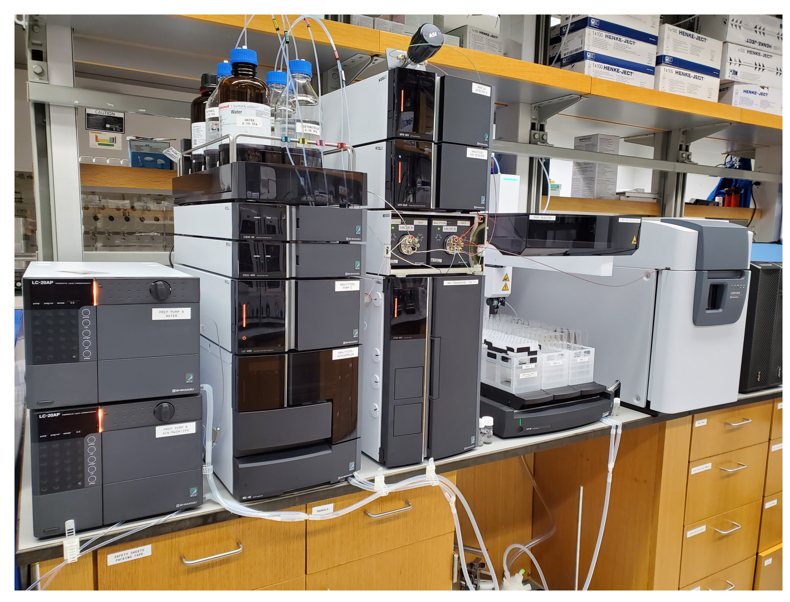
Shimadzu Nexera

Analytical/Preparative Hybrid HPLC Manual



For training and further questions contact

Morgan Bates

morganbates@ucsb.edu

Last Updated October 29, 2021

Contents

1 System Overview **2**

2 Waste management **3**

3 Start the software and turn on the instrument **3**

4 Analytical HPLC **4**

4.1 Purge the analytical pump 4

4.2 Turn on the oven and purge the autosampler 5

4.3 Analytical sample preparation 6

4.4 Create an analytical method 7

4.4.1 MS tab 8

4.4.2 Data Acquisition tab 9

4.4.3 Interface, Analog Output, LC Time Prog., Controller and AutoPurge tabs . . 9

4.4.4 Pump tab 10

4.4.5 Detector A and PDA tabs 13

4.4.6 Column Oven tab 14

4.4.7 Subcontroller A tab 14

4.4.8 Autosampler tab 15

4.4.9 Injector and Fraction Collector tabs 15

4.4.10 Download and Save the method 15

4.5 Run an analytical sample 15

4.5.1 Start a new batch 15

4.5.2 Modifying a running batch 17

5 Preparative HPLC **17**

5.1 General considerations in the use of prep HPLC 17

5.2 Purge the prep pumps 17

5.3 Prep HPLC sample preparation 18

5.4 Turn on the oven and purge the prep injector 19

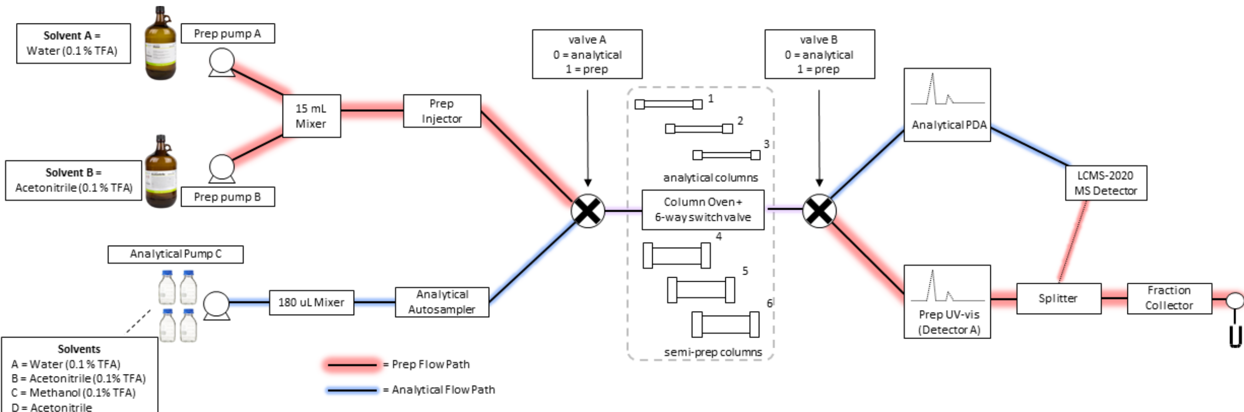
5.5 Create a prep method . . . 19
5.5.1 MS tab . . . 19
5.5.2 Data Acquisition tab . . . 20
5.5.3 Interface, Analog Output, LC Time Prog., Controller and AutoPurge tabs . . 21
5.5.4 Pump tab . . . 21
5.5.5 Detector A and PDA tabs . . . 22
5.5.6 Column Oven tab . . . 23
5.5.7 Subcontroller A tab . . . 24
5.5.8 Autosampler tab . . . 24
5.5.9 Injector tab . . . 24
5.5.10 Fraction Collector tab . . . 25
5.5.11 Download and Save the method . . . 28
5.6 Run a prep sample . . . 28
5.6.1 Set up a batch . . . 28
5.6.2 Modifying a running batch . . . 30
5.6.3 Early termination of a run . . . 31
5.7 Clean columns and shut down the instrument . . . 31

6 Analyzing the data **33**
6.1 Postrun program for analysis . . . 33
6.1.1 Viewing operation tips . . . 34
6.1.2 View MS chromatogram and spectra . . . 34
6.1.3 View the PDA data (Analytical) . . . 37
6.1.4 View the UV data (Prep) . . . 38
6.1.5 Modify the integration parameters . . . 38
6.1.6 Export the data . . . 39
6.2 Data Browser . . . 39
6.2.1 Comparing multiple files . . . 40
6.2.2 Overlay PDA and MS data (analytical) . . . 40
6.2.3 Layout Templates for comparing multiple files . . . 41

1 System Overview

The Shimadzu Nexera Hybrid HPLC system at BioPACIFIC MIP is a unique instrument designed with dual analytical and preparative flow paths. This design allows for the automated and easy interchange between an analytical tool for method development and fraction purity re-analysis and a preparative HPLC for fraction collection. This HPLC system also improves upon the typically UV-based fractionation process of prep chromatography by inclusion of an inline single quadrupole MS detector (LCMS-2020). Direct on-line mass spectrometry can help ensure only fractions containing the target compound are collected and potentially eliminate the need for subsequent offline MS analysis.

Automated switching between 3 analytical and 3 semi-prep columns and two valves along the system allow the user to choose whether to run samples on the prep or analytical side. The valve positions and column selection are set by the programmed method. The overall system layout is depicted in the schematic below.



Each prep pump feeds only one solvent to a 15 mL inline mixer that homogenizes the prep mobile phase. The solvent reservoirs for the prep line are 4 L solvent bottles located to the left of the instrument on the floor. Prep samples are introduced by the injector and separated by passing through one of the semi-prep columns housed in the column oven. The eluting peaks are analyzed by a dedicated UV-vis (referred to as Detector A). An inline splitter diverts a small portion of the preparative stream to the MS detector for analysis. Fraction collection can be done manually or triggered automatically by the UV-vis and/or MS detectors.

The analytical pump is a quaternary pump that uses four different solvents located in 1 L bottles on top of the instrument. There is a 180 μL mixer located within the pump module that blends the fluids. Analytical samples are introduced by the autosampler, separated by one of three analytical columns, and subsequently analyzed by a dedicated PDA and the LCMS-2020 MS detector.

In general, there are six steps for running the HPLC.

1. Start the software and ready the instrument
2. Design a method
3. Prepare a sample
4. Set up and run a batch
5. Analyze and export the data
6. Clean the columns and shutdown the instrument

These steps are covered in six sections, with steps specific to running analytical or prep HPLC described in sections 4 and 5, respectively.

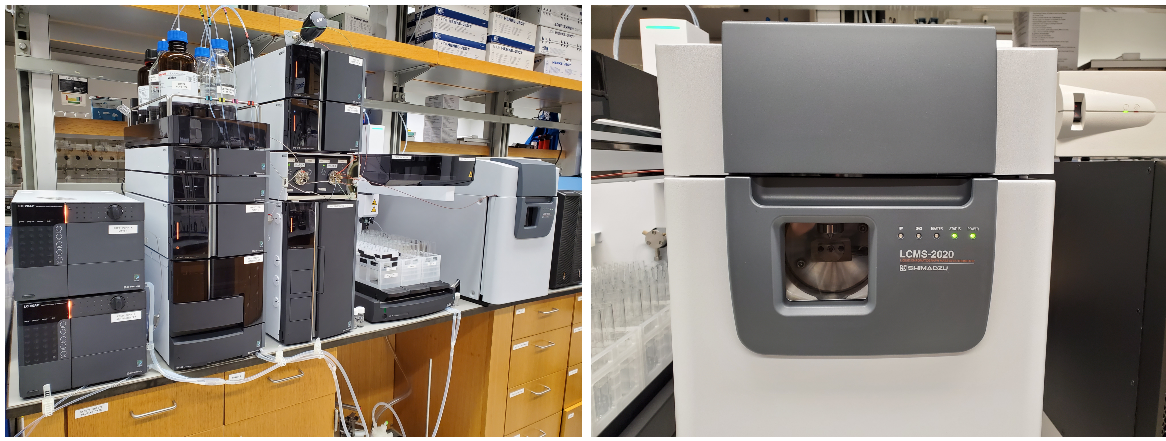
2 Waste management

Always check the solvent level in the receiving waste container prior to beginning a run. When the waste container is full, transfer the container to the hazardous waste pick-up area and check that the waste label is properly marked. Reinstall a new waste container with a new hazardous waste label attached. Depending on availability, three different types of waste containers are able to be used with the instrument.

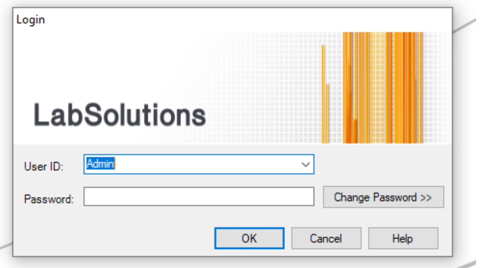
In the left photo, tubing is connected via a hose barb adapter for the container shown. The middle photo shows a reusable white 9 L container provided by EH&S that should only be used for HPLC eluent waste. The right photo shows how to connect a recycled 4 L solvent bottle as a waste reservoir. Spare caps with drilled holes for inserting the waste line are located in the secondary containment tub.

