IMSERC User Manual for Agilent GCMSD with DB5 Column

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IMSERC User Manual for Agilent GCMSD with DB5 Column (V1.0)

INTRODUCTION

Use of this instrument is allowed only by qualified users after receiving training by a staff member. Do not run this instrument without approval from IMSERC staff. Failure to do so may cause damage to the instrument, produce invalid data, and result in additional fees and/or removal of all IMSERC privileges.

This short set of instructions is meant to serve as a guide for 'routine' data collection on the instrument. Please read this standard operating procedure and acquaint yourself with the instrument. At every step of the operation, please pause, think, then proceed. If while using the system, something happens that you do not understand, please ***stop,*** and ***get help***. In any event, be completely prepared to justify your actions. The cost of even minor repairs is considerable.

The GCMSD is a general use instrument capable of analyzing small molecules for polar and non-polar molecules. It allows separation of volatile compounds and ionizes those compounds using electron impact (EI). The mass range for this instrument is limited to 800 Da. To keep the system simple and useful for a group of diverse users, a default method is designed to run routine samples (samples which are simple mixtures, are easily volatilized, and are in standard GC friendly solvents) in order to obtain a quick analysis of sample composition.

If there is a need for method further development such as resolution of sample components, contact IMSERC-MS staff and assistance will be provided to optimize the default method.

Your sample must:

1. Be volatile or semi-volatile.
2. Be diluted in a volatile solvent such as chloroform, methylene chloride, hexane, ethanol, methanol.
3. **NOT** exceed a concentration of ≤ 1 mg/mL
4. **NOT** exceed a boiling point of 300 °C.
5. Be compatible with DB5 column on instrument. See IMSERC-MS staff for questions/details.

You are responsible for maintaining instrument quality prior to and post sample analysis. It is best lab practice to always run a blank prior to running your sample. An IMSERC methanol wash vial is always kept in vial position 1 on the autosampler. You have the option to inject your own wash vial, but it must be compatible GCMSD. It is also good lab practice to inject a blank post sample analysis to ensure no carry over for the next user. The injection of the wash should be calculated in your time on the instrument. Do NOT leave the instrument and end your reservation while the instrument is running as it affects other users' reservations. If you are unable to reduce contamination, file a "Bug Report" and place a "Stop Sign" on the keyboard to notify subsequent users.

SAFETY

All users of IMSERC must review the general safety policies at http://imserc.northwestern.edu/about-policies.html.

Familiarize yourself with the location of standard safety stations like eye wash and shower stations found in just outside of BG76. Protective eyewear is required in this room, and gloves should be removed when using the computer.

Hazard	Location	PPE Required/Hazard Mitigation
Samples	BG70 – GCMSD autosampler	Eye Protection, Gloves
Methanol, Dichloromethane	BG70 – GCMSD rinse solvents on sample carousel	Eye Protection, Gloves
High Temperature	BG70 – GCMSD Inlet/Column oven	Do not touch

To become an independent user of this instrument, you must have the following safety training and certificates which are offered at https://learn.northwestern.edu:

- Laboratory Safety
- Personal Protective Equipment

Upon completion of the certificate, it will take an overnight to filter through the different systems and get into the files that NUCore uses.

Additionally, familiarize yourself with the location of standard safety stations like eye wash and shower stations found in the northwest corner of the room. Protective eyewear is required in this room, and gloves should be removed when using the computer.

DATA MANAGEMENT

Your personal data folder is created during training which must be located under your supervisor's group folder. See a staff member if you do not have a personal folder on this instrument yet. Your personal file folder must contain your Lastname_Firstname. Inside your personal folder is a folder for the year's data, which references the year the data was collected.

Every new year, you are responsible to create a new data folder and copy over your sample table. Be sure to also change your data file path to the new year, so that the data is collected in the new annual folder. See IMSERC staff if you require assistance.

Over time, files from previous years will be archived to a larger file server to save space on the local drive. If you do not use the designated file structure as outlined in this manual, it may result in a failure to back up your data. See IMSERC staff for clarification if you have any questions.

Once inside the 'annual' folder, you may label/identify your sample data according to your needs.

Example: PI name > Your Lastname_Firstname > YYYY> sampleinfo

Specific Example: Einstein > Currie_Marie > 2020 > test-abc-20200316

Data on this instrument are copied on 'imsercdata.northwestern.edu' under 'MS/GC_MSD_DB5' every 1 hour. Please follow instructions at http://imserc.northwestern.edu/about-general-faq.html#data for details about data access. Please remember to use the northwestern VPN when accessing data from a personal computer and disconnect from the drive after use when using public computers.

SOFTWARE

Data acquisition can be performed with Agilent Chemstation, and analysis is performed using Agilent's Chemstation processing software. Both icons to access each software are located on the instrument in the upper right corner of the computer screen.

You have the option to use the instrument computer for analyses, but you must reserve instrument time through NUCore.

SAMPLE PREPARATION

The table below outlines recommendations to ensure proper instrument care and sample analysis.

Detail	Recommendation	Consideration
Vials	Agilent Vial 5182-0714, Agilent cap 5185-5865	The Autosampler picks up the vial. With the wrong vial dimensions, the sample will drop during transport.
Solvents	Chloroform, methylene chloride, hexane, ethanol, methanol,	Volatile solvents recommended. Avoid DMSO and DMF.
Concentration / Purity	≤ 1 mg/mL	Use "Split" Injection. More dilute sample may use "Spitless" Injection. Remove non-volatile compounds. No ACIDS, BASES, catalysts, metals. Acceptable Range for column is pH 5-9.
Volume	400 µL	If volume available is too low, use Agilent vial spring loaded insert 5182-8872
Labeling	Name and solvent	Sample identification & safety / proper disposal

QUICK START ACQUISITION CHECKLIST

1. Begin your reservation in NUcore.
2. Load your Method.
3. Load your Sequence.
4. Confirm Data File Path is correct in the Sequence table.
5. Add sample(s) to your Sequence table.
    a. Update name of your Sample Name.
    b. Update the Vial position.
    c. Update the Date File.
        i. Should be an exact match of the 'Sample Name.'
6. Click "Okay."
7. *Save the sequence*.
8. Physically check the sample syringe.
    a. Plunger moves freely > Proceed to step 9.
    b. Plunger shows resistance > Contact IMSERC-MS staff for maintenance.
        i. If staff is unavailable, file a 'Bug Report' and place a 'Stop Sign' on keyboard.
9. Wash solvents are filled to fill line. Refill if necessary.
    a. Solvents are available for refill at user prep station at front of room BG70.
        i. Solvent A is methanol.
        ii. Solvent B is dichloromethane.
10. Run Sequence. (or POSITION and RUN for a subset of the sequence)
    a. Select the start position of the sample list and click OK.
11. Window prompt appears, click "Run Sequence."
12. Window prompt appears, "Process keywords before Starting Sequence?" Click, "Yes."
13. Injection of sample.
    a. If experiencing issues, see 'Instrument Troubleshooting' of User Manual.
14. Instrument will default to standby mode after the last sample run.
15. Analyze data using sample analysis software, Chemstation.
16. Save processed data, print.
17. End Reservation in NUcore.

DATA ACQUISITION

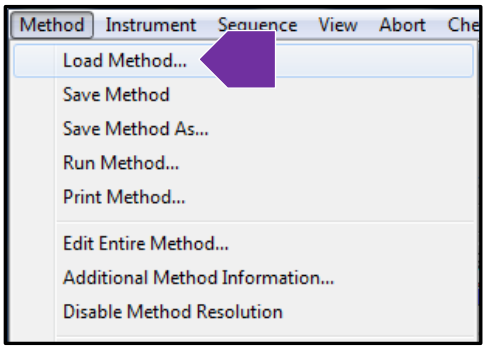
1. The computer screen is by default, deactivated. You must start your reservation through NUCore to be able to turn on the computer screen. If screen is already on, start your reservation through NUCore.

2. Make sure the GCMS acquisition software is open. You can select the GCMS icon 

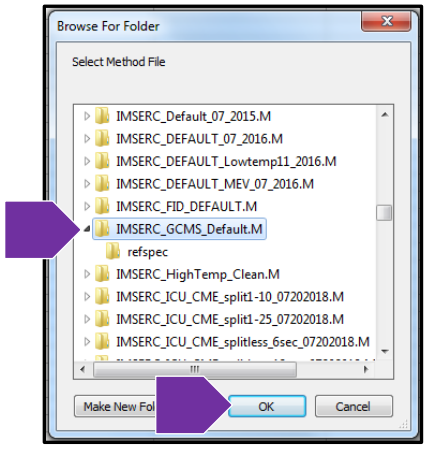
located on the top right screen or on the lower tool bar if it is open. Or double click the icon on the desktop to open the software.

3. **Load your method** by clicking "Method" and selecting "Load Method" on the top left tool bar. You can also select the load method icon  from the main window.

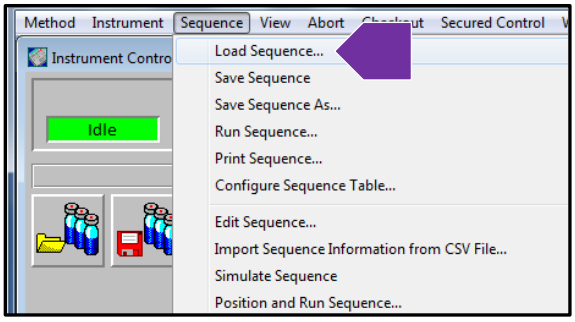
   Note: If you need to modify/edit your method, please see Appendix A after loading it. If you do not have a method set up, IMSERC staff will need to assist you through method setup. GC/MS parameters will be covered and their effect on analysis. The method development instructions to be covered are found in Appendix A.



