Standard Operations Procedure for HPLC

Upon turning on

- ✓ First, turn on the four plug points corresponding to the autosampler, quaternary pump, refractive index detector, and variable wavelength detector. Column compartment does not have a power button. Then turn on the switches given on each compartment.
- ✓ Always, open the HPLC online software followed by offline. Select either upload or download option.
- ✓ The window of the software will open then.

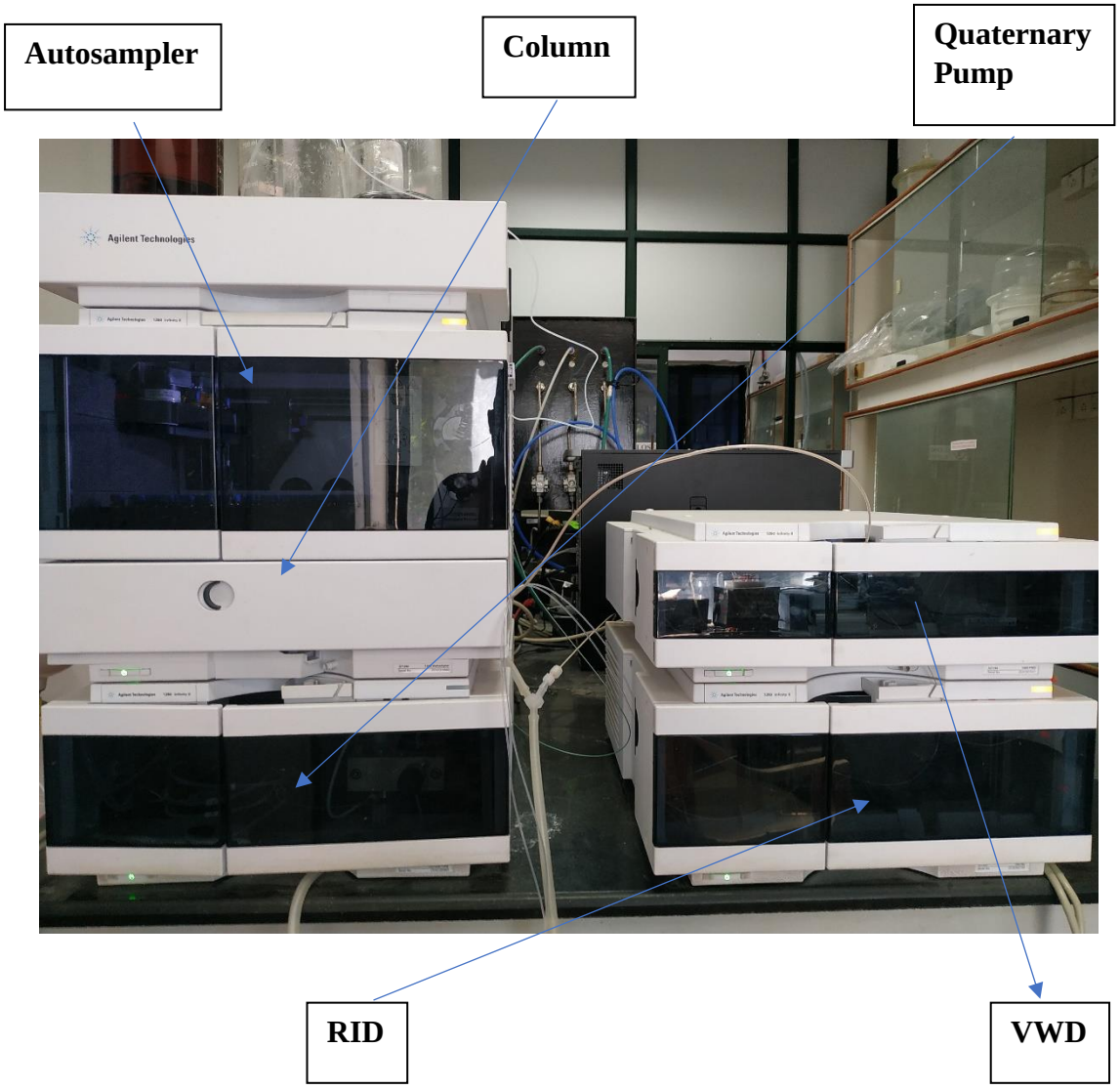

Fig 1. HPLC Instrument

Software Windows

- ✓ There are four five windows in the software screen, each one controlling the five hplc compartments.
- ✓ The compartments are autosampler, quaternary pump, column, refractive index detector, and variable wavelength detector.
- ✓ When you right click on the small window's corresponding to each compartment, several options will appear.
- ✓ Among them, the first one, '***control***' has option to turn on and off each compartment.