3 Start the software and turn on the instrument

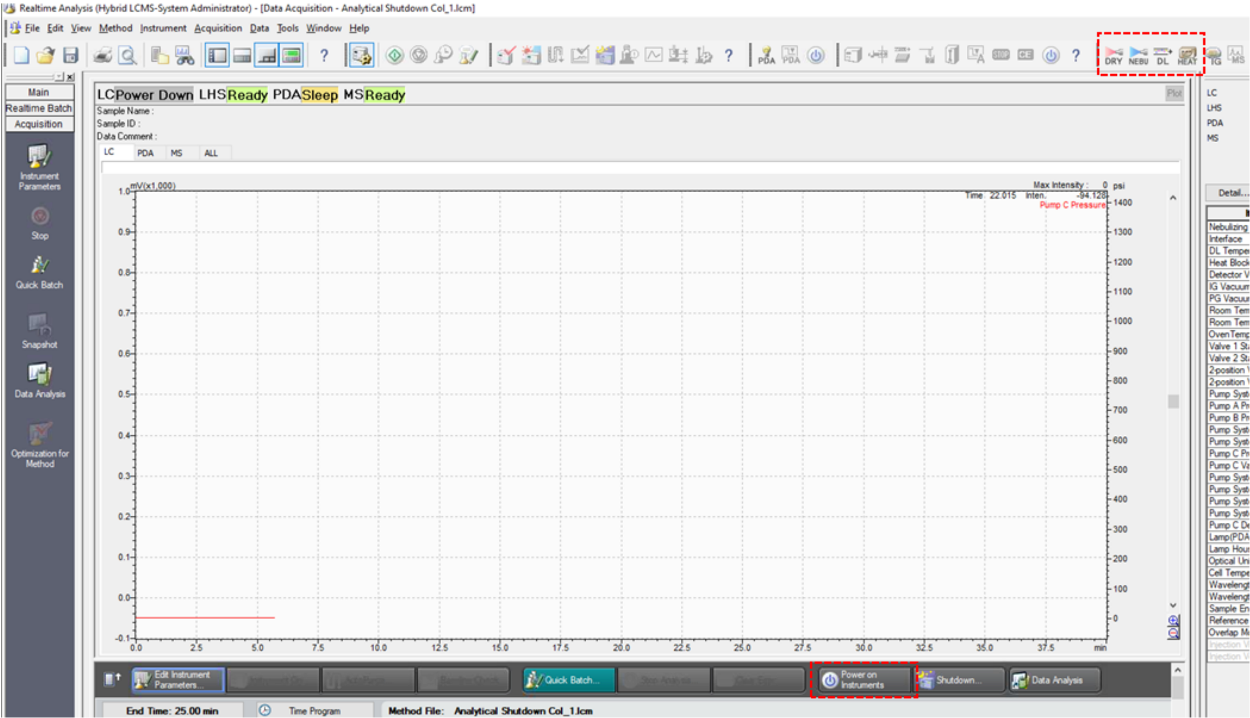
Each user will clean the columns that were used in their method and shutdown the instrument before leaving. When approaching the HPLC, it will be in a sleep state and look as such:



Double-click the Lab solutions icon on the computer desktop to open the software. Login user ID is "Admin". No password is required. Click OK.



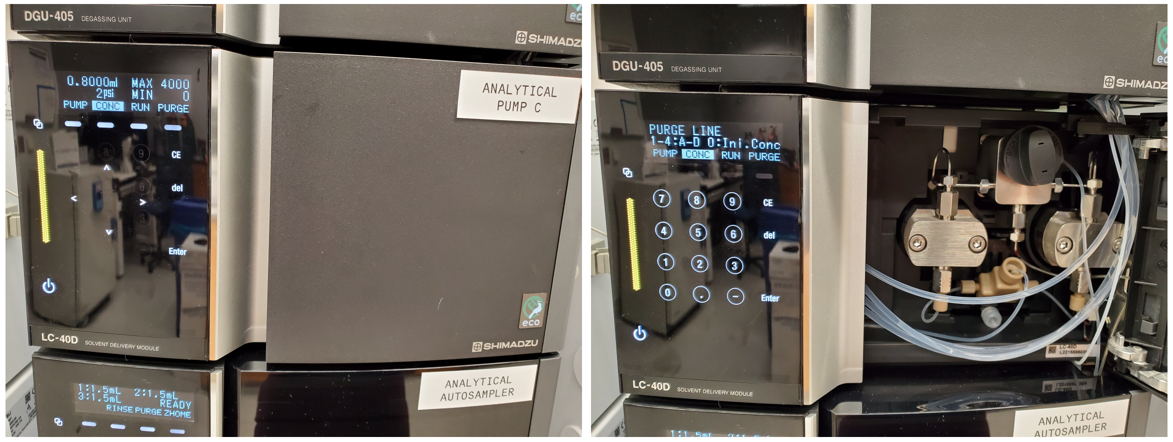
Start the real time analysis program by double-clicking Hybrid LCMS listed under Instruments. Two beeps will be heard as the system connects to the computer. The software will open to the following screen. Click Power on Instruments and the four icons DRY NEBU DL HEAT to ready the instrument



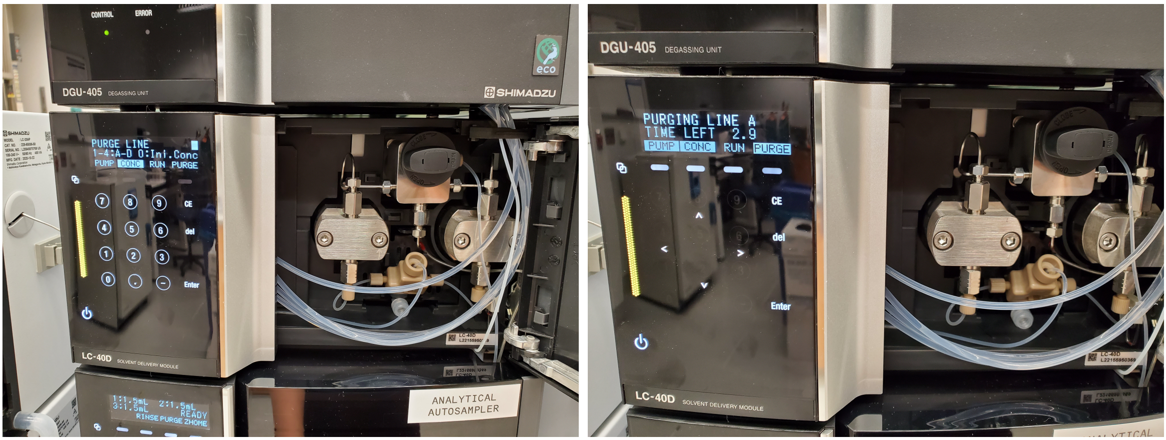
4 Analytical HPLC

4.1 Purge the analytical pump

Check that there is a sufficient amount solvent in the 1 L bottles located on top of the instrument. Purge the solvents that will be used, including solvent D (pure acetonitrile) since it will be used to clean the columns at the end of your run. To purge the solvents, open the door of the analytical pump and turn the handle of the black valve counterclockwise to open the purge valve.



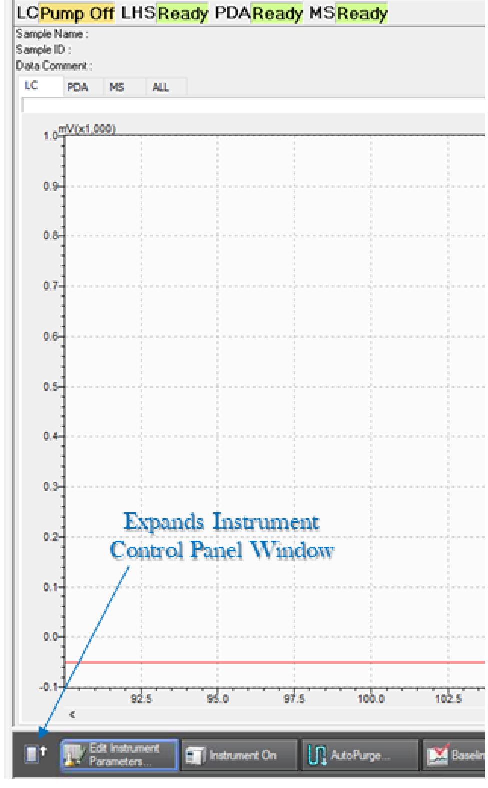
Press the purge button and type the number of the solvent line you would like to purge (e.g., 1 = Solvent A, 2 = Solvent B, 3 = Solvent C, 4 = Solvent D). Press Enter.



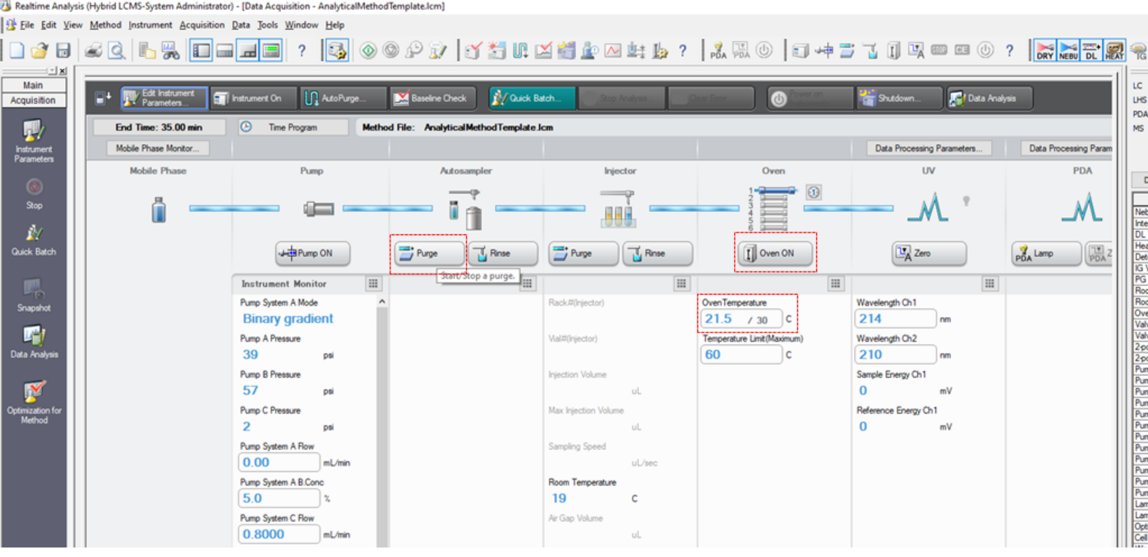
Repeat for each additional solvent line. Once the purging is complete, remember to close the black valve finger-tight. *Do not close too tightly or you may damage the pump.*

4.2 Turn on the oven and purge the autosampler

Return to the software and expand the control window of the system by clicking the icon with an up arrow as indicated in the figure below.