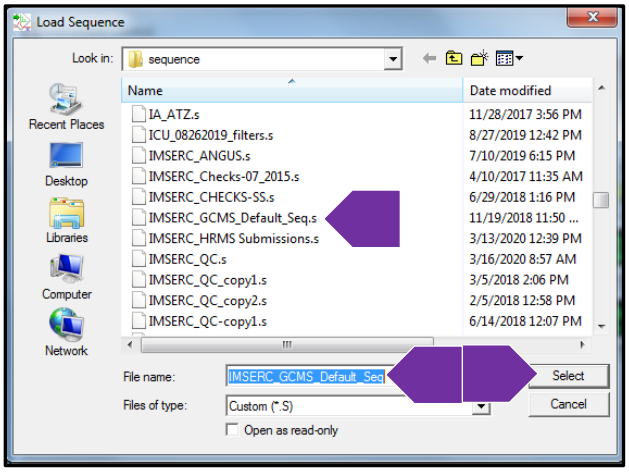
4. Locate your method. Then click “OK”.



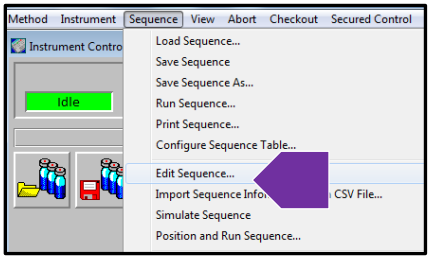
5. **Load your Sequence** table by clicking on “Sequence” on the top toolbar. Then click “Load Sequence.” Or select the “Open Sequence”  icon on the main window.



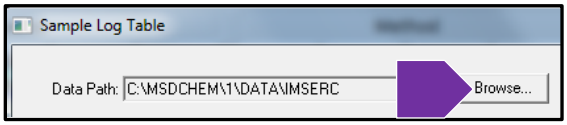
6. Select your own Sequence table or use the default Sequence table, “IMSERC_GCMS_Default_Seq.” Then click “Select.”



7. **Edit your sequence** table: Once you have loaded your Sequence table, you can edit it by clicking "Sequence" on the top toolbar. Then click "Edit Sequence." Or simply select the "Edit Sequence"  icon on the main window.



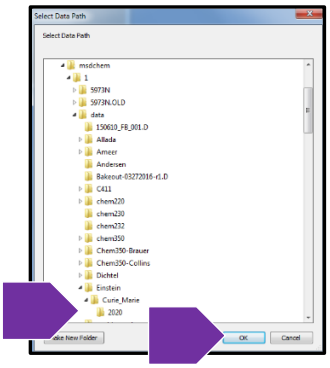
8. **Choose your Data Path.** Once inside your Sequence table, click "Browse." You will select the data path were your files will be collected.



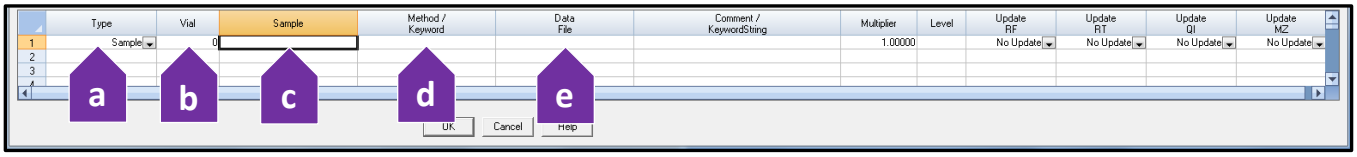
   a. Confirm the data path for your files are to be stored in the proper location as outline below. Then click "OK."

      i. C:\MSDCHEM\1\Data\PI Name\UserLastName_UserFirstName\YYYY.

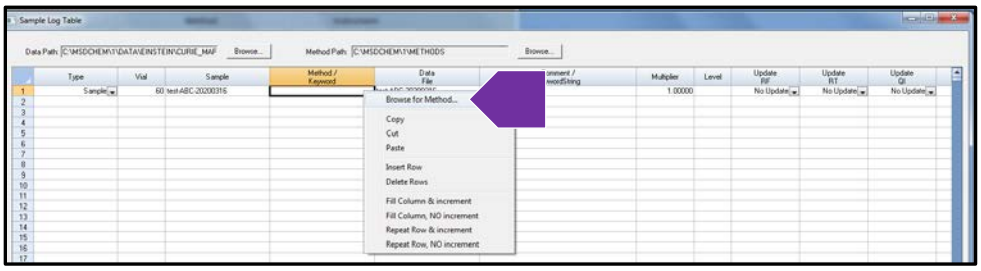
      ii. In the example below The PI Name is "Einstein," the username is Curie_Marie, and the data was collected in the year 2020. Create a new folder or ask IMSERC-MS staff if assistance is needed to create the correct data path.



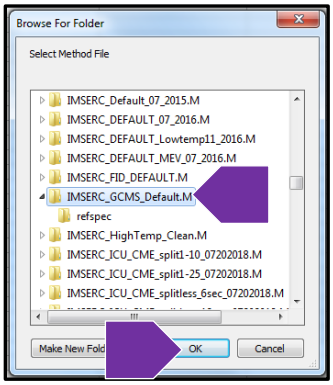
9. To **set up sample information**, in the Sequence table, you are required to enter / select:
    a. Sample type
        i. Either 'Blank' or Leave as 'Sample'
    b. Vial position
    c. Sample Name
    d. Method / Keyword
    e. Data File name
        i. It is recommended to copy the sample name and paste it over to the file name for consistency.



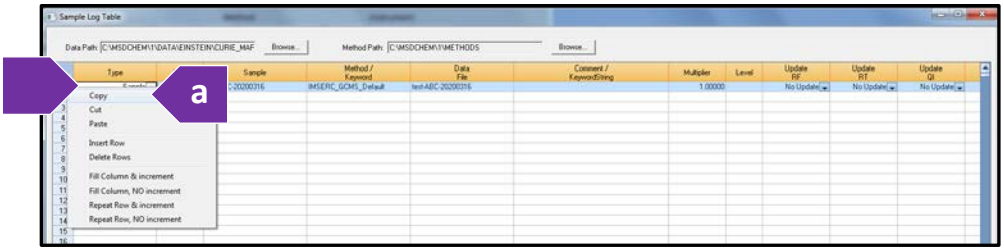
    f. To select your method, right click the Method / Keyword cell of your sample, then click "Browse for Method…"

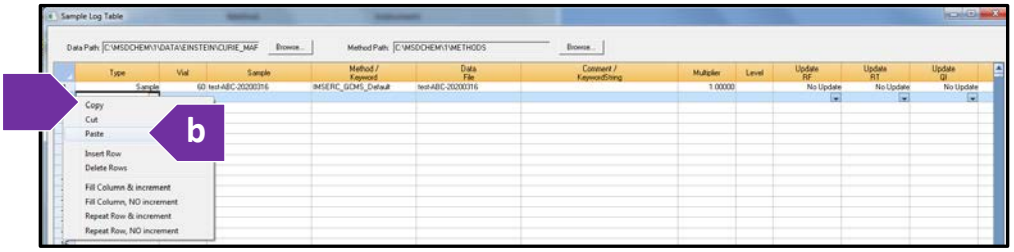


    g. All methods are located under MSDCHEM\1\Methods. Locate then select your method, then click "OK." If you do not have a method, select the default IMSERC method labeled, "IMSERC_GCMS_Default.M."
        i. Do **NOT** save/ load a method from anywhere other than MSDCHEM\1\Methods folder.

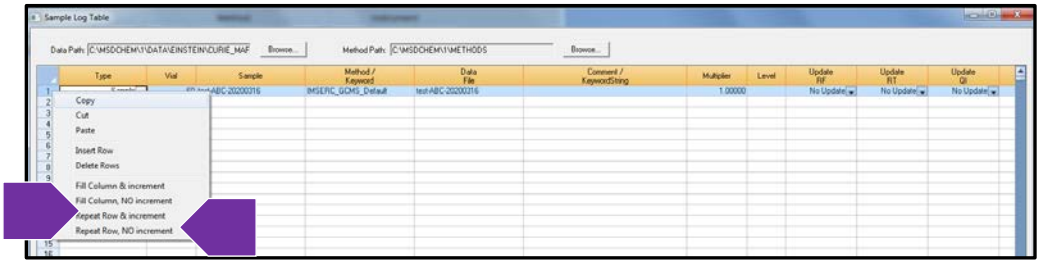


10. To **add another sample**, (a) right click the column you would like to copy and select “Copy.” Then (b) right click on the next available column, then click “Paste.” Remember to make necessary changes to vial number, sample name, data file, and method, if necessary.