- ✓ The next option when you right click is ***method***. The method parameters corresponding to each compartment is give there.

Autosampler:

- ✓ There are several parameters when you go to the method tab.
- ✓ Injection volume: refers to the sample injection volume. It can be kept a maximum of 100 ul.
- ✓ Needle wash: It is used to wash the injection needle after each injection. Either a dedicated vial as in wash vial option can be used and select the wash vial. Or, select flush port keep the maximum wash time, 100s.
- ✓ Stop time: Keep the stop time as the pump run time itself.
- ✓ Keep the advanced settings as such.

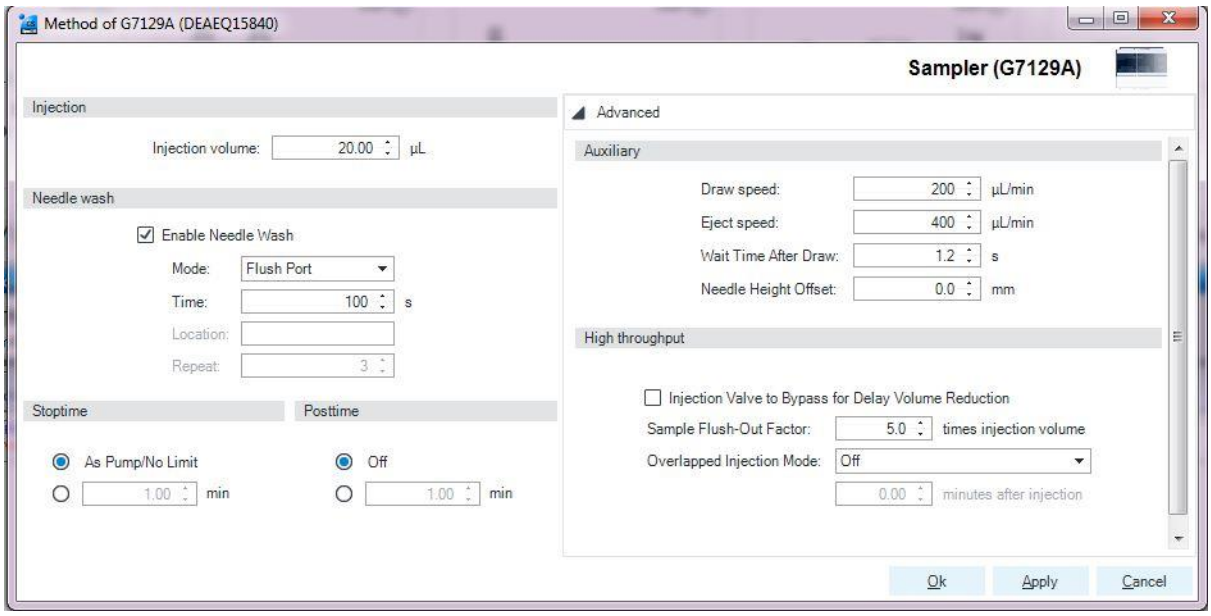

Fig 2. Autosampler method parameters

Quaternary pump:

- ✓ Flow: The maximum flow rate through the Hi-Plex H column is 0.7 ml/min. Don't set beyond that.
- ✓ Solvent: There are four solvent lines. Select the appropriate one for your analysis. 5mM H2SO4 is recommended for the analysis of carboxylic acids.

