

# HPLC Manual

## General Guidelines:

All samples must be filtered.

It is highly recommended that you check the separation by TLC and/or column chromatography before attempting an HPLC run.

HPLC grade solvents only.

Test tubes for the fraction collector are 18x150 mm (stock #4711) and hold approximately 25 mL each.

No gradient elutions for runs using an RI detector. UV gradients are possible.

Do NOT exceed the maximum pressure for each column. The small column limit is 3000 psi and the large 40 mm column is 2000 psi. It is recommended that you use the lowest pressure for the separations. High pressures have increased wear on the components. The pumps are currently set for a maximum of 2000 psi.

NEVER run the column dry. Make sure there is enough solvent in the reservoir.

Sparge the solvents by bubbling through with He for about 10 minutes before equilibration of the RI detector.

Equilibrate the columns by running 5x and 20x the column volume full of solvent for RP and NP columns, respectively. The column volumes are approximately 11.6 mL and 182 mL for the small and large columns.

Ramp the pumps up or down over a period of time (<5 mL/min). Do NOT suddenly shut them off since you can damage the column.

The Scale up factor for the 10 mm scout column to the 41 mm prep column is 17.23. Multiply the sample size and flow rate by this figure when attempting to scale up from the analytical column to the preparative column.

Equilibrate the UV detector for 1/2 hour. When you are not running a sample, turn the UV lamp OFF. This is done by pressing LAMP on the UV-1 detector and following the instructions. The RI detector needs to be equilibrated for at LEAST 1-2 hours before running a sample. This is accomplished by flushing solvent through both the reference cell and the sample cell. Use the PURGE key to do this.

Run tests on the detectors and vary their sensitivities to see if you have a good baseline and a good signal from the detector. This is most easily accomplished by injecting a sample straight in and bypassing the column (position 6 on the selector). In this way, you can easily determine what settings you need.

If running UV detection on your sample, run a UV-VIS spectrum first to see where the maximum absorbance is. Do NOT assume that 254 nm is a good setting. Check first, and make your life a whole lot simpler.

The A/B switch is for the fraction collector. If you use the fraction collector mode for separation of peaks, make sure you have right setting for the detector you are using.

Concentrations for the HPLC are best around 25-50% w/v (g/mL). The more concentrated the samples are, the better (even neat), AS LONG AS THE SAMPLES ARE NOT TOO VISCOUS OR HAVE SOLID PARTICULATES IN THEM.

The analytical column can run about 1-4 mg of sample per run. The preparative column can handle between 80-320 mg of sample per run. This is sample dependent, as well as how much separation you get using the column and mobile phase. Larger amounts of sample can be run, but they can overload the column and may not be adequately separated.

It is best to play with the HPLC variables such as mobile phase and flow rate to get the best separation. Don't just use the previous settings that someone else left. A good way to check is to use TLC and see how the separation goes. You can even buy reversed phase TLC plates to test RP chromatography on the instrument.

**\*\*\* (EXTREMELY IMPORTANT) \*\*\* FLUSH THE INJECTOR PORT AFTER EACH INJECTION. THIS IS THE BEST WAY TO PREVENT BUILDUP AND/OR BLOCKAGES IN THE HPLC INJECTOR PLUMBING. \*\*\***

**\*\*\* (EXTREMELY IMPORTANT) \*\*\* AFTER A DAY'S WORK, MAKE SURE TO FLUSH OUT THE COLUMN(S) WITH 100% POLAR MOBILE PHASE (ETHYL ACETATE) AT A LOW FLOW RATE FOR ABOUT AN HOUR TO MAKE SURE THAT ALL SPECIES HAVE BEEN REMOVED FROM THE COLUMN. AFTERWARDS, RUN NONPOLAR MOBILE PHASE (HEXANES) THROUGH THE COLUMN FOR STORAGE. FOR COLUMN LONGEVITY, IT IS BEST TO STORE NONPOLAR SOLVENTS IN THE COLUMN AND HPLC PLUMBING.**

If you have any problems or are unsure of injecting a sample due to instability problems, excess viscosity, appearance (ppt?), etc, please ask experienced HPLC users for advice.

Remember, good maintenance and proper use lead to long instrument life.

# OPERATION

## Instrument Startup

Turn all the instruments ON. This includes computer, pumps, solvent stirrer, UV detector, RI detector, and fraction collector. It is essential to turn on the fraction collector so the waste can be properly directed.

## RI Detector

Turn on using the switch on the back. Make sure the solvent is degassed/sparged  
To equilibrate the detector:  
Pump > 10 mL solvent through the sample cell. Press PURGE and pump solvent through reference and sample cells for at least 1 hour (2 or more may be required.). Press AUTO ZERO to set the detector readings at zero. Press RANGE and UP/DOWN buttons to set selectivity.(256 is least sensitive, 1 is most sensitive) 32 is good start point. Press AUTO ZERO to set readings again to 0. Press FINE ZERO for a final adjustment to zeroing. Once detector is stable and the readings are somewhat constant, press PURGE again to send flow only through the sample cell. The INTEGRATOR OUTPUT sends signal to computer, the RECORDER OUTPUT sends a signal to the fraction collector.