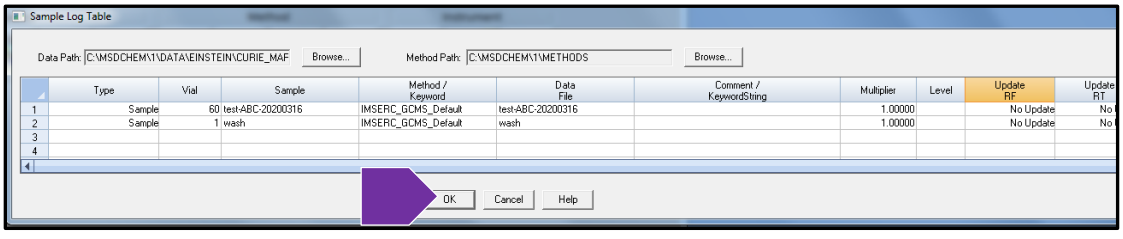




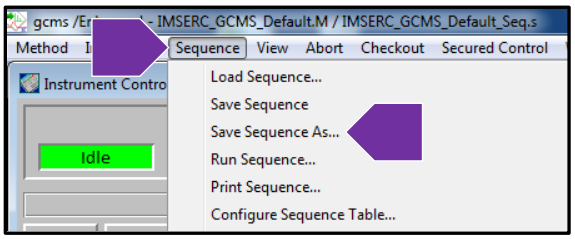
11. Alternatively, another quick way to add a sample is to right click the sample, then click “Repeat Row, and Increment” or “Repeat Row, NO increment.”



12. Once you completed all changes to your Sequence table, click “OK.”

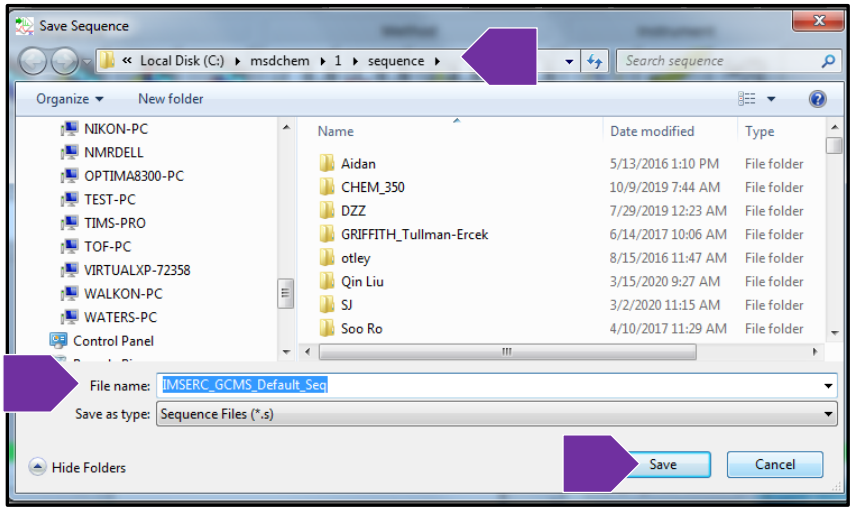


13. To **save your sequence** table, click "Sequence" on the top toolbar, then select "Save Sequence As…"

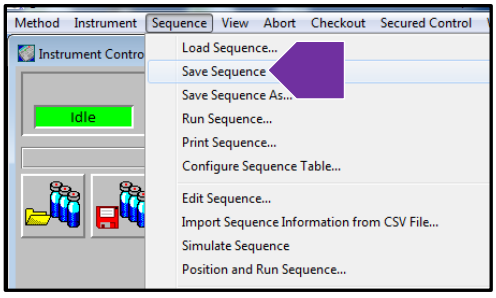


a. Enter a new File Name specific to you, then click "Save."

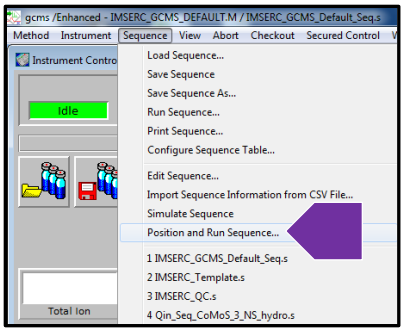
i. It should be saved under Local C:\msdchem\1\sequence.



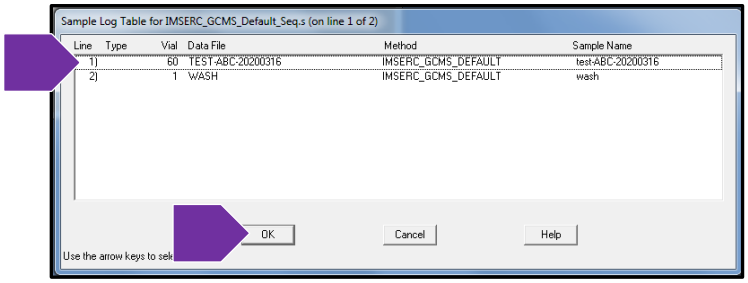
14. If you have already saved your sequence table under your specific name, you can simply click on "Sequence" on the top toolbar, then select "Save Sequence." Or alternatively, you can click on the 'Save Sequence'  icon from the main window.



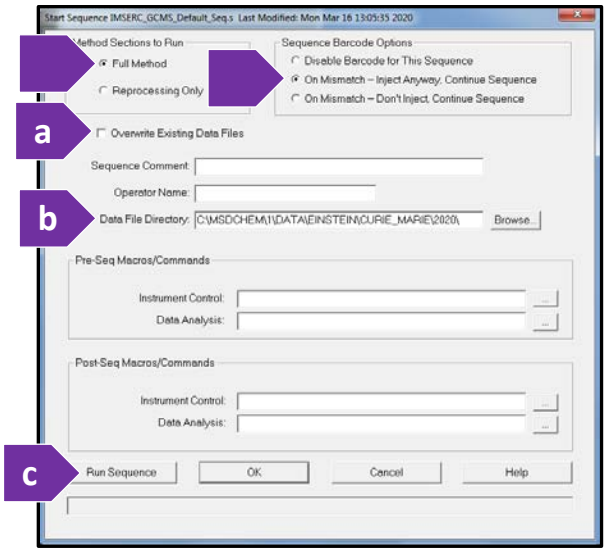
15. **IMPORTANT: Before running the sequence**, you will perform a quick maintenance check on the syringe and syringe wash vials located in the injector.
    a. Check the sample syringe.
        i. Plunger moves freely > Proceed to step 9.
        ii. Plunger shows resistance > Contact IMSERC-MS staff for maintenance.
            1. If staff is unavailable, file a 'Bug Report' and place a 'Stop Sign' on keyboard.
    b. Wash solvents are filled to fill line. Refill if necessary.
        i. Solvents are available for refill at user prep station at front of room BG70.
            1. Solvent A is methanol.
            2. Solvent B is dichloromethane.
16. **To run your sample(s)** click "Sequence" then select "Position and Run Sequence."



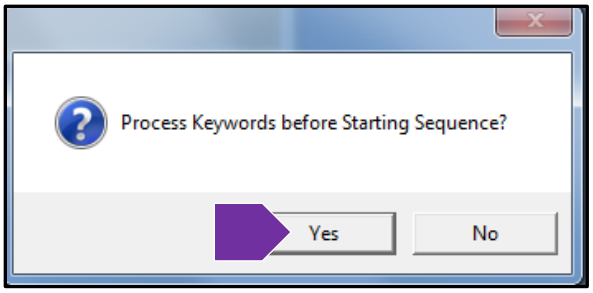
17. A window will appear. Click on the line where you would like the sample list to start running. Then click "OK."



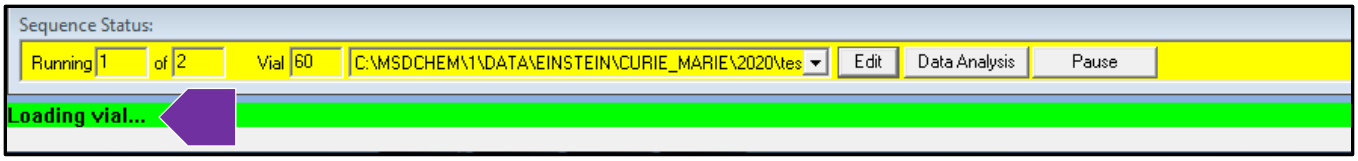
18. Another window will appear. Confirm the selections match what is listed below. (a) Check “Overwrite Existing Data Files” based on whether you want to do so or preserve them. (b) Confirm the Data File Directory follows the mandatory file path for data collection. (c) Click “Run Sequence.”



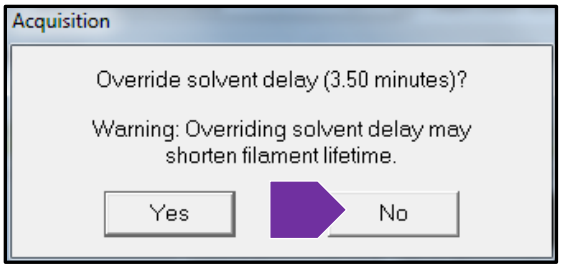
19. Another window appears, “Process Keywords before Starting Sequence.” Click “Yes.”



20. The instrument status will change from “Waiting on GC…” to “Loading Vial…” It will pick up your sample and move it to the carousel for injection within a few seconds of Loading vial message appearing. If you experience any issues, see ‘Instrument Troubleshooting’ of User Manual.



21. A final window appears asking, “Override solvent delay (X.XX minutes)? Click “NO” or Leave it alone.
    a. **NEVER** override the solvent delay. Failure to comply will result in damage to GCMSD filament.
    b. The solvent delay is also programed into your method. Doing nothing / not clicking, will run the method default, utilizing the solvent delay.