Turn on the oven, adjust the set-point of the oven (if required), and begin to purge the autosampler using the icons indicated below. Purging the autosampler will take ∼ 10 min. To efficiently use this time, begin to set-up a method and/or prepare your samples.



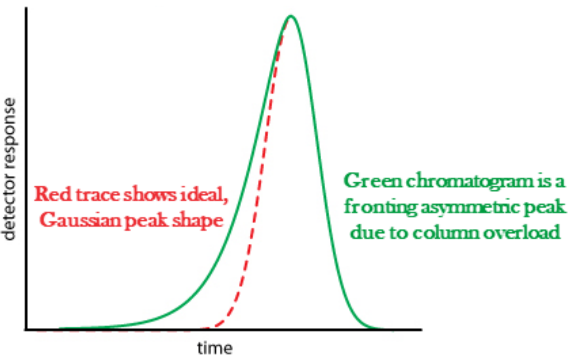
4.3 Analytical sample preparation

Always filter samples using the provided disposable 0.20 or 0.45 μm syringe filters. Hydrophilic PVDF and hydrophobic PTFE membrane materials are both suitable for filtering aqueous solutions, but sometimes more hydrophobic samples (peptoids/peptides) can adsorb to the PTFE surface when poorly solvated in water; in which case, the PVDF membranes should be used.

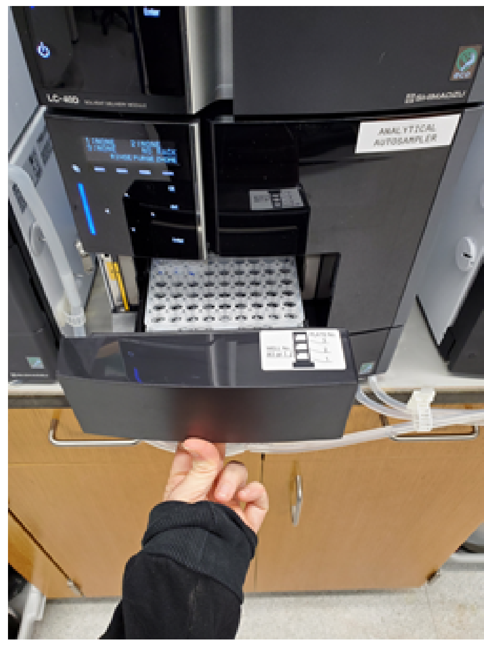
Vials of the mobile phase solvents with TFA additive are provided near the instrument to prepare samples for injection. Samples should be dissolved in 100% aqueous solution. If the sample is insoluble in 100% water, add organic modifier (acetonitrile) but keep it to a minimum to prevent premature elution of the sample from the column.

Sample concentrations of 0.001 to 0.01 mg/mL are generally adequate for injection volumes of 10 μL. However, $\sim$0.1 mg/mL has been necessary for large peptides/peptoids (>50-mer). If you are not obtaining enough signal from your sample at a given concentration, you can opt to increase the injection volume up to 100 μL. As a rough guideline, the max sample capacity for a 4.6 mm diameter analytical column is 0.2 mg. **Avoid overloading.** Too much sample should not be injected. If peak shape changes by diluting the sample 10 to 100 folds or by decreasing the injection volume to one fifth, there is a possibility of sample overloading.

Overloading is also indicated by the fronting of peaks as illustrated:



Open the autosampler tray and place the prepared samples inside, noting the tray number and labelled position number within the tray.



4.4 Create an analytical method

Navigate to **File** → **New Method File**. Select **AnalyticalMethodTemplate** to start developing a new analytical method. This template method applies a 5-95% B (acetonitrile) gradient

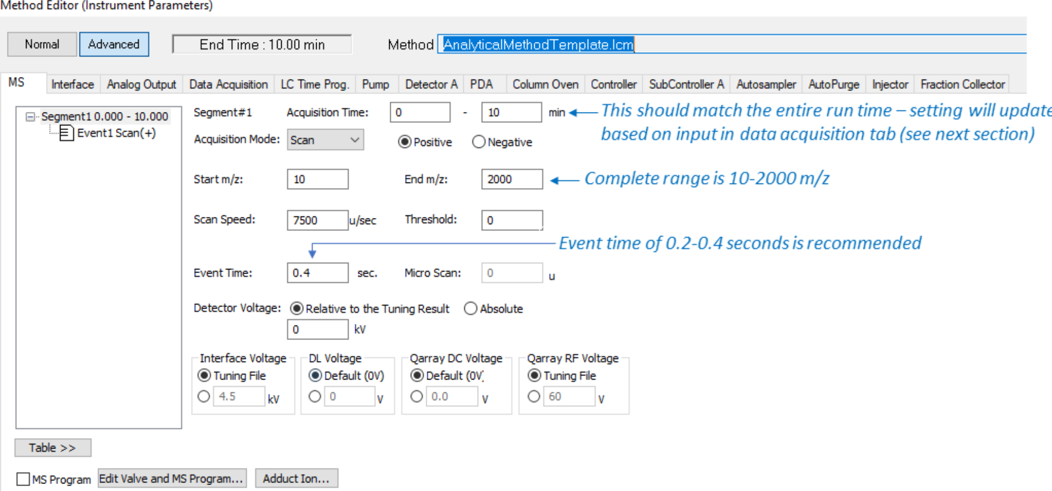
over 45 min with a 3 min hold to flush the column and 2 min hold to re-stabilize at the initial condition.

The Method Editor window will appear, containing a series of tabs that represent different modules of the HPLC. If the template is employed to design a method, most of these tabs will not require modification, but all are documented in this manual for completeness.

4.4.1 MS tab

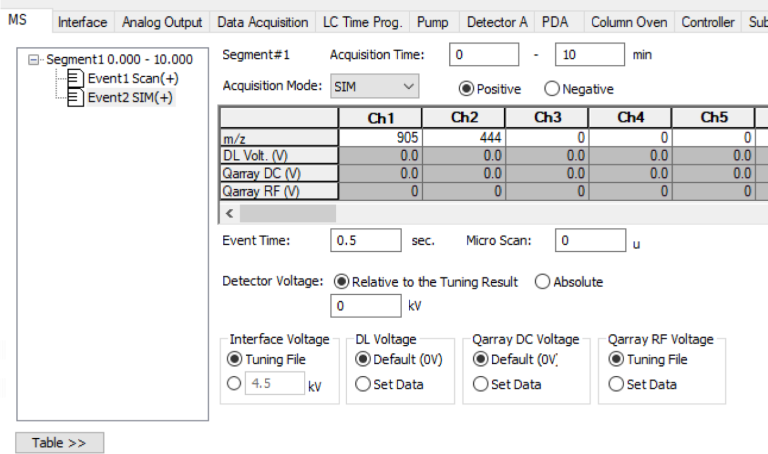
Important: Only use positive mode for solvents containing TFA or damage to the MS detector may occur. If you would like to use negative mode, ask for assistance in switching the system to an appropriate solvent and additive.

On the MS tab, the acquisition time specifies over what time the MS detector will scan the m/z range and record data, generating a chromatogram of the total ion count (current). The acquisition time should equal the length of time required for the entire run, which includes time for the gradient plus 5 additional minutes to wash the column and return it to the initial condition.



In the above example, the mass spectrometer is set to scan over a pre-defined mass range of 10-2000 m/z and record full mass spectra. This range can be narrowed to increase instrument sensitivity if the analyte m/z is known. Be aware that compounds can acquire multiple charges, and the more charged compound may actually be observed in greater relative abundance.

Alternatively, additional events can be added to increase detection sensitivity for a specified m/z, such as selective ion monitoring (SIM). To add a SIM event, right click on Event1Scan(+) located within the left panel, and click “Event Add”. Change the acquisition mode from scan to SIM and specify the m/z values of interest in Ch1, Ch2, etc.



4.4.2 Data Acquisition tab

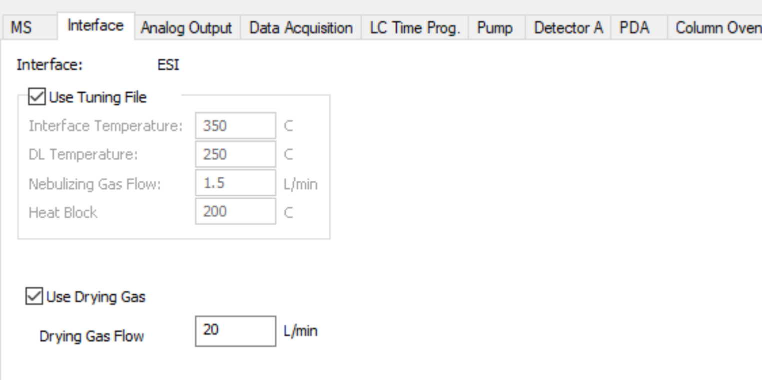
The data acquisition tab tells the detectors to acquire data for the duration of the run and to set the frequency of measurement for the UV (prep) and PDA (analytical) detectors. The LC Stop Time should equal the entire run time. For a 5-95% gradient applied over 45 min, LC Stop time should equal 50 min, since it includes time for cleaning and stabilization of the column.

Click **Apply to All acquisition time** so this setting will be carried over and applied to all other tabs (including the MS tab described above). It is not recommended to adjust the sampling rates for the PDA or UV detector in order to avoid data files becoming too large or the data being too sparse.

4.4.3 Interface, Analog Output, LC Time Prog., Controller and AutoPurge tabs

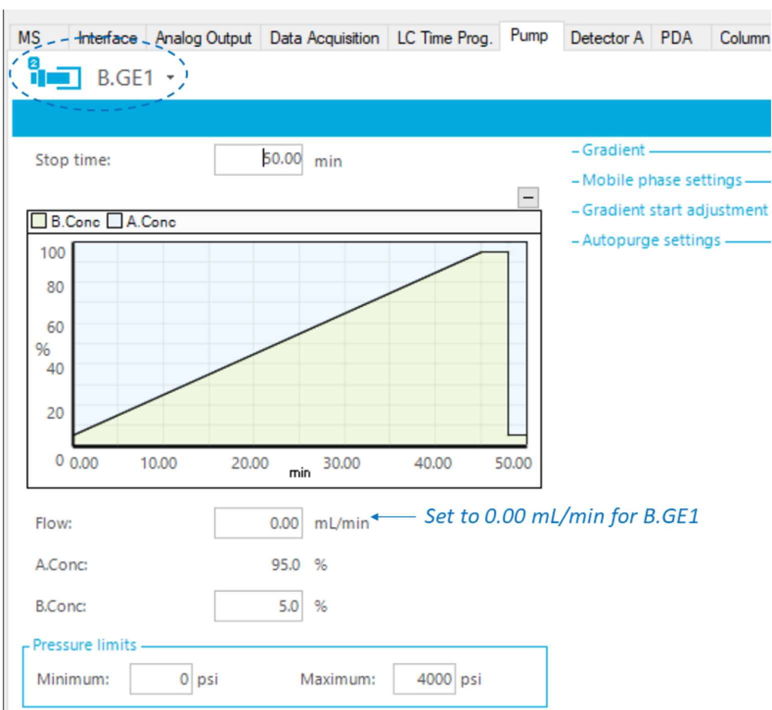
No changes should be necessary on the interface, analog output, LC Time Prog., Controller, or AutoPurge tabs. For the interface tab, confirm that the image below matches the settings in

the method.