- ✓ Pressure limit: Pressure is the overall system pressure, and it varies linearly with the pump flow rate. Has kept the maximum as 100 bar. Above this pressure, system will shut down.
- ✓ The advanced settings is for gradient elution which cannot be done on RID.

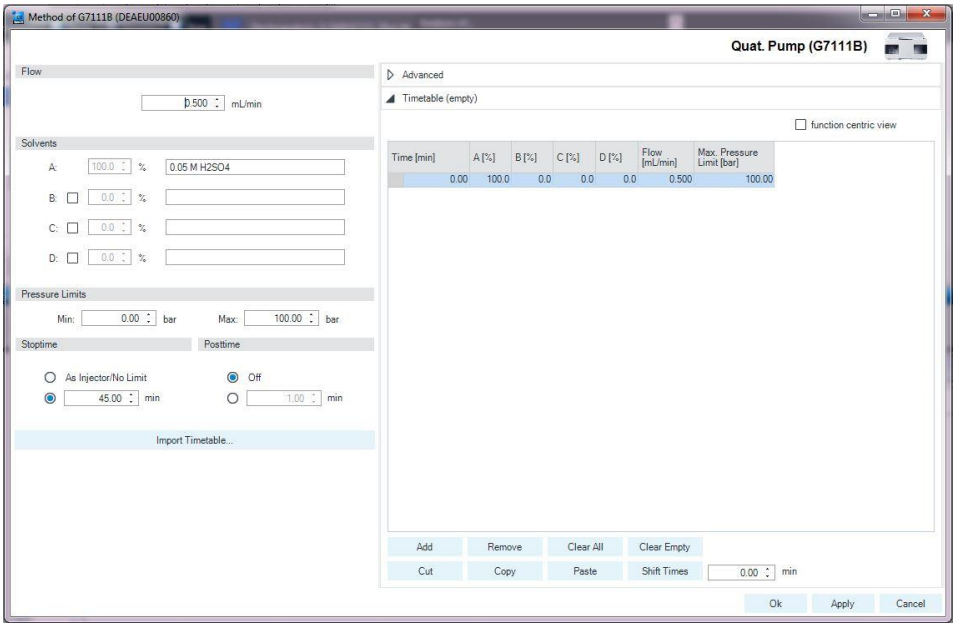

Fig 3. Quaternary Pump method parameters

Column:

- ✓ Temperature: Kept it as 50C.
- ✓ Valve position: There are no valves in this configuration.
- ✓ Stop time: Ticked same as that of the pump.
- ✓ Enable analysis: Ticked when temperature is within 0.8 C

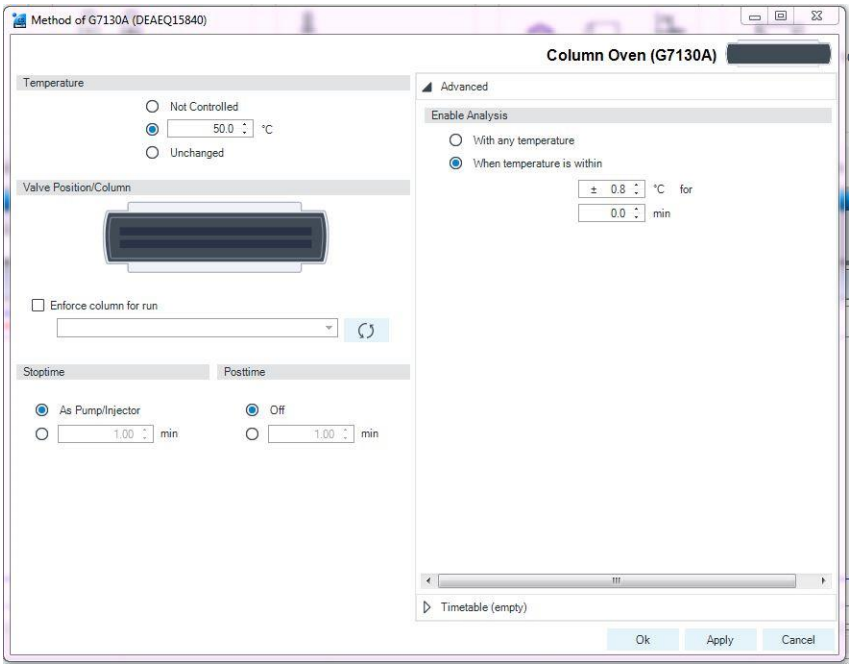

Fig 4. Column method parameters

VWD:

- ✓ VWD uses a light signal to detect chromophores in the analytes. It can be used if you have ketones/carboxyllic acids/aldehydes.
- ✓ Signal: Single wavelength is given. Acquisition frequency is given as 10 Hz with a peak width of 0.05 min. It is good enough to record sufficient data points.
- ✓ Keep the advanced settings as such.
- ✓ VWD is not the primary detector.

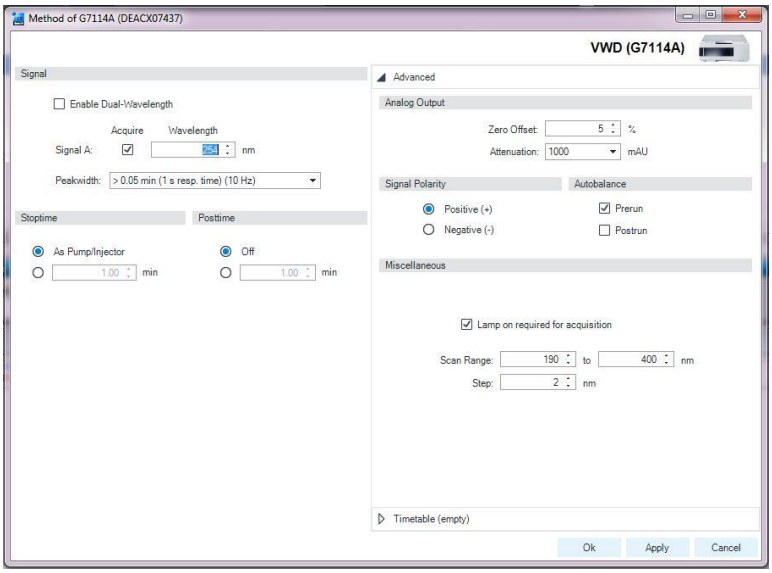

Fig 5. VWD method parameters

RID:

- ✓ RID is the primary detector in this HPLC configuration. It is a highly sensitive detector which works based on the difference in refractive index of your sample and analyte.
- ✓ Optical unit temperature: Keep it same as the column temperature. Another baseline will not be good.
- ✓ Signal: Acquisition frequency and peak width are set.
- ✓ Advanced settings are already set.

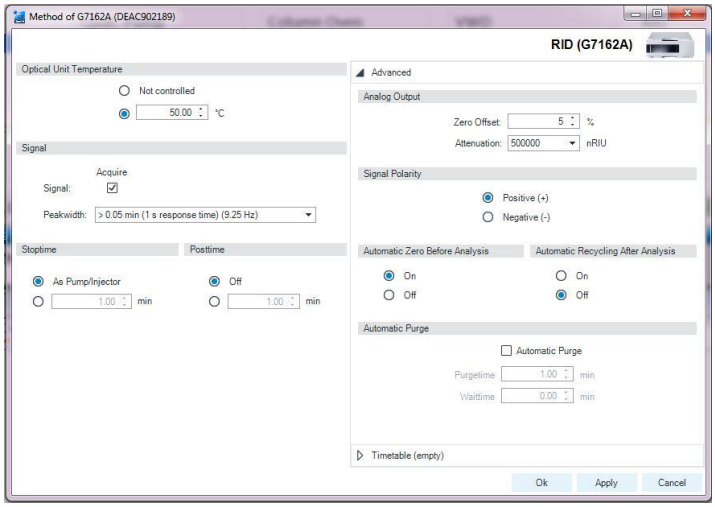

Fig 6. RID method parameters

Getting the instrument ready.