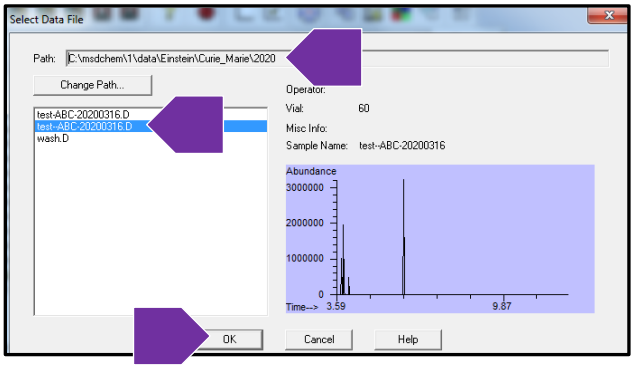


DATA PROCESSING

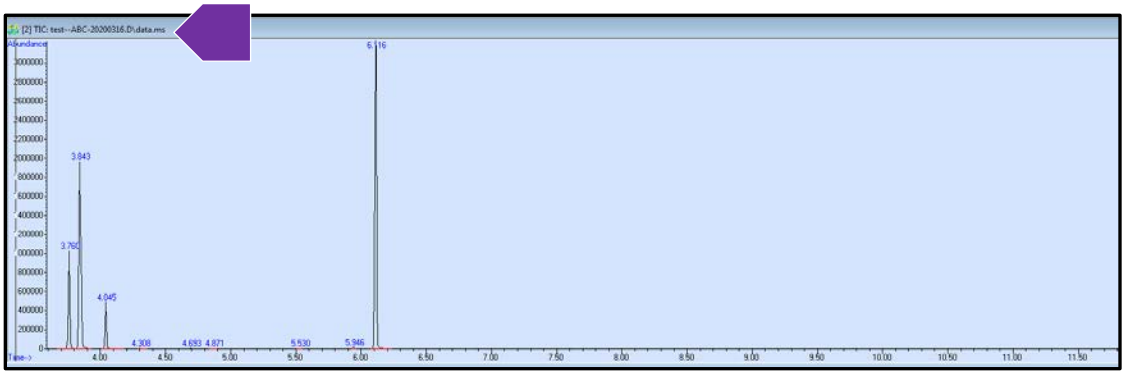
OPENING A DATA FILE

1. Open the GCMS Data Analysis by clicking on the icon 

on the top right of the computer screen, or check if it is already open
2. You have 2 ways to look at your data: **Snapshot** and **Open File**.
    a. To preview an actively running sample, you can click on the **snapshot** icon  . You will be unable to see any data for the duration of the solvent delay since no data is being collected.
    b. To open an acquired sample file, click on the '**open file**' icon  .
3. Locate your sample file by selecting the data file path that was chosen in you sequence table. Highlight you file, then click "OK."

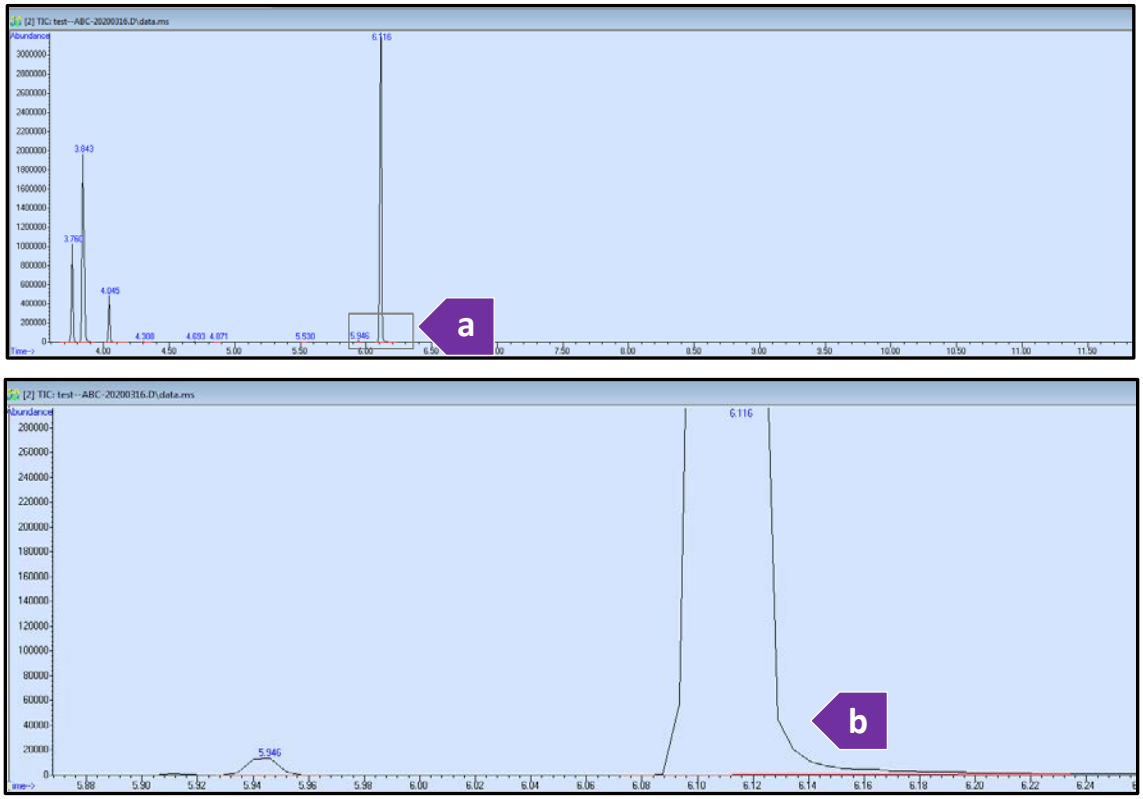


4. Once you open your file, the Total Ion Chromatogram (TIC) will appear.

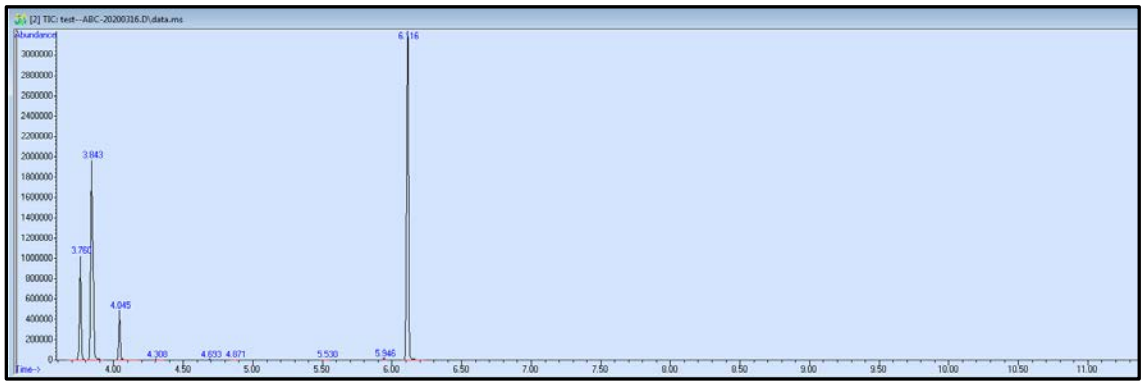


ZOOM / UNZOOM

To Zoom, (a) start from an area you would like to zoom into. Hold down the left mouse button. While still holding the left mouse button, highlight your area of interest. (b) After you release the left mouse button, you will see the zoomed in area.

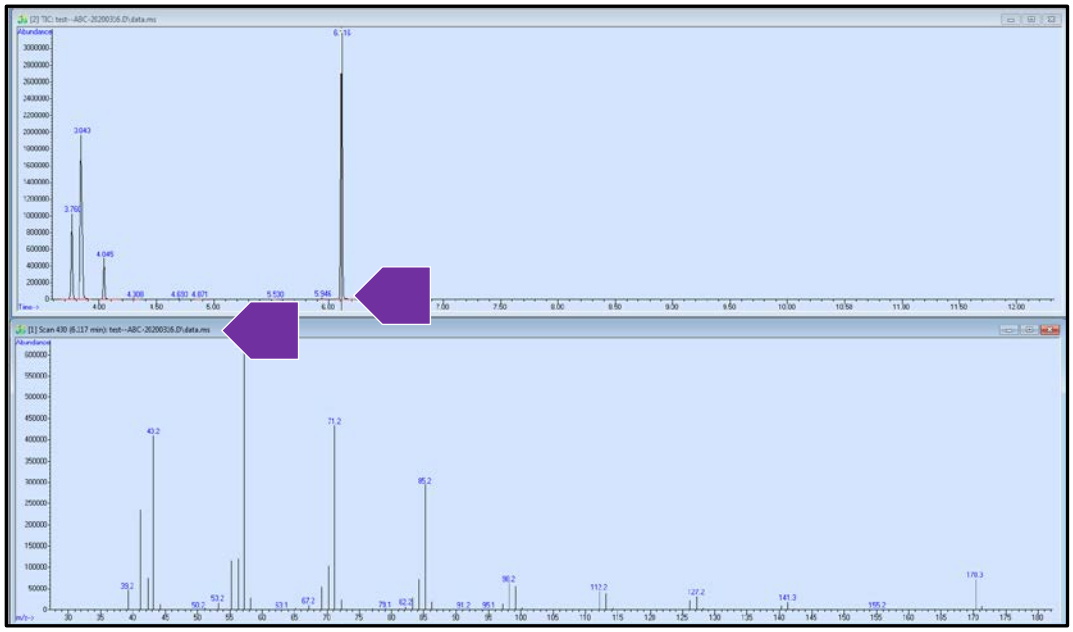


To Unzoom, double click the left mouse button. Each time you double click, it will return to the previously zoomed in view, until the full TIC is displayed again.