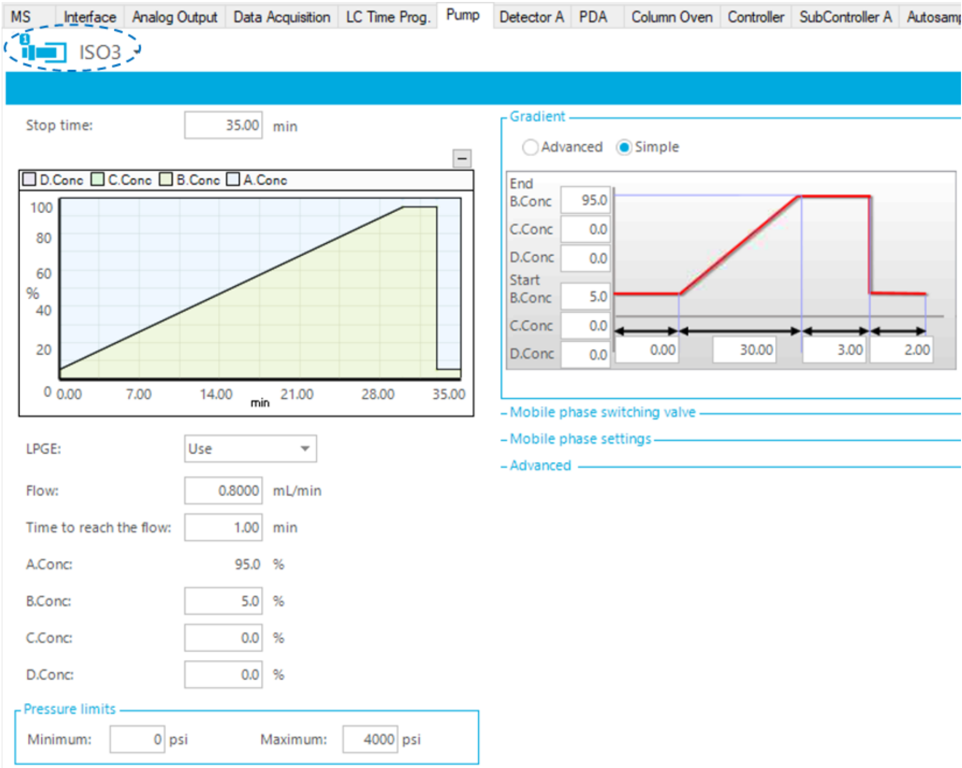


4.4.4 Pump tab

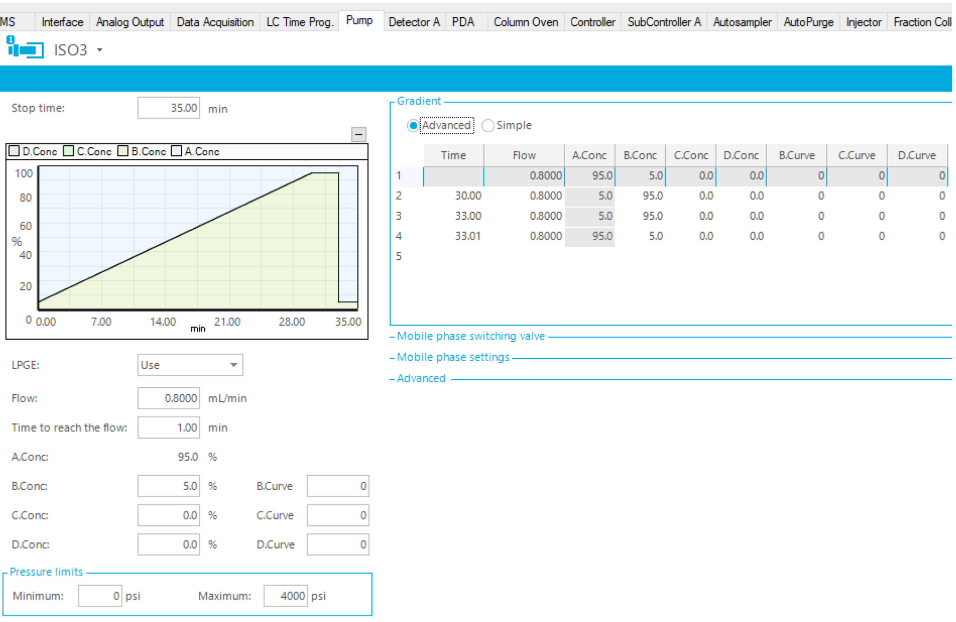
The pump tab defines the gradient that will be applied during the sample run. When the tab is selected, it by default shows settings for pump B.GE1, which refers to the two prep pumps. Ensure that the flow rate is set to 0 mL/min as shown below.



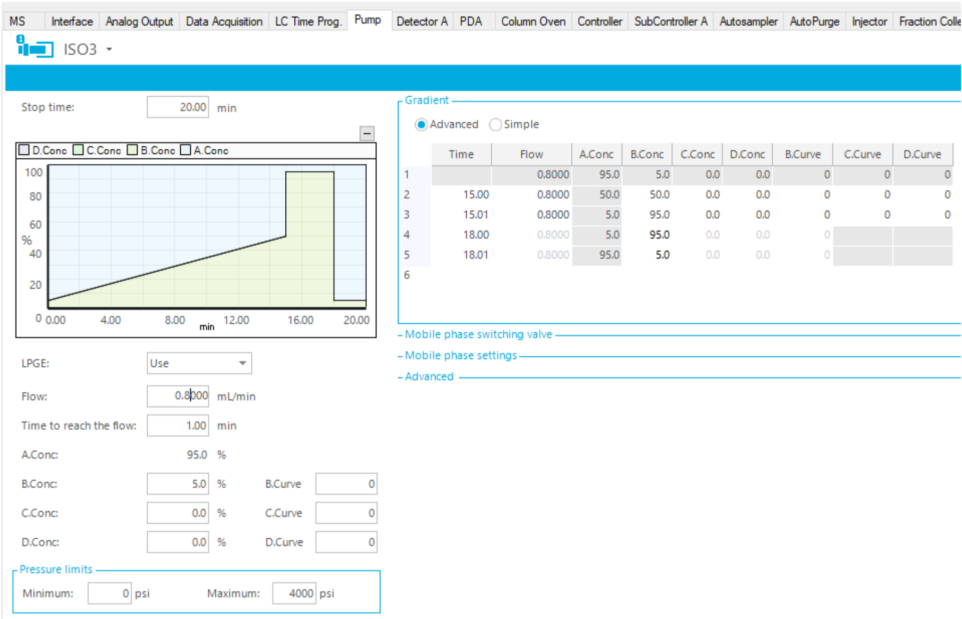
Click on B.GE1 to use the dropdown and switch to ISO3, which is the quaternary analytical pump. Analytical flow rates should be within the range of 0.5–1.2 mL/min with a gradient steepness of 0.5–2.0 % acetonitrile/min. Time to reach flow protects the columns from rapid changes in pressure and should be set to 1 min. Under the gradient menu, a simple gradient from 5–95%B can be programmed for the method using the graphical entry on the right shown below. Always include an isocratic hold for 3 min at 95 %B and for 2 min at 5 %B at the end of the gradient to clean and stabilize the column.



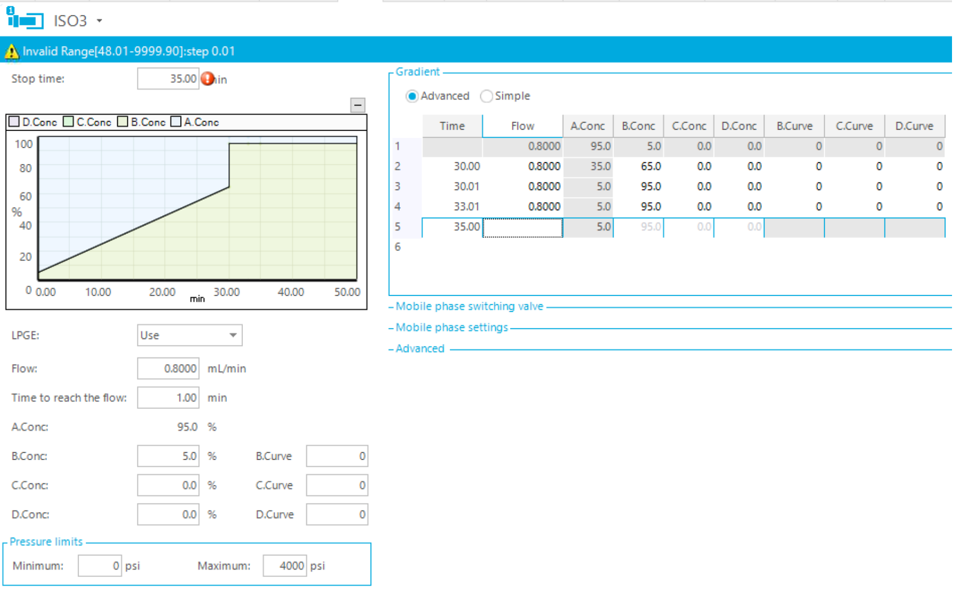
Gradients of greater complexity can be defined via Advanced gradient mode. An example of the above gradient in table form is shown below:



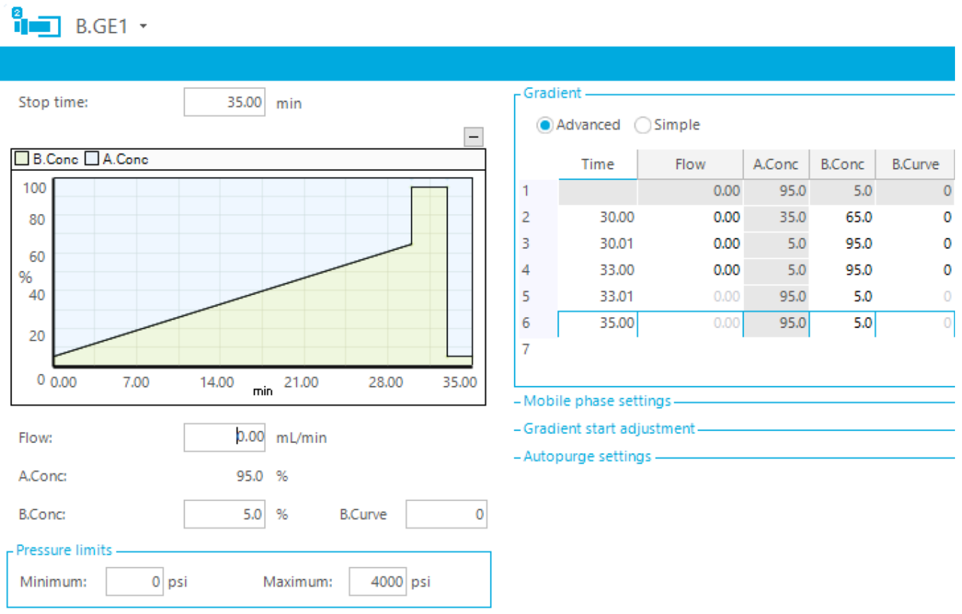
This table format is useful for shortening the run time by applying gradients over a narrower range of interest because **a subsequent isocratic hold at 95% B to clean the columns at the end of the run is still required.** A 2 min hold at 5% B follows in order to restabilize the column at the initial condition. An example of an advanced gradient table implementing these holds is shown below:



Note that when trying to reduce the gradient range for the ISO3 (analytical) pump by modifying the table, you may observe this error message about an invalid range:



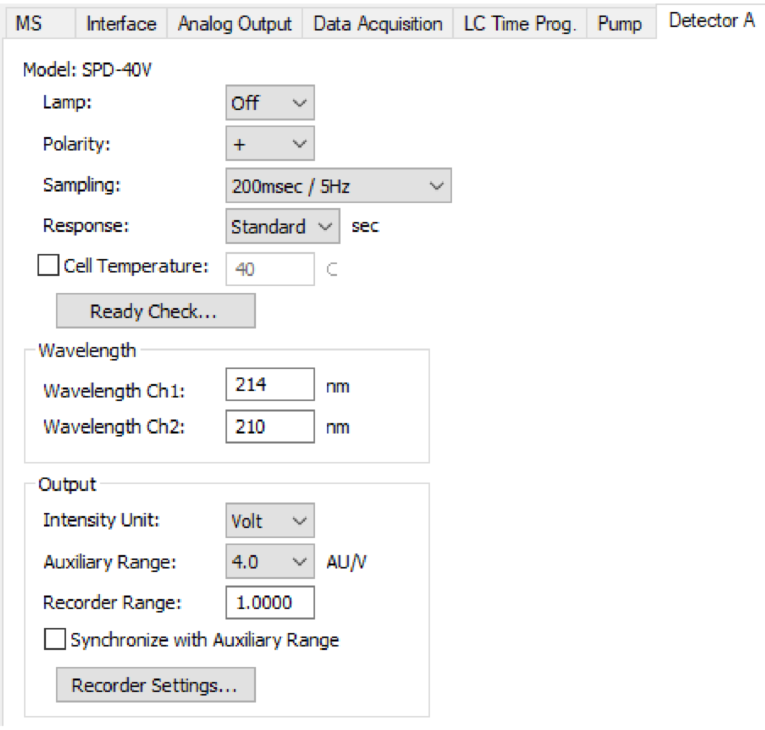
This is due to a bug in the software and is fixable by also modifying the gradient table for pump B.GE1, as shown below:



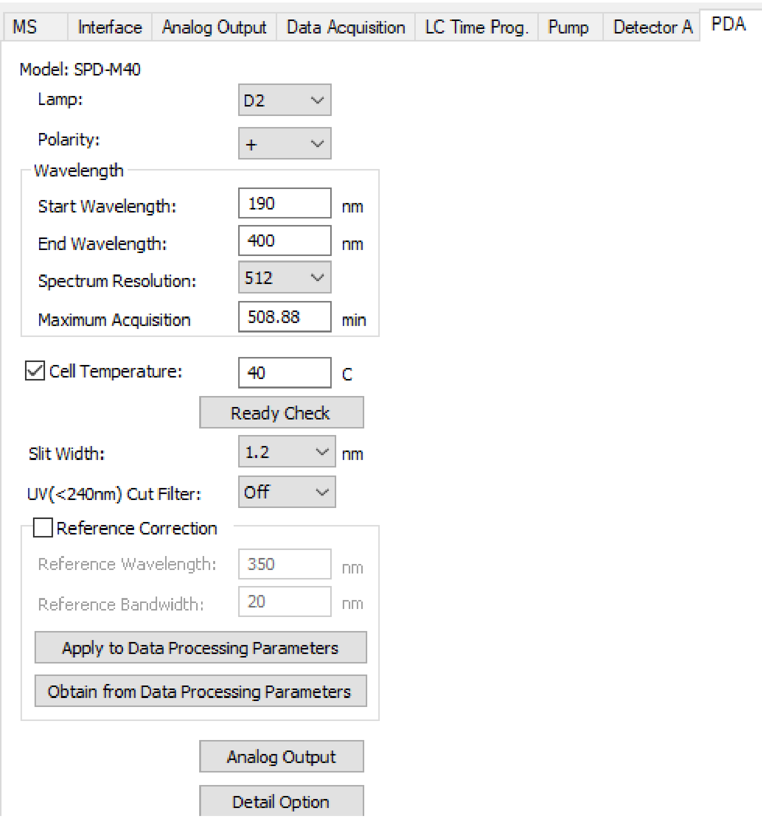
Remember to change LC Stop Time and click "Apply to All acquisition time" in the Data Acqusition tab if reducing the run time of the gradient!

4.4.5 Detector A and PDA tabs

Detector A refers to the prep UV detector. Ensure this lamp is turned off as shown below.



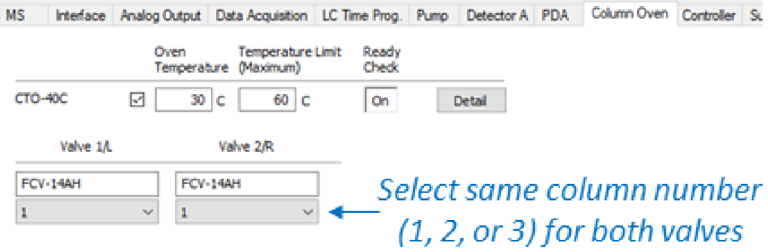
The PDA is the analytical UV-Vis detector that is capable of monitoring all wavelengths (190–800 nm) simultaneously. The example below shortens the range of interest to 190–400 nm to reduce the file size and employs the deuterium (D2) lamp only. The tungsten (W) lamp or the D2 & W lamps can be used to monitor wavelengths of 371–800 nm or 190–800 nm, respectively.



4.4.6 Column Oven tab

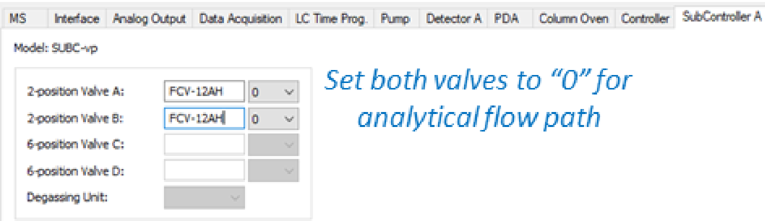
Set the column temperature. Note that the max column temperature allowed is 60 °C. Select the same analytical column (1, 2, or 3) for both valves, in accordance with these designations:

1. XBridge BEH C18 Column, 130Å, 5 µm, (4.6 mm x 150 mm)
2. XBridge BEH C18 Column, 300Å, 5 µm, (4.6 mm x 150 mm)
3. XBridge Protein BEH C4 Column, 300Å, 5 µm, (4.6 mm x 100 mm)



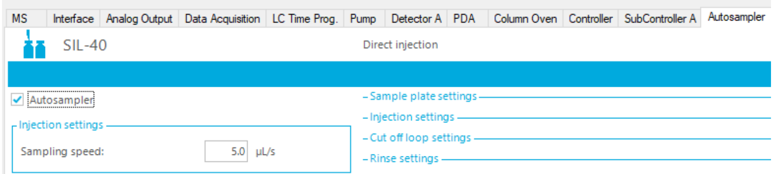
4.4.7 Subcontroller A tab

In the Subcontroller A tab, select the zero position for both valves so the analytical flow path is employed.



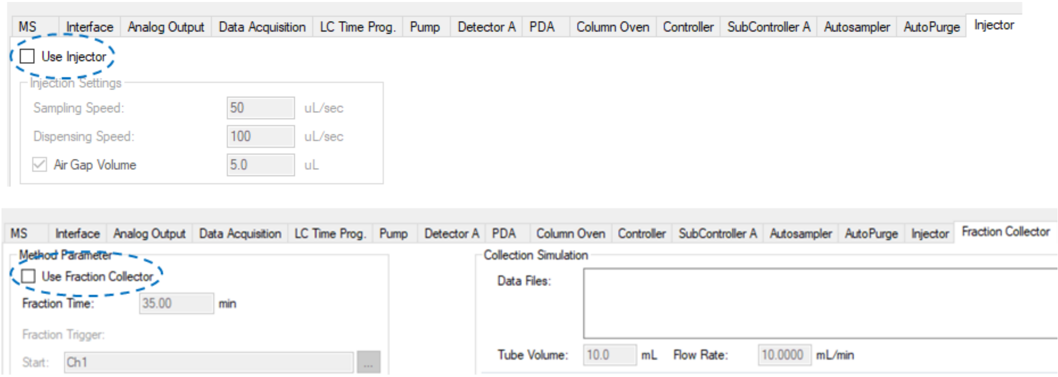
4.4.8 Autosampler tab

Ensure that the analytical autosampler is enabled (box checked). No other setting modifications are necessary.



4.4.9 Injector and Fraction Collector tabs

Ensure that the prep Injector and Fraction Collector are disabled (boxes unchecked) on their respective tabs.



4.4.10 Download and Save the method

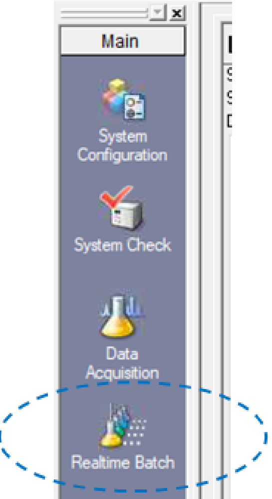
Once all the method tabs have been reviewed, click Download and Close to download the method to each module of the system and exit the Method Editor. Save the method to your data folder for future use by navigating to **File → Save Method File As...**

Create or locate your folder in the **C:\LabSolutions\Data** directory and save the method file using a naming convention that describes the method. For example, the filename "analytical_5-95ACN_30min_col2.lcm" has a good naming structure because it indicates an analytical method employing a 5–95% gradient of acetonitrile over 30 min using column 2.

