doi/full/10.1021/ac4001223*

The voltage surrounding this central electrode can be varied, allowing for resonance of different masses. This allows for integrated detection of resonant ions during each orbit, substantially improving limits of detection and resolution[1]. Due to this design, orbitrap has many practical applications to analytical chemistry. First, orbitrap gives substantial potential for quantitative analysis. The extremely high resolution of the orbitrap MS leads to the ability to differentiate very similar peaks, and additionally is able to quantify complex ions at substantially lower concentrations than similar high resolution mass spectrometers. Some authors even speculate that as low as single ion detections could be attained through orbitrap[2] This high resolving power in complex samples has additionally been extensively applied to peptide sequencing, as samples can be rapidly differentiated to identify unique peptide sequences[3][4]. This is an effective instrument for high molecular weight molecules, that otherwise require additional instrumentation, but

[1] https://www.sciencedirect.com/science/article/pii/S1387380612001923
[2] https://pubs.acs.org/doi/full/10.1021/ac4001223
[3] https://www.mcponline.org/article/S1535-9476(20)30167-5/fulltext
[4] https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/full/10.1002/mas.20186

additionally solves problems in small molecule mass spectroscopy, which otherwise requires multiple phases of mass spectroscopy. Specifically, the ability to identify and differentiate small molecules such as metabolites and drugs in biologic systems is easily accomplished with orbitrap MS. This can therefore be effectively applied to biologic study of small molecule targets and prevalence, and to identify metabolites of target drugs[5].

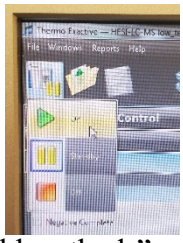
Sample Preparation:

Samples should be prepared at concentrations not more than 100 µg/mL. The easiest way to achieve this concentration is to dissolve 1 mg of sample in 1 mL of solvent, transfer 0.1 mL of solution to an HPLC vial, and add 0.9 mL of solvent. These samples should be in a relatively inert solvent if possible, such as Ethyl Acetate. All sample must be completely dissolved, or else the sample should be filtered to remove any precipitate. Precipitate in the sample can plug the column in the LC, which could irreversibly damage the column. These samples are ideally purified prior to introduction to the LC, and should not contain non-volatilizable salts, as these can accumulate in the ionizer.

Starting the Instrument:

The entire instrument is controlled by the computer, with the exception of the orbitrap source selector, which allows for a switch between LC introduction and direct injection. Direct injection should only be used with explicit permission from a professor. At this point, also double check the solvent reservoirs on top of the LC, and make sure they are at least ¼ full. If they are low, notify Dr. Roberts immediately, and verify there is enough solvent to run the instrument. If the instrument is

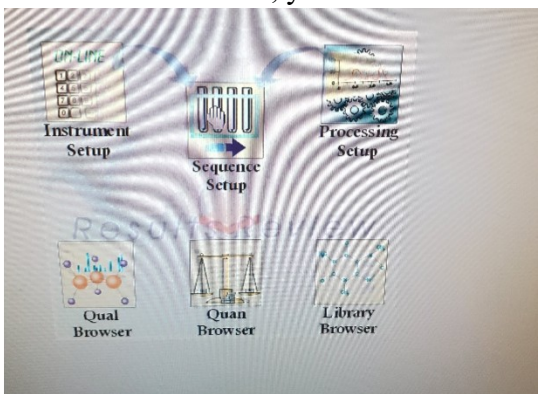
- First, ensure the source selector on top of the orbitrap shows a "2"
    - If it needs changed, use the arrows to change the source.
- Turn on Nitrogen Flow at the tank to the right of the computer. Ensure there is enough nitrogen in the tank to run for the duration of your samples.
    - Ideally above ¼ on the pressure gauge on the right
- Go to the computer, and start the Exactive Tune program, and the Xcalibur program
- Open Exactive Tune, and change the instrument status to "On"