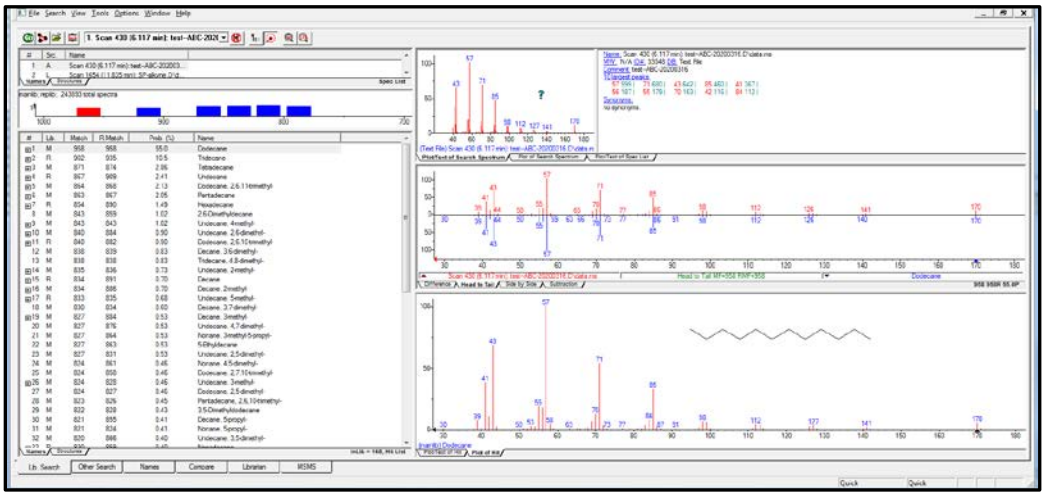
EXTRACT SPECTRA

To obtain a spectrum, double click the right mouse button on the peak of interest. You can click the right mouse button, hold down, and drag the mouse across to collect and sum multiple scans.



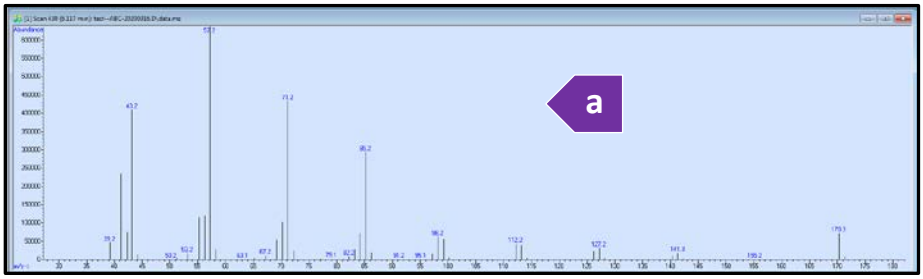
GC LIBRARY

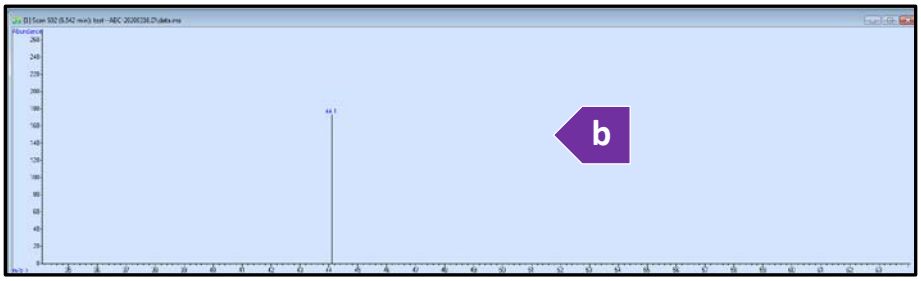
To view the GC Library database match, double click the right mouse button in the spectrum window. The Library match will appear in a new window. See Appendix B for more details regarding the GC Library.

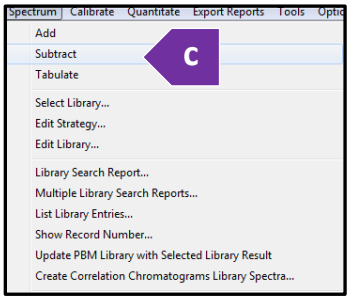


BACKGROUND SUBTRACT

To background subtract, (a) Double click the right mouse button on the chromatogram to obtain the spectrum of interest. (b) Double click using the right mouse button on the background to get the background spectra. (c) Then click on “Spectrum” at the top of the toolbar. And select “Subtract.”

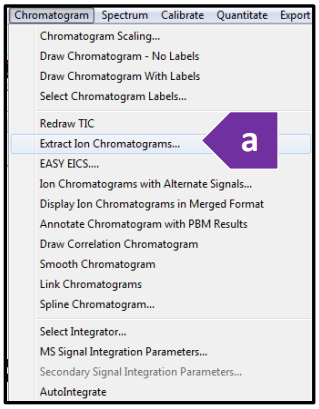


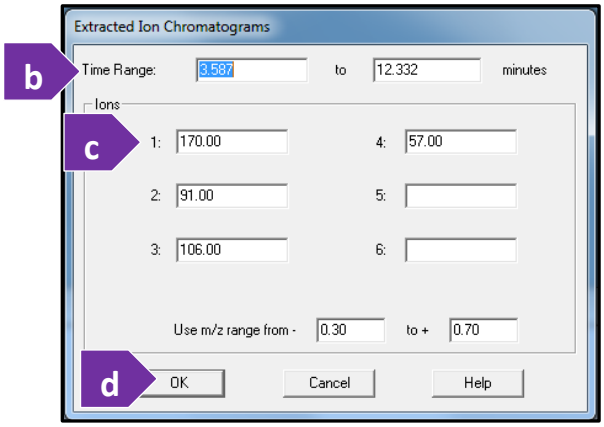


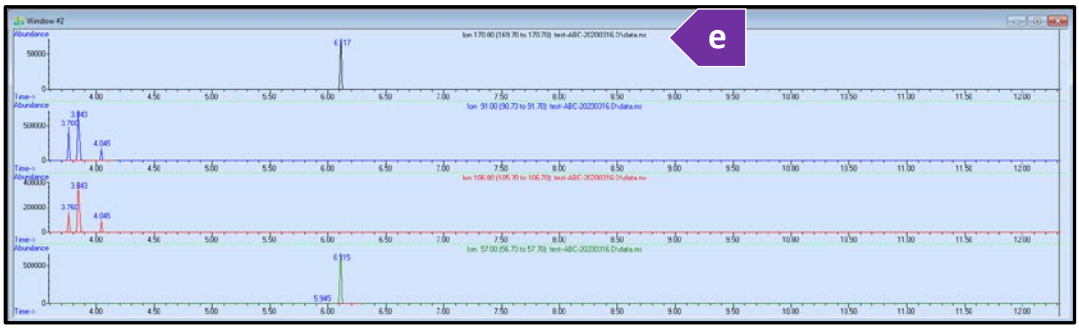


EXTRACTED ION

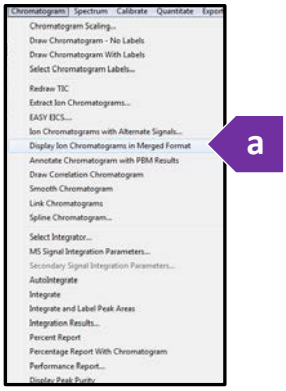
To view specific targeted ions otherwise known as “extracted” ion in the chromatogram, (a) click on “Chromatogram” at the top of the toolbar, then select “Extracted Ion Chromatogram.” (b) Enter the time range or you can select the entire length of the TIC. (c) Enter ions you want to view, (d) then click “OK.” (e) The ions will appear in separate windows. This is useful if you would like to integrate them separately.

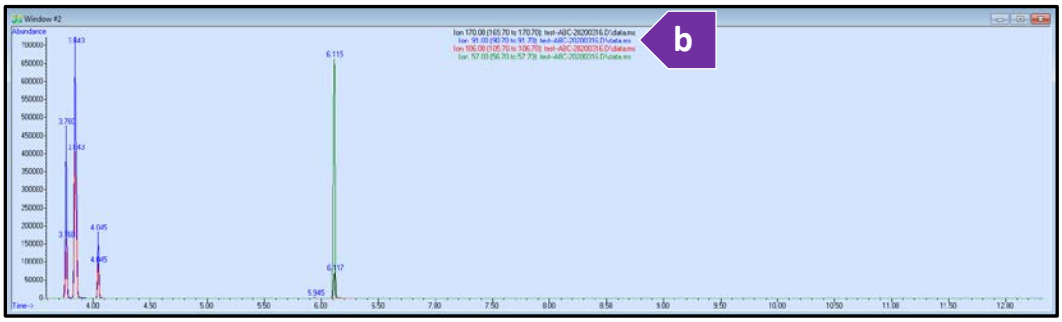






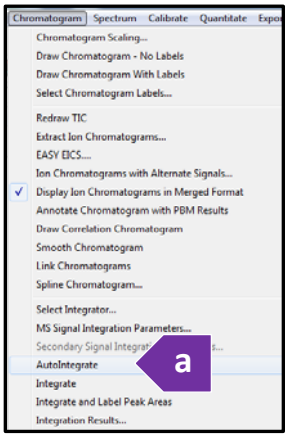
To view the ions together, overlaid on top, (a) click on “Chromatogram” on the top toolbar, then select “Display Ion Chromatogram in Merged Format.” (b) You will be able to view the overlaid extract chromatograms, which are color coded. Your selected list of ions will appear at the top.