- ✓ Upon turning on, all the compartments will have a small illumination of orange light which indicate that the instrument is not ready
- ✓ Look at the solvent line above the sample compartment and check for bubbles on the line till pump compartment. Make sure bubbles are not there.
- ✓ If bubbles are there, don't put the software in analysis methods. Keep shutdown mode. Open the purge valve (one full rotation) on the pump compartment, and in the pump window in the software, keep a high flowrate of 3-4 ml/min. It is very important that the purge valve is open at this point to direct the flow to waste. If the valve is not open, and column will damage.

Setting the method:

- ✓ Once the main software window is open, system, will likely be in shutdown mode. Check for bubbles in the solvent line from the solvent jars till the pump. If bubbles are there, ***open the purge valve*** by 1 ½ rotation anticlockwise. Then go to pump the method window of pump and keep a flow rate of 3-4 ml/min until you can see the bubbles are removed from the entire line. This time, solvent will go the waste line. It will not enter the column.
- ✓ Once the bubbles are gone, stop the flow to zero. ***Close the purge valve***. Once urge valve is closed, flow will be directed to column. Load the desired analysis method. If the compartments are not on, click on the green coloured on button on top of each compartment/ go to control and turn on.
- ✓ First turn on Autosampler. Before turning on the pump compartment, go to method, make the flow rate zero. Then turn it on. Then go to method again, slowly increase the flow rate to the desired level by 0.1 at a time. After, if the software asks the method is modified, needs to be saved, say no. Then turn on the remaining compartments.
- ✓ The message here is '***Eluent flow through column should be on where column heater is on'***. Otherwise, column will be damaged.
- ✓ In the RID, the reference cell needs to purged with the eluent to clean the reference cell and to obtain a good baseline. So go to control, in the purge reference cell option, keep it on for 50-60 minutes. Or after the RID temperature reached the desired level, keep purge reference cell on for 10 to 20 minutes.

Method Development:

- ✓ You can develop your own methods for analysis by varying the method parameters. Always save the new method in a new name.
- ✓ Load a particular method. Then go to method, Save as a new method name. Then, Go to Method again and edit entire method. Make the necessary changes, then the new method is ready.

Initiating a sequence analysis:

- ✓ Click on sequence, select sequence parameters, in the subdirectory, make a new folder or select an existing folder where the data will be saved.
- ✓ Go to sequence, then sequence table. Select the vial position, enter the sample name and select the method. At the last line, you can keep ***shutdownJan2021*** method. Then

system will shutdown after the analysis of all the samples. If you keep standby method, flowrate decreases to 0.1 ml/min and remaining parameters will be the same.

Data analysis.

- ✓ Open the HPLC offline software. You can select open a sequence and view the data of both the detectors. Use the integrate tool to integrate the peaks.
- ✓ Calibration can be done in HPLC itself. Run a series of calibration standards in HPLC and open the sequence file in the offline window. Integrate all the necessary peaks.
- ✓ Open the file of the least concentration sample. Go to calibration ⟶ calibration setting. Type mM. Also, put include origin. Press OK. In the upcoming window, select level one. Press OK. Now, in the table that appears, corresponding to the peak positions, type level 1. Type the compound names corresponding to the peak positions. Press OK. Now open the 2mM data file, similarly, integrate all the peaks. Now, go to Calibration ⟶ Add level, enter level 2. In the calibration table, type level 2 in the rows corresponding to the 2mM compounds, press OK. Similarly do it for all the concentartions.
- ✓ Now, Go to Method ⟶ Save Sequence Method. Again Method ⟶ Update Master method. Now the master method is updated and you can use this method to autodetect and quantify samples.
- ✓ After running an unknown sample using this master method, integrate all the calibrated peaks, go to Report ⟶ Report settings. Select In the quantitation settings, select ESTD. Now again go to Report ⟶ Generate Report. At the of the report, you can find the quantification.