## UV Detector

Front Panel:

Set wavelength ( $\lambda$ ) using the computer.  
Set RANGE. This sets recorder range, the signal to the fraction collector. It does NOT affect the signal to the data trace. The values go from 0.005 to 20 AUFS (absorbance units full scale). 0.005 is most sensitive, 20 is least sensitive. A good starting point is about 0.1. Check to see what levels of AU you are getting when you run a sample. Hit AUTO ZERO to zero detector and offsets.

~ (tilde)Menu:

Note: The Recorder sends signals to the fraction collector (1V max).

Go to SET RCDR.

Recorder response time (RCDR:RT), set at 0.2 sec

Recorder auto range (is OFF normally), but if you are running highly absorbant samples on the preparative column, you may want to set it at ON

Note: The Integrator sends signals to the computer data trace. (1 V max)

Go to SET INTG. (\*\*\*\*Very Important\*\*\*\*)

Integrator Range (INTG RANGE), normally set about 1V/FS (1 absorbance unit per full scale). Basically, this sets the scale of the signal set to the computer screen. The larger number, the less sensitive the setting. Values range between 01.-20AU/FS. This is the setting you will probably change the most, since you can amplify or attenuate the signal with this. Play around to get a good signal.

Integrator response time (INTG RT), set at 0.02 sec is optimal.  
Autozero should be ON, Mark should be OFF.

## Pumps

Turn on using the switch on the front panel, it should say TIME/FLOW/PRESSURE and after the computer selects the pumps, REMOTE, % A and %B. All operations should go through the computer, not the controls on the pump panel.

Features: We have a 200 mL pump head size. The max pressure is set for 2000 psi to save the columns from damage.

Priming:

The pumps are self-priming. If you need to prime, open up the prime valve on the right side of the pumps on the mast. Set ramp flow to 150 mL/min and pump solvent through at that rate through for 2 minutes, making sure there is no bubbles in the solvent lines.

## Computer Setup

### METHOD EDITOR (ME) PROGRAM

Select the HPLC icon. Select **Method Editor** (ME). Under the **System** menu, select **Set Up**. Make sure that max flow is set at 200 and the detector is UV-1. This is all you have to do with ME.

## DATA ACQUISITION (DA) PROGRAM

*Under the **Control** Menu:*

Use the HPLC icon and select **Data Acquisition** (DA). Under the **Control** menu, select **Deselect Pumps**, then under the **Control** menu, **Select Pumps**. The pumps should read REMOTE on the front console.

Under the **Control** menu, select **Manual control**. Type in the % B you desire (% A will automatically adjust), the desired Flow Rate, and the desired Ramp Time.

A menu should appear that allow you to ramp the solvent ratios to the ratio that you will be using. The system should be to 80% non polar solvent. The solvent polarity should be increased slowly. Pump A pumps non polar solvent; pump B controls the polar solvent. You need to change only the percentage of B as the percentage of solvent A will automatically change. Set the percent B as roughly determined by TLC test. Set the ramp time approximately as follows: 10% B: 10 minutes, 20% B: 20 minutes, etc. Set the acquisition time in the range 45-60 minutes, or approximately where you think the peaks will elute. Remember that both short and long run times may not give adequate separation due to too rapid elution and peak tailing, respectively.

On the pump mast:

The six position valves (#2 and #3) should always be in the same position and control the flow to the columns. Position 1 is to the analytical column, Position 2 is to the preparative column, and position 6 is to bypass the columns (used for priming and testing sensitivity of the detectors).

Now check the pump status by selecting **system** and **system status**. It should say that the system is idle:

Pump A local stopped; Pump B local stopped

To start the pump flow, Select **Start Ramp**. You should hear the pumps engage and begin to pump. Check to make sure that the waste flow is going out to the jug. If it is necessary to stop pumping, ramp down the flow rate to 0 mL/min. Do NOT use **Stop Pumps** unless it is a dire emergency! The shock of suddenly stopping flow can damage the column packing.

*Under the **System** Menu:*

System Status. This indicates flow rate, which pumps are on-line, and which contacts are on. Contacts 1,2,3,4 should be on.

Device Status. 1 and 2 are the two main pumps. 12 is the computer interface. Leave everything alone. Don't try and modify settings.

Pump Parameters. 9 refill, 5 comp. Leave this alone.

*Under the **Data** Menu:*

**Show Data Trace** (\*\*\*)Very important\*\*\*). This is the on-line monitoring of the signal. This shows you the chromatogram on the fly. You can adjust the signal amplitude and zoom in/out the peaks.

Hands: A moves up/down the UV trace, B moves up/down the RI trace, and A+B moves both traces in unison. Double click on hands to return trace to the bottom of the screen.

Slide Rule: Increases and Decreases the amplitude of the spectra (signal and noise). Function is pretty obvious.

Peaks: Click on to zoom in and zoom out the spectra.

Expanding the chromatogram. Click and drag, drawing a box on the picture to expand the chromatogram.