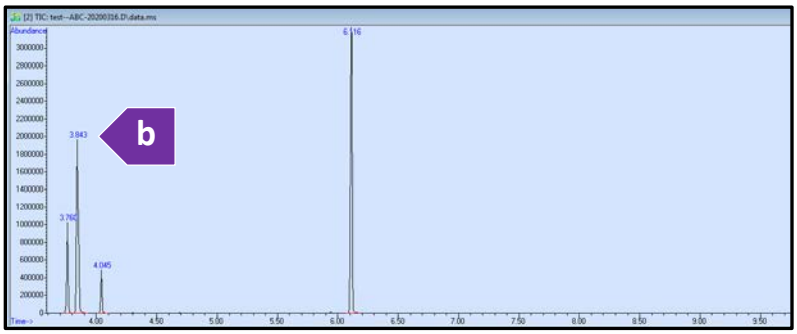




INTEGRATION / PEAK LABELING

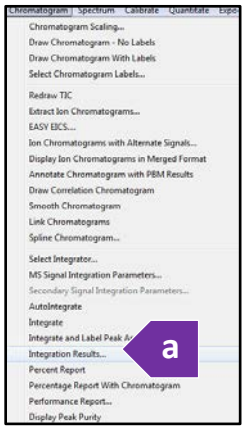
To integrate peaks, (a) Click “Chromatogram” at the top toolbar, then select “Autointegrate.” (b) The peaks will now have labeled retention times above them.

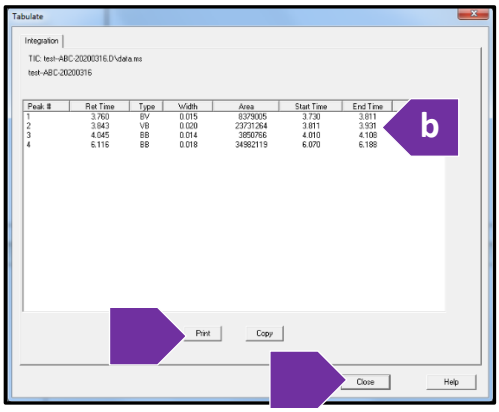




To view the peak areas from the integration, (a) click “Chromatogram,” then select “Integration Results...” (b)

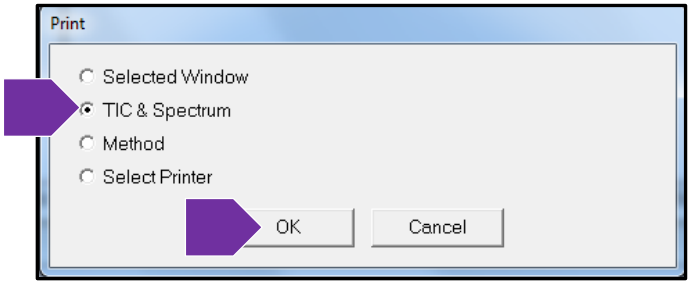
The integration results will be displayed for each peak, and you will have the option to print or close.





PRINTING CHROMATOGRAM AND SPECTRA

1. To print the chromatogram (TIC, EIC) and/or the Spectra, click the print icon  .
2. A window will appear. You can choose to print both TIC and the visible Spectrum together in one page, or Choose "Selected Window", then choosing which one to print. Choosing selected window, will allow you to print only the TIC (window 2) or only the spectrum (window 1). After you confirm your print choice, click okay to print.



PUBLICATION

EXPERIMENTAL SECTION

Mass Spectrum data was collected on the Agilent 6890 Series GCMSD using processed using Enhanced Chemstation Version E.02.02.1431 for data acquisition. Data was processed using Agilent Chemstation. The mass spectrometer was configured with an Agilent 7683 Series Injector and auto sampler, and 5973 Network Mass Selective Detector.

The GC utilized helium carrier gas fitted with a 30M, J&W-DB-5MS column with an internal diameter of 250 µm and film thickness of 0.25 µm. The sample was run using direct injection of the sample at ***<<insert your injection volume here >>*** µL injection volume. Sample was injected a split ratio of **<<insert split ratio here>>.**

Please note: *You may want to add GC Oven temperature program: Initial temperature, Hold time, Temperature ramp, Final temperature, and hold time at Final temperature.*

ACKNOWLEDGEMENT

All results gained from the use of this instrument, and used in publication must use the following acknowledgement:

"This work made use of the IMSERC at Northwestern University, which has received support from the Soft and Hybrid Nanotechnology Experimental (SHyNE) Resource (NSF ECCS-1542205), the State of Illinois, and the International Institute for Nanotechnology (IIN)."

TROUBLESHOOTING

NUCORE / RESERVATION

1. The instrument shows an error - The lower right corner has red error label, and/or the instrument front lights turn red:
    a. Fill in Bug Report.
    b. CONTACT IMSERC STAFF:
        Saman Shafaie sepehr@northwestern.edu
        Arsen Gaisin arsen.gaisin@northwestern.edu
        Gabby Allison gabrielle.allison@northwestern.edu
        Ben Owen benjamin.owen@northwestern.edu
    c. Put “Stop Sign” on instrument keyboard if staff is unavailable.
2. The computer Screen will not Turn On?
    a. Begin Your reservation in NUcore to initiate access to the instrument
3. There is an error with my reservation?
    a. If you have already started your reservation using NUcore, please logoff by selecting the error reporting option and a brief description about the issue.
    b. If you have not started your reservation using NUcore, please report problems with the instrument at http://imserc.northwestern.edu/contact-issue.html add place the ‘Stop’ sign near the instrument computer. ‘Stop’ signs are located at XXXX and online at the link above.
    c. Email or talk to a staff member.

INSTRUMENT

1. The instrument shows an error - The lower right corner has red error label, and/or the instrument front lights turn red:

a. Fill in Bug Report.

b. CONTACT IMSERC STAFF:

Saman Shafaie sepehr@northwestern.edu

Arsen Gaisin arsen.gaisin@northwestern.edu

Gabby Allison gabrielle.allison@northwestern.edu

Ben Owen benjamin.owen@northwestern.edu

c. Put "Stop Sign" on instrument keyboard if staff is unavailable.

2. I started my sequence. The software is saying LOADING VIAL in the status message line, but the instrument appears to be frozen and won't injection my sample?

a. This is a common error when the instrument is in standby over a long period of time.

i. Make sure to SAVE your sequence.

ii. Restart the computer and log back onto the IMSERC account.

iii. Open your sequence, and start your sequence again adhering to all prompts as listed in the protocol.

1. 1. Make sure to choose to override existing data file in the sequence run pane before clicking Run Sequence, because the instrument errored and technically collected data on your first sample.

SOFTWARE

For issues with software, contact IMSERC-MS Staff. Or file a "Bug Report" and place a "Stop Sign" on keyboard.

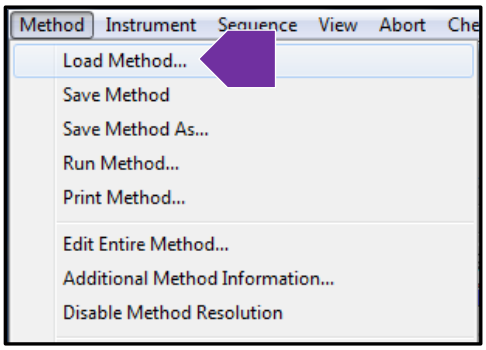
APPENDICES

APPENDIX A: EDITING METHODS

1. Open the GCMS acquisition software or check that it is already open. To open the software, you can select the GCMS icon 

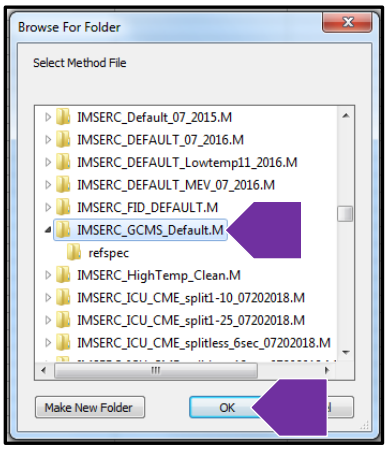
located on the top right screen or on the lower tool bar.

2. Load your method by clicking "Method" on the top left tool bar, or by clicking on the "edit method" icon  on the main screen.

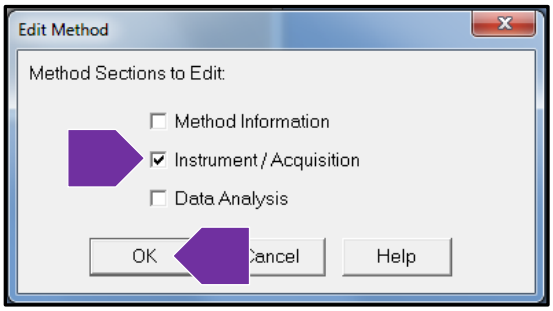


3. You may only edit your own method. If you are using the default method named "IMSERC_GCMS_Default," you must immediately "Save as" and rename the method unique to you.