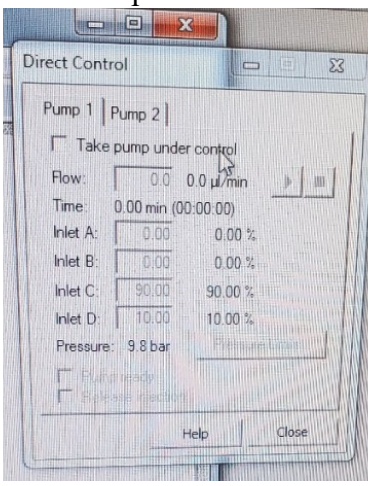
- Then go to "File", click load tune file, double click "shortcut to old methods", and pick the appropriate tune file.

[5] https://www.annualreviews.org/doi/full/10.1146/annurev-anchem-071114-040325#_i20

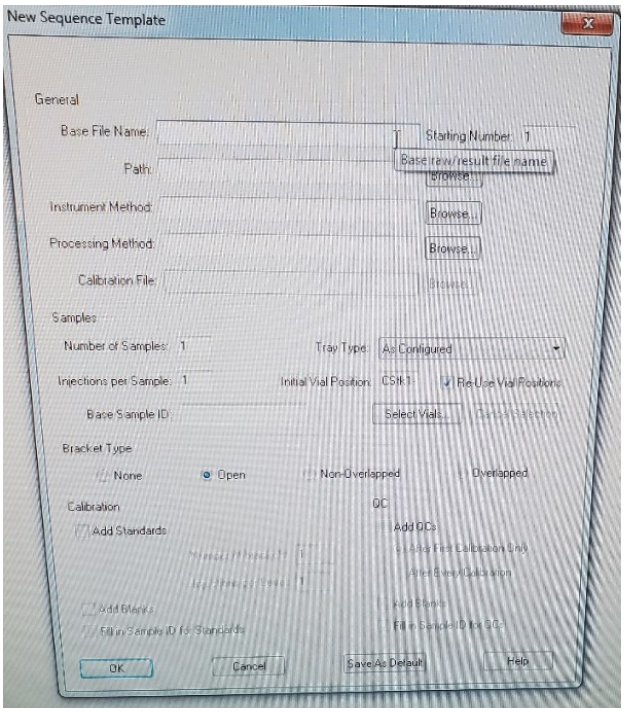
- The Mass Spectrometer should now be set up, so navigate to the Xcalibur Program to set up the run
- In the Xcalibur menu, you should see the roadmap view:



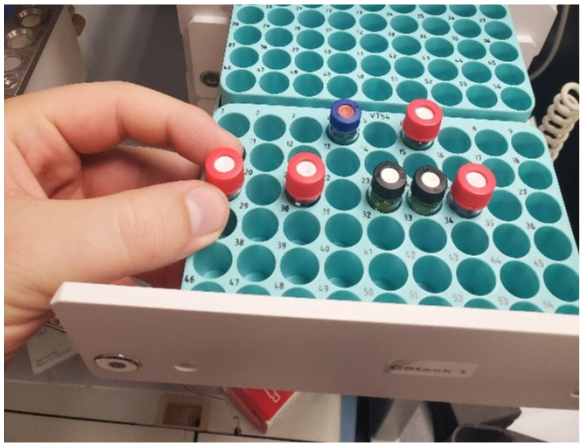
- Click instrument setup, which will bring up the parameter setup for the instruments
- In the upper right hand corner, select “File – Load – Shortcut to Old Methods – LC Methods” and select an appropriate method. These have been developed by labs previously, and if a new method needs to be developed a professor should be consulted.
- Once the file is loaded, go to the Accela 1250 Pump, and on the top toolbar select “Accela 1250 Pump” and then click “direct control”, bringing up the following window



- Check the “Take pump under control” box
- Change “Inlet D” to 100 %, and then change “Flow” to 250, and press the Play button. This will prime the column and remove contaminants prior to the run.
- Leave this window open, as you will need to return to it and turn off direct control before you begin the run.
- Return to the Xcalibur roadmap view, and now select “Sequence Setup”
- In the Sequence window, select File – New from the top toolbar
- This will bring up the file setup window:



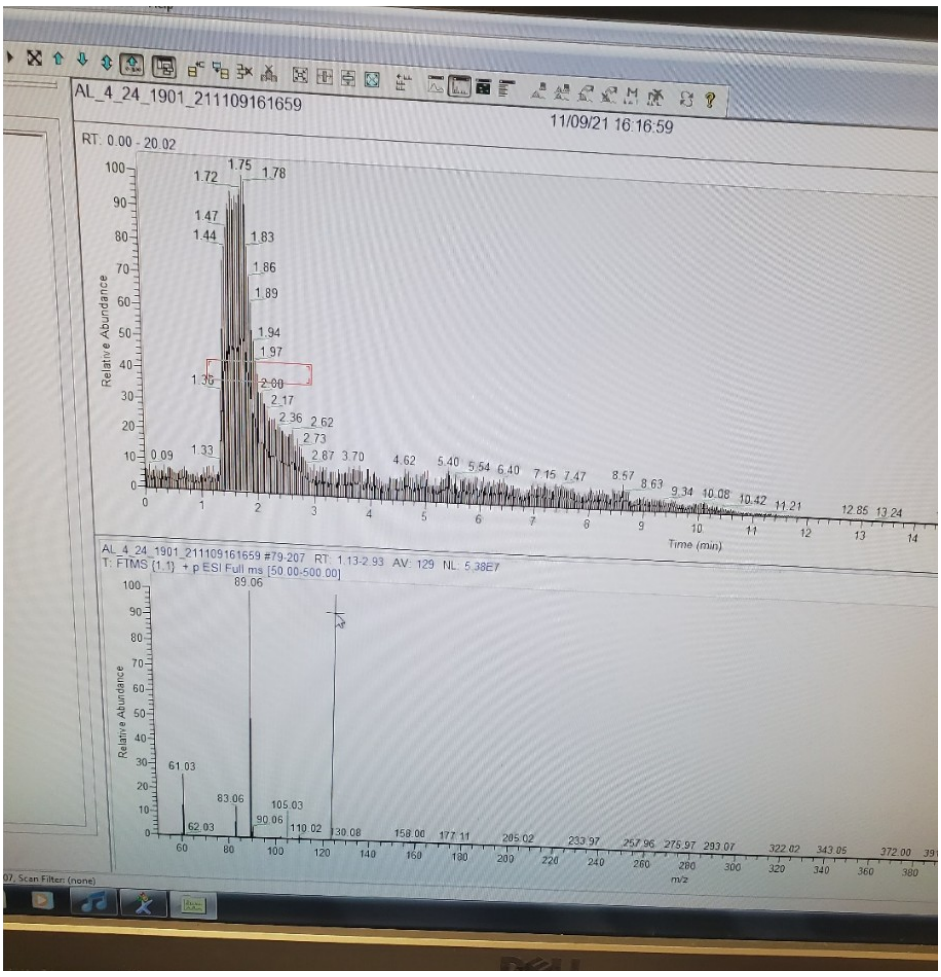
- First pick the name you would like the file data to be saved under
- Then go to “Path” and click browse. This will bring up all of the labs that use this instrument. Select the appropriate lab, double click the folder, and press “ok”
- Then go to instrument method and click browse. Select the same instrument method that was loaded in the instrument setup steps.
- Click the “select Vials” and double click the position of the vial your sample is in. The default drawer for the autosampler is the top drawer, in the front holder.
- Click “OK”
- In the top toolbar, go to “File – Save As – Xcalibur – Data - *Your lab* - *File Name*”
- Now return to the pump control window
- Change “Inlet C” and “Inlet D” to the percentages that will be used at the start of your sequence. This is often 90% for Inlet C and 10% for Inlet D. Wait 10 minutes.
- After 10 minutes, uncheck the “Take pump under control” option
- Return to sequence setup and click “Run Sequence” ***IMPORTANT NOTE***
    - Before clicking run sequence, be ready to go to the autosampler and hold the vial in place after the needle is inserted, as shown below. The autosampler has a tendency to pick up vials, and if they are jammed into the injection port it can damage the instrument.