4.5 Run an analytical sample

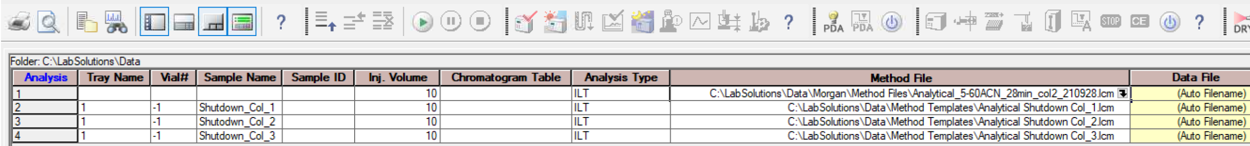
4.5.1 Start a new batch

To run a sample, select "Real-time Batch" located under the main menu.



Create a new batch file, by clicking **File** → **New Batch File**. Select **Analytical Batch Template**. The template has one line entry for a sample followed by three lines corresponding to shutdown methods for each column. These shutdown methods automate flushing each column with acetonitrile so that they are stored without TFA after use.

Note that position vial# -1 results in no injection. Delete shutdown method entries for columns that are not used.


Folder: C:\LabSolutions\Data

Analysis	Tray Name	Vial#	Sample Name	Sample ID	Inj. Volume	Chromatogram Table	Analysis Type	Method File	Data File
1					10		ILT	C:\LabSolutions\Data\Morgan\Method Files\Analytical_5-60ACN_28min_col2_210928.lcm	(Auto Filename)
2	1	-1	Shutdown_Col_1		10		ILT	C:\LabSolutions\Data\Method Templates\Analytical Shutdown Col_1.lcm	(Auto Filename)
3	1	-1	Shutodwn_Col_2		10		ILT	C:\LabSolutions\Data\Method Templates\Analytical Shutdown Col_2.lcm	(Auto Filename)
4	1	-1	Shutdown_Col_3		10		ILT	C:\LabSolutions\Data\Method Templates\Analytical Shutdown Col_3.lcm	(Auto Filename)

Line entries for additional sample runs can be added by inserting rows above the shutdown methods. To insert a row, highlight a line entry and right click. Select "Insert Row".

Input the tray and vial number location of the sample within the analytical autosampler. The sample name will become the filename for the data.

An injection volume of 10 μL is typical but can vary from 1–100 μL as needed. The directory of the method should match that of the method file created in the previous section (or some other previously created method that you want to use).

The example batch below shows how one can run two different samples (located in positions tray 1, vials 3 and 4). The first sample will be ran twice using two different methods - one specific for column 1 and one specific for column 2. The second sample is run also using the method developed for column 2. Because only columns 1 and 2 are used, the shutdown method entry for column 3 is deleted.

Folder: C:\LabSolutions\Data

Analysis	Tray Name	Vial#	Sample Name	Sample ID	Inj. Volume	Chromatogram Table	Analysis Type	Method File	Data File
1	1	3	MWB_1-11_col1		10		ILT	C:\LabSolutions\Data\Morgan\Method Files\Analytical_5-60ACN_28min_col1_210928.lcm	(Auto Filename)
2	1	3	MWB_1-11_col2		10		ILT	C:\LabSolutions\Data\Morgan\Method Files\Analytical_5-60ACN_28min_col2_210928.lcm	(Auto Filename)
3	1	4	MWB_1-11_col1		10		ILT	C:\LabSolutions\Data\Morgan\Method Files\Analytical_5-60ACN_28min_col2_210928.lcm	(Auto Filename)
4	1	-1	Shutdown_Col_1		10		ILT	C:\LabSolutions\Data\Method Templates\Analytical Shutdown Col_1.lcm	(Auto Filename)
5	1	-1	Shutodwn_Col_2		10		ILT	C:\LabSolutions\Data\Method Templates\Analytical Shutdown Col_2.lcm	(Auto Filename)

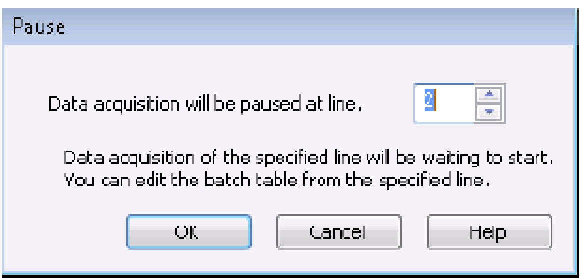
To start the batch, highlight all rows, and click the green play button. Save the batch file in your folder using an informative filename such as the date. The system will proceed to run all the samples, clean the columns, and automatically shutdown at the end of the batch.

Monitor the data in real-time using the PDA, MS, or All viewing windows. To switch the top display in the "All" viewing window from LC to PDA (if necessary), right click on the display and select "Display settings".

The LC tab shows changes in pressure of pump C (the analytical pump) and can be useful for assessing whether PDA signal changes are due to the presence of sample or some fluctuation in pressure.

4.5.2 Modifying a running batch

A running batch can be paused to edit the Batch Table. To pause a batch, click the (Edit table/Restart) icon. Set the row where the batch should pause and click OK.



Data acquisition will stop at the selected row. Add, insert, or delete entries in the batch table by right-clicking on the row and selecting the row action. **Do not delete entries that have already run.** Click the save button on the toolbar. Click the (Edit table/Restart) icon to resume the batch.

5 Preparative HPLC

5.1 General considerations in the use of prep HPLC

While analytical chromatography focuses on developing chromatography conditions that optimize resolution vs time, prep chromatography focuses on developing conditions that offer the most practical balance of yield, purity, and throughput (i.e., how much material can be purified in each injection cycle).

In analytical chromatography, small quantities of samples are loaded onto the column to ensure that the amount of sample loaded does not affect the resolution. The amount of sample that can be loaded without band broadening occurring depends on the size of the column. In contrast, in prep purification it is common practice to exceed the sample capacity and "overload" the column in order to increase yield and throughput. Prep chromatogram peaks are therefore typically broadened due to column overload, and the chromatogram is not as well resolved as analytical chromatograms.

In prep chromatography, several fractions collected across the peak of interest are analyzed for impurities. Based on the analytical results, fractions with little or no impurities are pooled together. Fractions near the beginning and end of a peak which may contain significant impurities are discarded. Choosing which fractions to pool and which to discard is a matter of balancing purity and yield.

Prep chromatography uses large volumes of solvent and requires significant time. Therefore a good workflow would use analytical chromatography to first establish elution conditions that give the best separation. Fractions collected across the peak during a prep run will be re-analyzed by the analytical method to determine maximum yield and purity for pooling. Fractions are usually dilute enough to re-analyze on the analytical side without further dilution being necessary.

5.2 Purge the prep pumps

Switch the B solvent line from the 4 L pure acetonitrile bottle to the 4 L bottle of acetonitrile with 0.1% TFA additive. Check that there is a sufficient amount of solvent in both the A and B bottles for your runs.

Purge the A and B prep pumps until any bubbles observed upon purging are removed. To purge the solvents, open the front door of the pump modules and turn the handles of the black valve counterclockwise to open the purge valves. Press the purge button.

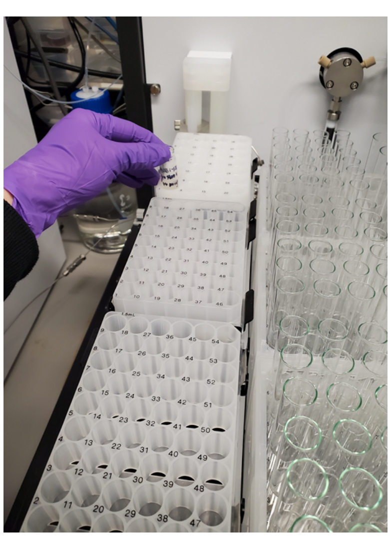
Watch for any bubbles in the lines and let these pass through the piston heads of the pumps for removal. Press the purge button again to stop purging. Remember to close the black valve finger-tight. *Do not close too tightly or you may damage the pump.*

5.3 Prep HPLC sample preparation

Always filter samples using the provided disposable 0.20 or 0.45 μm PVDF or PTFE syringe filters. Vials of the mobile phase solvents with TFA additive are provided near the instrument to prepare samples for injection. Samples should be dissolved in 100% aqueous solution. If the sample is insoluble in 100% water, add organic modifier (acetonitrile) but keep it to a minimum.

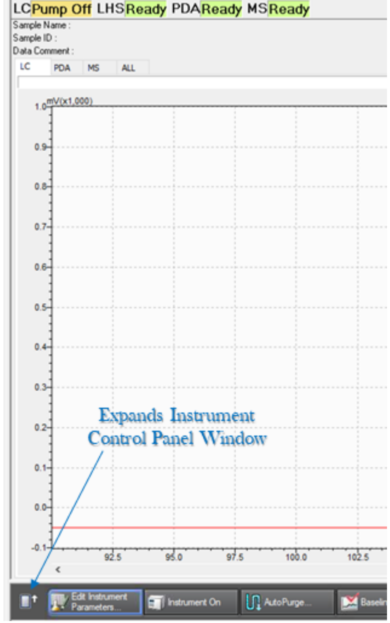
Prep injection volumes can be varied between 50–2000 μL. Sample concentrations and injection volumes should be tested to determine the max sample load where adequate fraction purity is still achieved. It is best practice to maximize sample concentration rather than opting to inject a larger volume of a more dilute sample. As one example to note, peptoids have been purified with a sample load of 60 mg (e.g., 1 mL injection of a 60 mg/mL solution) on a 19 mm ID column.

Place the filtered sample in an open vial onto the prep injector tray, noting the tray number and labelled position number of the vial.

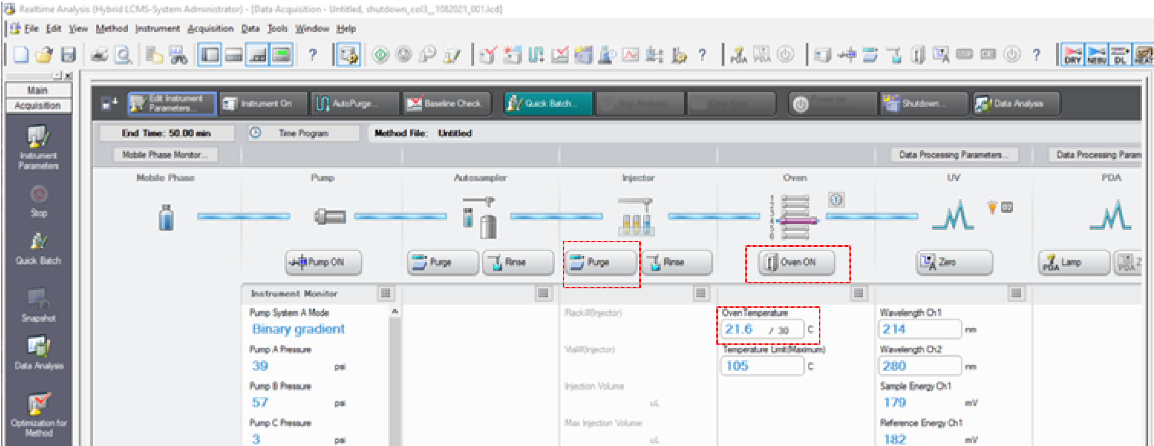


5.4 Turn on the oven and purge the prep injector

Return to the software and expand the control window of the system by clicking the icon with an up arrow as indicated in the figure below.