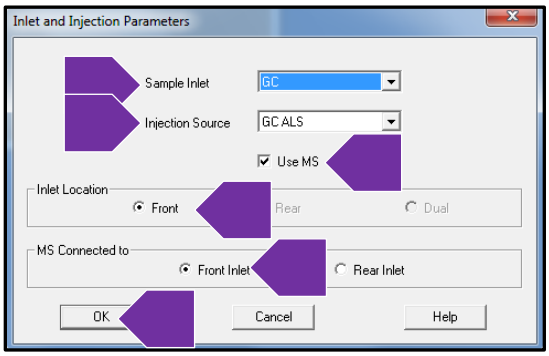
4. Locate and select your method. Then click "OK."



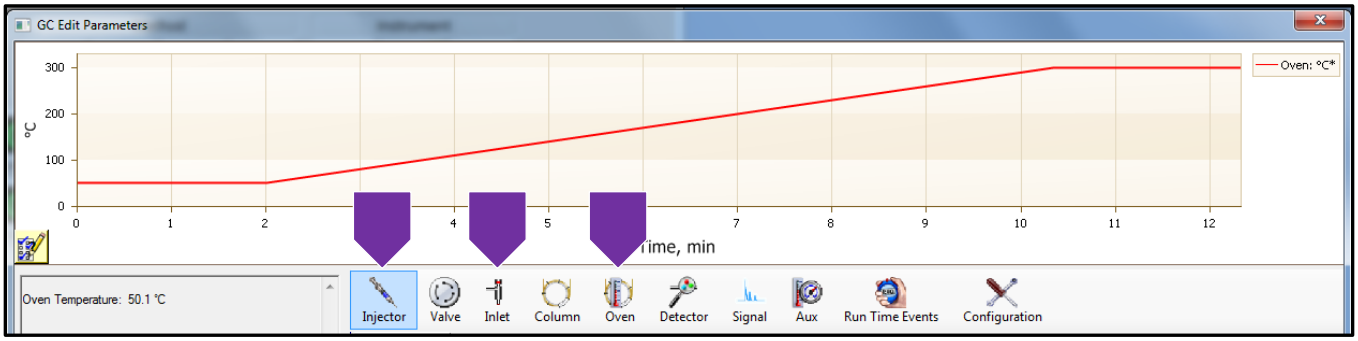
5. In the next window, only select "Instrument / Acquisition." Then click "OK."



6. Confirm the settings in the "Inlet and Injection Parameters" window, then click "OK."



7. The "GC Edit Parameters" window will appear. In this window you will notice several tabs that allow you to edit different components that make up the GC method. Injector, Valve, Inlet, etc. The only tabs you will edit consist of the "Injector," "Inlet," and "Oven." As you make changes to the method, **do NOT click "OK" until all changes to the GC parameters are done**, otherwise the method editing window will close.

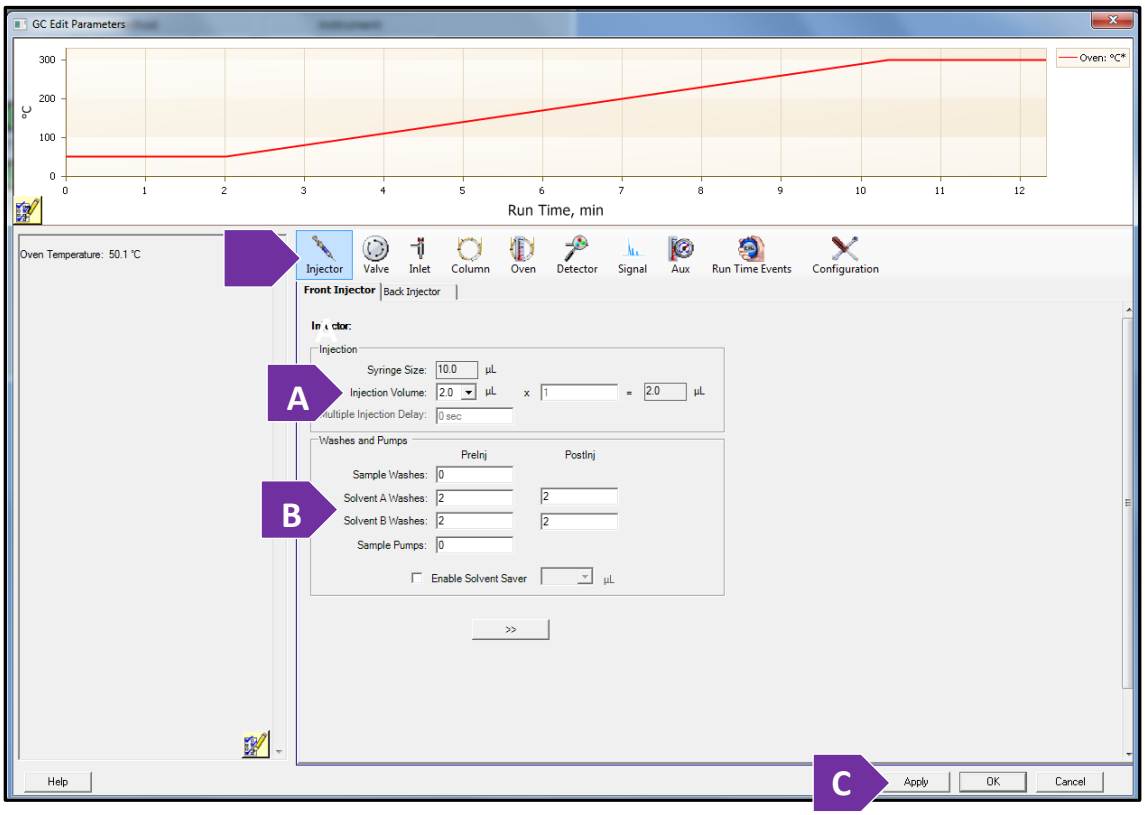


8. Select the "Injector" tab. You can change:

a. The Injection volume. This which is the amount of sample taken through the syringe. Recommended limits are within 1-5 µL. Do not use less than 1 µL.

b. The solvent washes and their frequency before and after injection. Solvent A and B are located on the carousel of the injector. Solvent A is filled with methanol and solvent B is filled with dichloromethane. You can select how many times the needle is washed before it injects your sample “PreInj” and after is injects your sample “PostInj.”

c. Click “Apply” after you have made your edits.

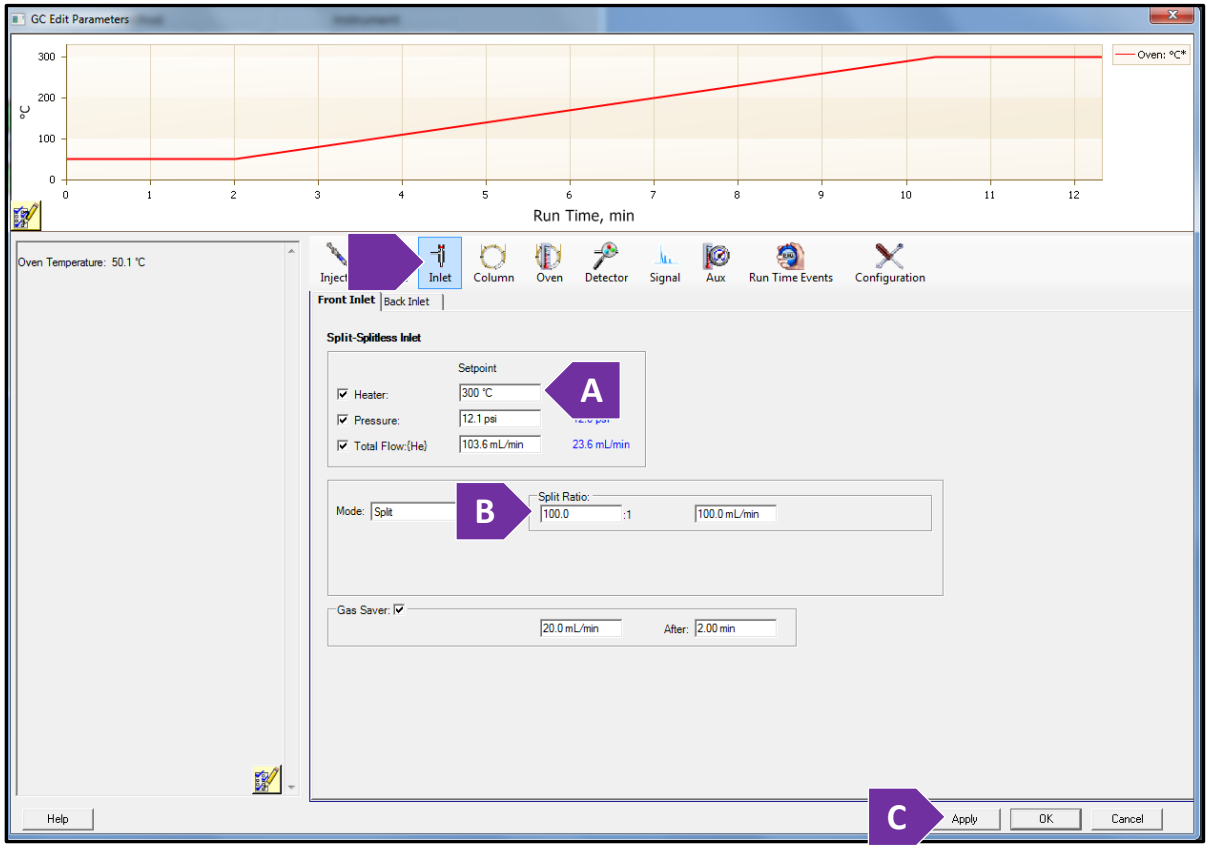


9. Select the “Inlet” tab. You can change the split ratio of your sample. This means for every X units of gaseous sample sent to waste; 1 unit is delivered onto the column.

a. It is important to point out the Setpoint of the heater. This is the inlet temperature setting. The temperature is set at 300 °C to vaporize all the target analytes for analysis. If inlet temperature is reduced, depending on their boiling points, certain compounds will not be vaporized / introduced onto the column.

b. If your split ratio is unknown, it is recommended that you start at 100. You can make further adjustments from there. Stay within the limits of 10-200 when choosing a split ratio.

c. Click “Apply” after you have made your edits.