- The sequence will now run, which takes roughly 20 minutes, depending on the loaded method.

Data Analysis

- The data was saved as part of the run setup, so now go to the instrument roadmap, and select “Qualitative Analysis”
- Go to the top toolbar and select “File – Load - *your lab* - *your filename*
- This should load in a display that looks like the following:



- You can now select peaks, or larger areas, by clicking and dragging in the upper display
- The MS data will appear in the lower box, but by default only displays two decimal places.
- To display more decimal places, right click the MS box and select “display”
    - Go to “Labels”, ensure “Mass” is checked, and change the “Decimals” box to the appropriate number of decimals. For the organic labs, at least 4 are required.
- If you are looking for a very specific peak, you can right click the MS box, select “Ranges”, and in the box at the top that says “Mass Range” define a range of atomic

weights to display and press OK. This will restrict the shown peaks to only peaks within the defined range.

- Once the desired information has been found, you can exit the qualitative browser. The file is already saved from the run, so no more saving should be required unless the file is edited.

Instrument Shutdown

This instrument should never be manually turned off unless directly under the instruction of a professor, but the pump should be turned off in the software and the MS put in standby mode, as described below

- Return to the "Instument Setup" box through the Xcalibur Roadmap, and once again select the Pump, and click "Direct Control" under the pump menu in the top toolbar
- Check the "Take pump under control" box, and change "Inlet D" to 100%, press play, and wait 5 minutes.
- After 5 minutes, change the flow rate to 0, and press the stop button to the right of the play button. The flow rate should display zero. After this deselect "take pump under control" and exit this window.
- Return to the Exactive Tune Software, and change the Instrument Status from "On" to "Standby"
- Go to the Nitrogen tank, and turn the knob on top to the closed position.
- Close all windows

Parameters

There are few parameters that are changed on a regular basis for this instrument. The MS tune file should not be changed except by highly trained individuals. The solvents used in this instrument are very high purity, and need to be prepared via special procedures. We currently use buffered water and Acetonitrile, so if your compound isn't eluting at an appropriate speed, you can change the gradient elution protocol to adjust the ratios of solvents. Otherwise, parameters should only be changed as part of method development, which will not be covered in this operating procedure.

Common Issues

- Instrument is not switching from “Standby” to “On”
    - Look at the box on the right of the MS box in Exactive Tune. If you see any Red or Blue circles, there is an issue in the instrument. This is preventing the instrument from starting, and will require maintenance. Notify Dr. Roberts.
- Instrument is not starting a run after clicking “Run sequence”
    - This can happen when a method has been loaded incorrectly, so start by re-loading your method.
    - This can also happen when the instrument communications protocols have been reset. Attempt restarting the computer after shutting down the instrument as described in Instrument Shutdown
    - If the Instrument setup box is displaying the Accela PDA, the instrument is looking for a component that is no longer present. This will require re-configuration by a professor.
- Sequence was run, but no peaks eluted
    - Double check that the injection port on top of the MS is set to a “2”, which indicates MS injection from the LC.
    - If this is set appropriately, and a peak still doesn’t show, run your sample on the HPLC-UV with comparable solvents to verify the elution time of your compound, and adjust the method accordingly
- The Autosampler picked up a vial, and it was placed onto the injection port
    - Attempt to stop the sequence in the software, and immediately find a professor
- The three lights in Exactive Tune are not Green
    - A yellow or red light indicates an issue with the instrument. If the third light is yellow, hover over it to see the message. A “Calibration Due” message should be noted, but is otherwise still usable. Any other message, or a red or yellow light in any other position should be reported to a professor.