Turn on the oven, adjust the set-point of the oven (if required), and begin to purge the injector using the icons indicated below.



5.5 Create a prep method

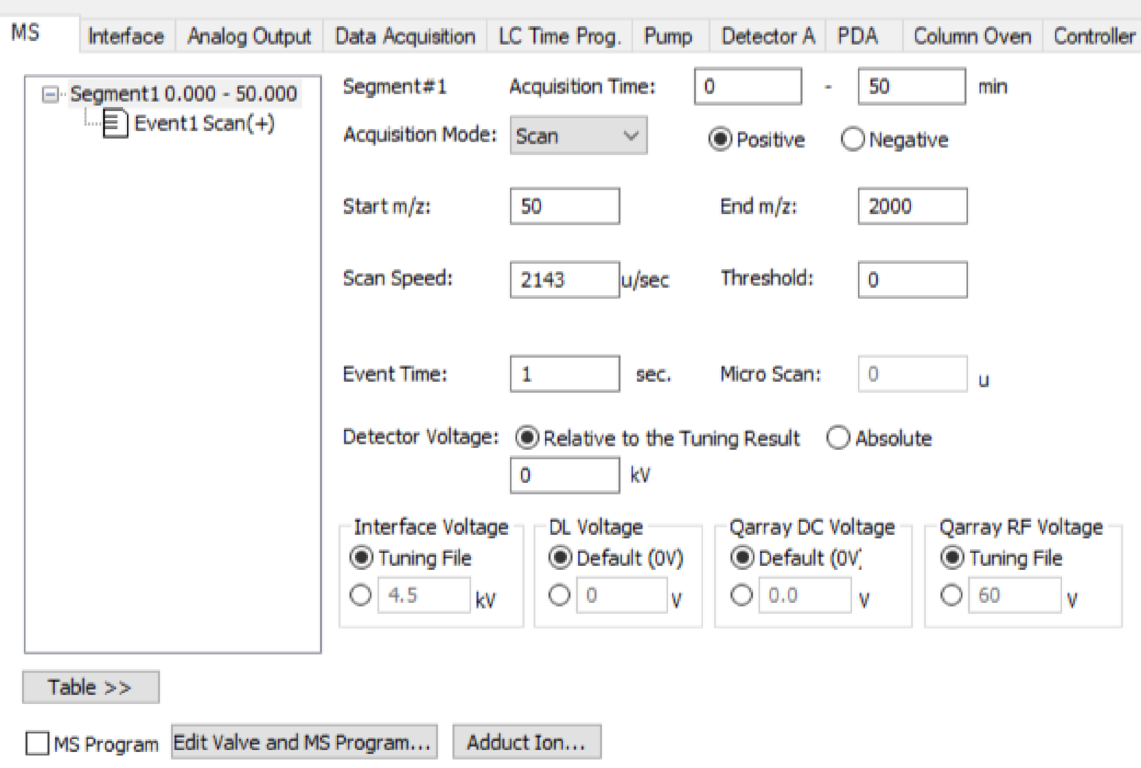
Navigate to **File** → **New Method File**. Select **PrepMethodTemplate**, and the Method Editor window will appear. If the template is employed to design a method, most of these tabs will not require modification, but all are documented in this manual for completeness.

This template method applies a 5-95% B (acetonitrile) gradient over 45 min with a 3 min hold to flush the column and 2 min hold to re-stabilize at the initial condition.

5.5.1 MS tab

On the MS tab, the acquisition time specifies over what time the mass spec will scan the m/z range and record data, generating a chromatogram of the total ion current (TIC). The acquisition time should equal the length of time required for the entire run, which includes time for the

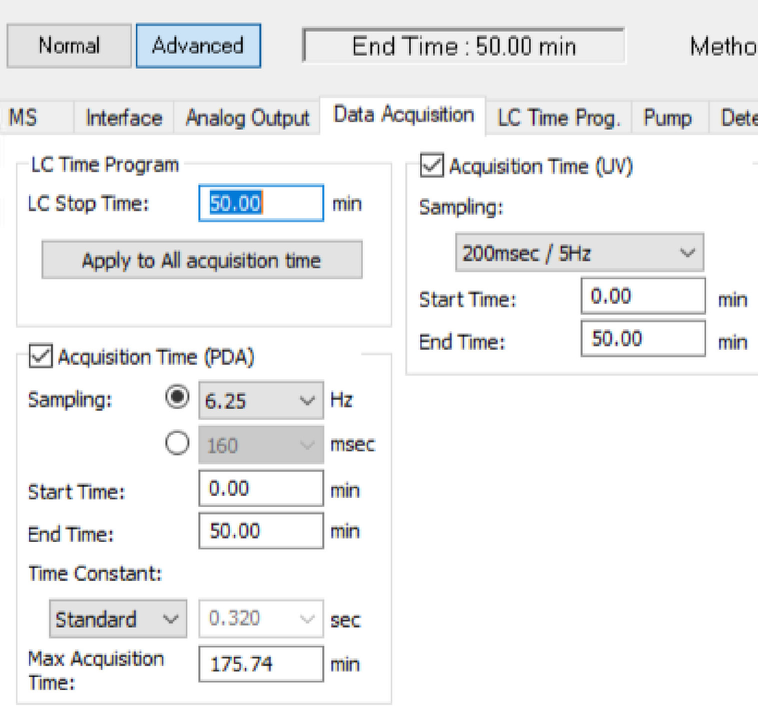
gradient plus 5 additional minutes to wash the column and return to the initial condition of 5 % B (Acetonitrile with 0.01% TFA).



In the above example, the mass spectrometer is set to scan over a pre-defined mass range of 10-2000 m/z and record full mass spectra. Check that the event time is set to 1 second.

5.5.2 Data Acquisition tab

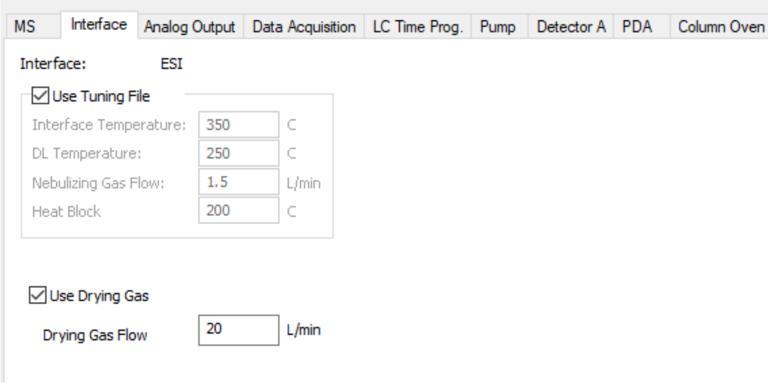
The data acquisition tab tells the detectors over what time to acquire data and sets the frequency of measurement for the UV (prep) and PDA (analytical) detectors. The LC Stop Time should equal the time it takes for the run. For a 5-95% gradient applied over 45 min, LC Stop time should equal 50 min, since it includes time for cleaning and stabilization of the column.



Click **Apply to All acquisition time** so this setting will be carried over and applied to all other tabs (including the MS tab described above). It is not recommended to adjust the sampling rates.

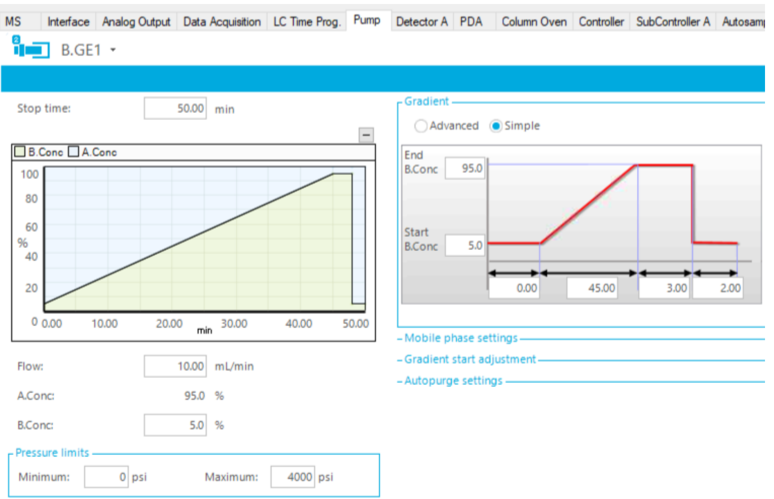
5.5.3 Interface, Analog Output, LC Time Prog., Controller and AutoPurge tabs

No changes should be necessary on the interface, analog output, LC Time Prog., Controller, or AutoPurge tabs. For the interface tab, confirm that the image below matches the current settings in the method.



5.5.4 Pump tab

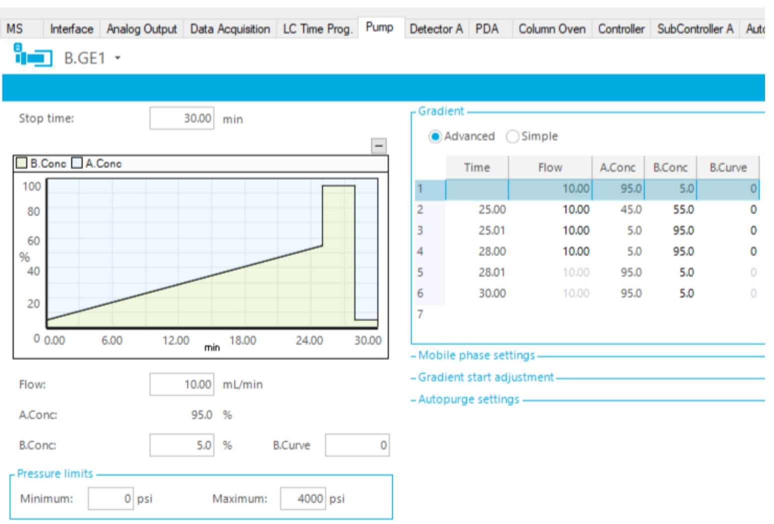
The pump tab defines the gradient that will be applied during the sample run. It will by default show settings for pump B.GE1, which refers to the two prep pumps.



Flow rates of 10-30 mL/min are appropriate for the semi-prep columns installed. To maintain the quality of separation in analytical chromotgraphy, the prep flow rate is increased to ∼17x the analytical flow rate, in accordance with the increase in column cross-sectional area. Commonly used rates include 0.6 mL/min for analytical and 10 mL/min for prep, or 0.8 mL/min for analytical and 15 mL/min for prep.