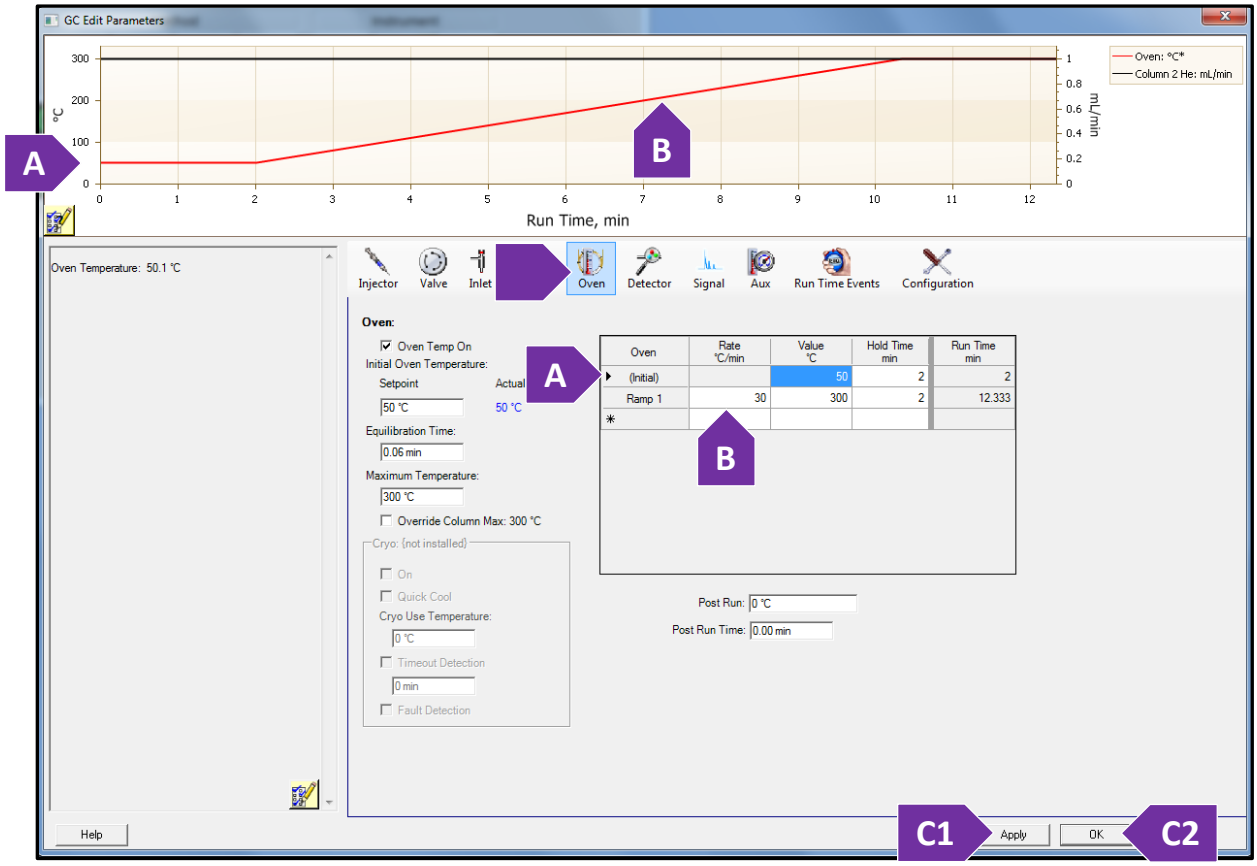


10. Select the “Oven” tab. Here you can adjust the temperature settings of your gradient.

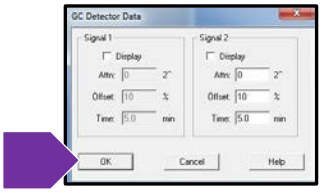
a. The initial settings tell you the starting temperature of the gradient. The hold time is how long (in minutes) that temperature will hold.

b. Depending on the rate of temperature increase (°C) per minute, will determine how the long method will be to reach the final temperature set in the method.

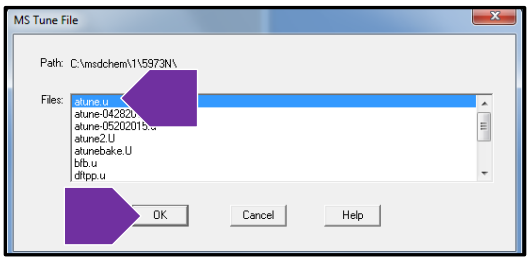
c. *(1)* Click "Apply" after you have made your edits, and then *(2)* click "OK."



11. The “GC Detector Data” window appears. No changes need to be made here since we are not using the GC detector, but the Mass Spectrometer instead. Select “OK.”

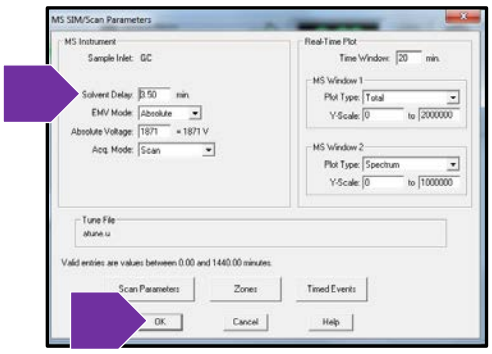


12. The “MS Tune File” window appears. Select “atune. u” and click “OK.”

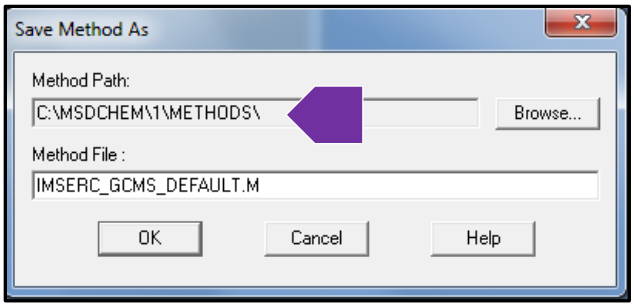


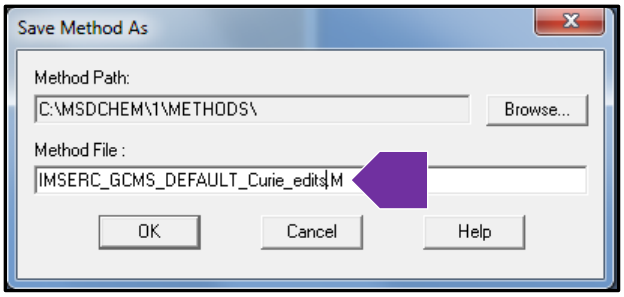
13. The “MS SIM/Scan Parameters” window appears. Verify the length needed for your ‘Solvent Delay.’ Then click “OK.” Note that, 3.5 minutes is the method default. 2.5 minutes is appropriate for lower boiling point solvents like methanol, dichloromethane, ethanol, and hexane.

    a. The solvent delay shuts off the detector to protect the MS filament from the effects of high concentration of solvent introduced to the source from your sample. The detector will be inactive for this amount of time until it has passed through the column. Depending on your choice of solvent, you may need adjust this time. **ALWAYS use the solvent delay**. Failure to use the solvent delay will results in damage to the filament. Please talk to IMSERC staff to make adjustments or setting up for other solvents and mixtures not defined in the manual.



14. Here you will have another opportunity to save your edited method. **All methods must be saved in the methods folder**. If you do not save your method in this folder, the instrument will fail and lock up when you attempt to run your sample. **DO NOT save over the default method.** It is at your discretion to uniquely name your edited method.





APPENDIX B: GC LIBRARY

How do I use the GC Match Library?

1. Look at the compound list and the match probability (Prob. %).
    i. It will match your spectrum to the closest entry. Your compound may NOT be in the library, but the library will still produce the closest match.
    ii. The closest entry may give you part of the structure, or it may be unrelated, if it is a very poor match.
2. Check the actual comparison of your spectrum (red spectrum) with the library match (blue spectrum). Compare which peak lines match up as well as the expected intensities from head to tail.
    i. You will be able to pick up small differences, which are not expressed in the matching algorithm used on the Prob. %.
3. You need a good spectrum for a good match. This means:
    i. A robust signal.
    ii. Resolved compounds (no overlap or coelution with other compounds).
    iii. A clean background.
4. The Prob. % may provide more than one entry for a compound. Review all entries and perform a comparison. This will provide and idea of what is potential variability of fragmentation data.
    i. You will notice slight variations on the intensities when comparing the entries. Using the data in this fashion will eliminate the habit of making judgments on the data based on just a probability % without looking at the raw data.

APPENDIX C: QUANTITATION METHODS

See IMSERC-MS staff.

REVISIONS

V1.0 2020/03/19	• SOP conversion to new template. New graphics uploaded; additional information added.