**Show Report.** Gives peak retention times and height/area, as well as the type of peak present.

**Monitor Channels.** Shows the signal outputs. A is UV, B is RI, C should be empty, D is fraction collector.

**Data Parameters.** Sets up all parameters for the spectra.

Acquire Data? Check boxes: A for UV detection, B for RI detection, C leave empty, D is if you want the fraction collector on. Event Mark: check if you want the fronts and tails of the peaks marked.

Sampling interval. Use 0.1 seconds for channels, A,B, and D.

Process Data? Check A for UV

Positive Peaks Only? Check if you don't want negative peaks integrated

Peak Width. 30 seconds is the default, which is OK. 60 seconds also works.  
Quantification Area. set minimum peak area at 10,000. maximum should be 1,000,000 uV, which is equal to the full scale of 1V. Check if you want to Report Peaks < minimum area or height.

Drift Limit/dSlope Threshold/Tangential shim. The default settings are OK, but you can also use the Auto Set function.

**Processing Mode.** Sets special processing for peaks. Not needed for our use.

**Display Parameters.** Also sets parameters for showing the spectra.

Display. Check boxes A for UV, B for RI if you want to observe those data traces.

Range. use 1,000,000 uV for 1V full scale.

Offset. Use the 75,000 default value.

Mark start/end of peak. Check to see the marks on the trace

Baseline. check to see dotted line drawn across the peaks where baseline should be.

Report Format. Not used in the manual data collection mode.

Sample Notes. Ignore here.

### Fraction Collector:

There are four programs that have been saved into the fraction collector's memory, each with a different separation method. They are as follows:

- A: Time- changes collection tubes every X seconds
- B: Time Windows- collects samples in a certain, set time window only
- C: Slope Detection- collects peaks when the slope reaches a certain value
- D: Level Sensing- collects once the detector moves off the baseline by a set percent

You most likely will have to edit the program to accommodate how many seconds of the solution you want pumped in each tube. In most cases, this should be done by using 20-25 mL for each tube and then figure out how many seconds this take at the flow rate used.

To load/edit a program:  
Select LOAD by pressing key C.  
Press the Key assigned to the program desired.  
If you are going to run the program, hit RUN twice.

If you are going to edit the program, press EDIT (key A):  
To modify the seconds of collection, per tube, hit key A (TIME/VOL/PEAK). Now hit collection mode TIME (key A). Depending on the program, you may or may not use the windows/peak detection program (program A doesn't, B, C and D do) Keep the rack size at 18 mm (72 tubes) for the test tubes. Type in the fraction size in seconds for the run. This is the key part here. Do the calculation! Compute using the flow rate how much time it takes to fill up a test tube.

If you are using peak detection (program B), you must also enter (in the first screen) PEAK (button B) to change the time windows you are using and for program C and D, the slope sensitivity (normal is fine) and the threshold level (10-20% is fine). The menus should be self-explanatory. If you need to go back a menu, hit the STOP button.

There are several options for collection:

- A. Test Tubes
- B. Beakers/large 400 mL bottles
- C. Through the drain valve and into 4L bottle

Remember, the test tubes are loaded in a zig-zag configuration.

There is a myriad of options for collection for the fraction collector, and if you want to know more, look it up in the manual.

## RUNNING A SAMPLE

Making an injection:

The #1 valve is the injection port. For 5 mL injections, move the valve to the UP position to load, load your sample into the UPPER injection port, and then move the valve to the DOWN position when you are injecting the sample. For 100 uL injections, move the valve to the DOWN position to load, then inject your sample into the LOWER injection port, and then move the valve to the UP position to inject.

Make sure to flush out the injection ports with solvent before and after injection to clean it and make sure there is no debris left in the port

Use an appropriately sized syringe for most injections. **ALWAYS FILTER YOUR SAMPLE BEFORE INJECTION.** Particulate matter can plug up the tubing and/or wreck the columns. Filters are available in the lab. Do it!

(\*\*\*Very important\*\*\*) Immediately after sample injection, on the computer, Select **Data, Show Data Trace.** Hit **Start Acquisition.**

Go to the Fraction Collector. Enter the appropriate program of collection. Press **Run, Run** to start the program. If you want to stop the fraction collector, hit PAUSE.

Collect and/or analyze your sample.

### Shutting down the instrument (EXTREMELY IMPORTANT)

Before shutting down, flush out the injection port. Ramp up to a more polar phase (100%) than you were using for the separation to make sure all the sample is properly flushed out. Then, flush the column with non polar solvent for storage (0% B). Now, shut down all components of the instrument.

### Printing your chromatogram

Go To Save Data As \_\_\_\_\_. Save file. Then when out of HPLC program, click on icon of the saved spectra, which loads **Reprocessing (R)**. Under the control menu, click on **Reprocess, Report**. This should print your chromatogram.

### Problems?

Technical support, parts, etc: call Varian Instruments

Call 1-800-FOR-HPLC

Ask for Wilson or Dan at Tech support

Ask for Bob Bookbinder at sales rep