An initial gradient steepness of 2.0 % acetonitrile/min is recommended. Slower gradients can then be implemented if necessary to improve resolution/purity.

The gradient can be modified in Simple or Advanced gradient mode. Advanced gradient mode (see below) displays the programmed gradient in table form. The example below narrows the gradient to a smaller region (5–55% B) where the targeted compound elutes, thereby limiting solvent waste and reducing the run time. The required holds at 95% and 5 % B for 3 and 2 min, respectively, in order to ensure the column is clean and ready for the next injection.



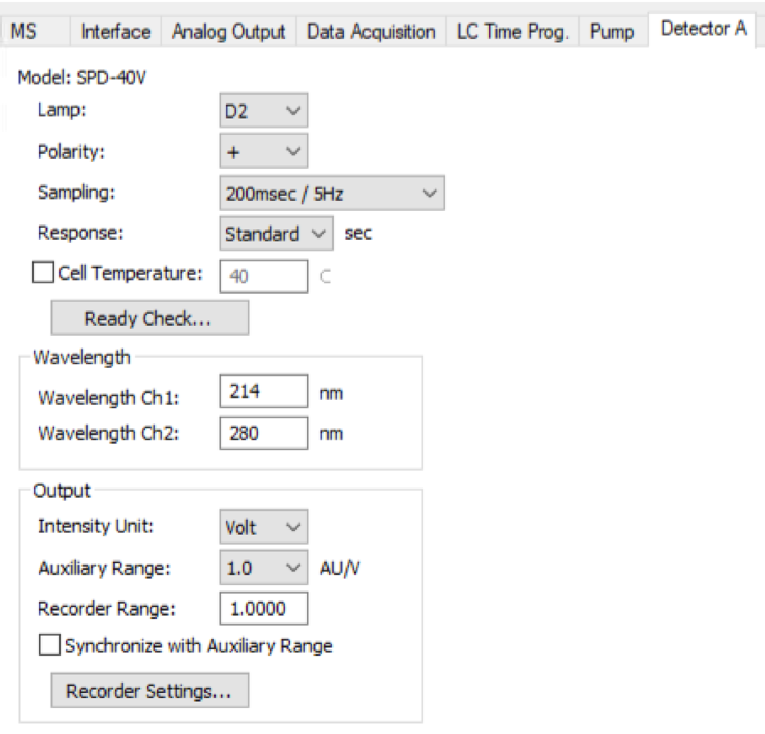
	Time	Flow	A.Conc	B.Conc	B.Curve
1		10.00	95.0	5.0	0
2	25.00	10.00	45.0	55.0	0
3	25.01	10.00	5.0	95.0	0
4	28.00	10.00	5.0	95.0	0
5	28.01	10.00	95.0	5.0	0
6	30.00	10.00	95.0	5.0	0
7					

To reduce the compositional range of the gradient, modify the entries in the table in advanced mode. The Stop time will be updated automatically. **Remember to then also change LC Stop Time and click "Apply to All acquisition time" in the Data Acqusition tab!**

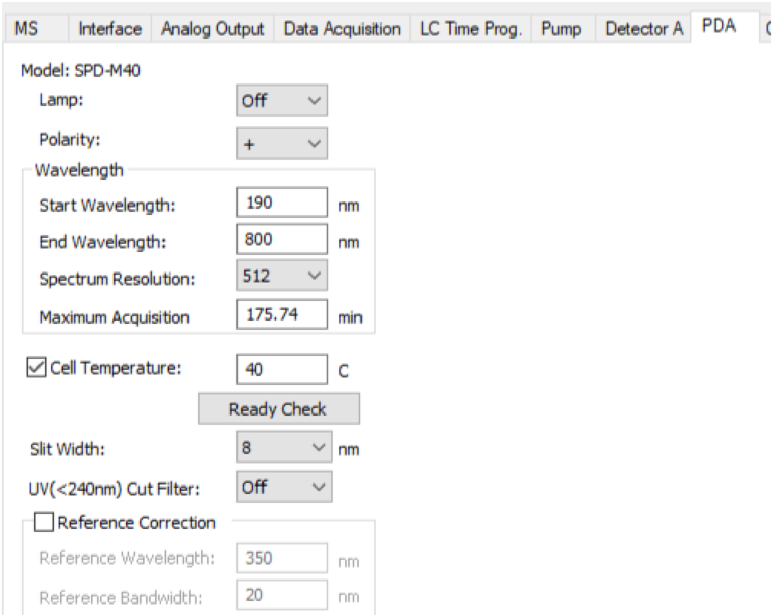
5.5.5 Detector A and PDA tabs

Detector A is the prep UV Detector. Choose the D2 lamp for monitoring wavelengths 190–370 nm or the W lamp for wavelengths 371–1000 nm. For monitoring peptoids or peptides, use a

Ch1 wavelength of 214 nm. Although two wavelengths (Ch1, Ch2) are monitored, the Ch1 signal is used for triggering fraction collection.



Check that the PDA lamp is turned off.

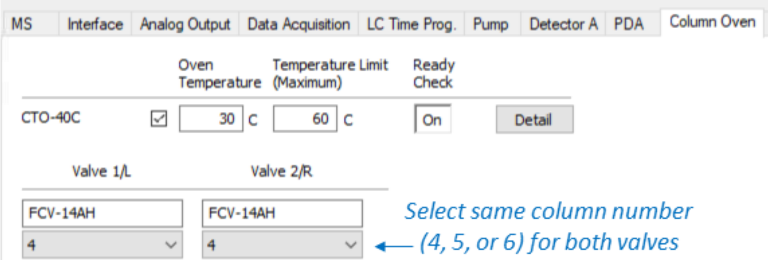


5.5.6 Column Oven tab

Set the column temperature. Note that the max column temperature is 60 °C. Select the same prep column (4, 5, or 6) for both valves, in accordance with these designations:

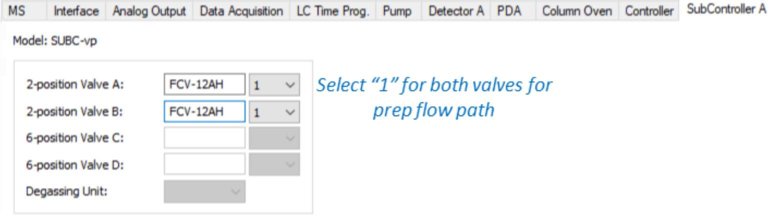
4. XBridge BEH C18 OBD Column, 130Å, 5 µm, (19 mm x 150 mm)

5. XBridge BEH C18 OBD Column, 300Å, 5 µm, (19 mm x 150 mm)
6. XBridge Protein BEH C4 OBD Column, 300Å, 5 µm, (19 mm x 150 mm)



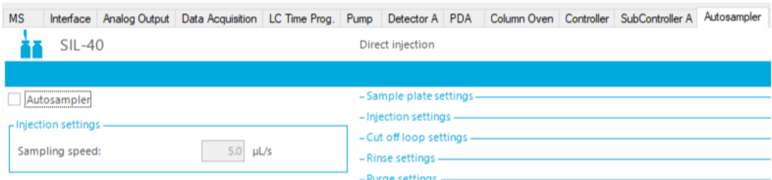
5.5.7 Subcontroller A tab

In the Subcontroller A tab, set the valves to the "1" position to select the prep flow path.



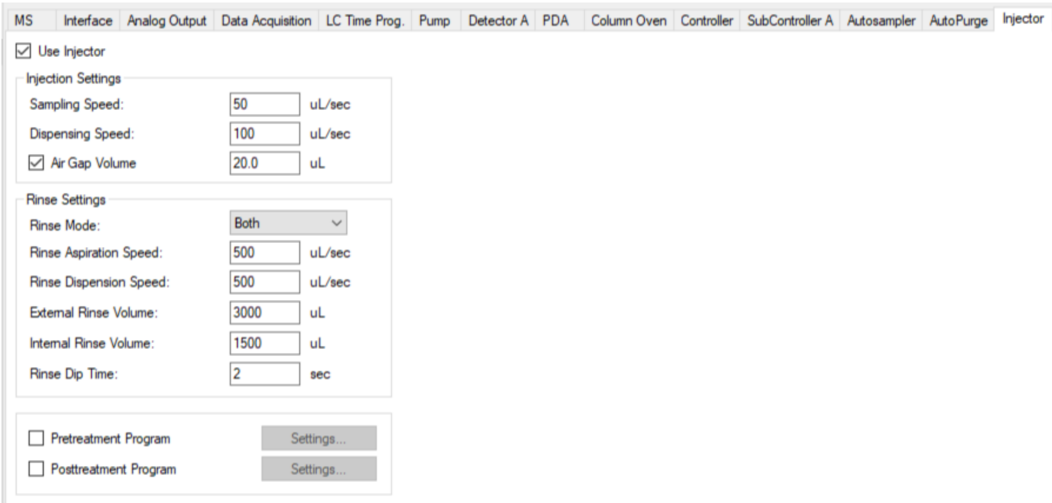
5.5.8 Autosampler tab

Ensure that the analytical autosampler is disabled (box unchecked).



5.5.9 Injector tab

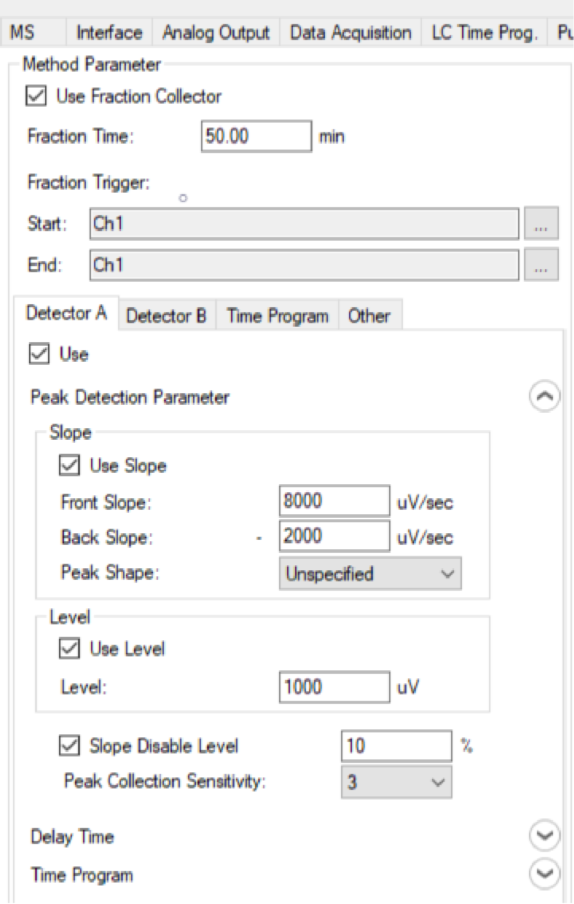
Ensure that the prep injector is enabled (box checked).



5.5.10 Fraction Collector tab

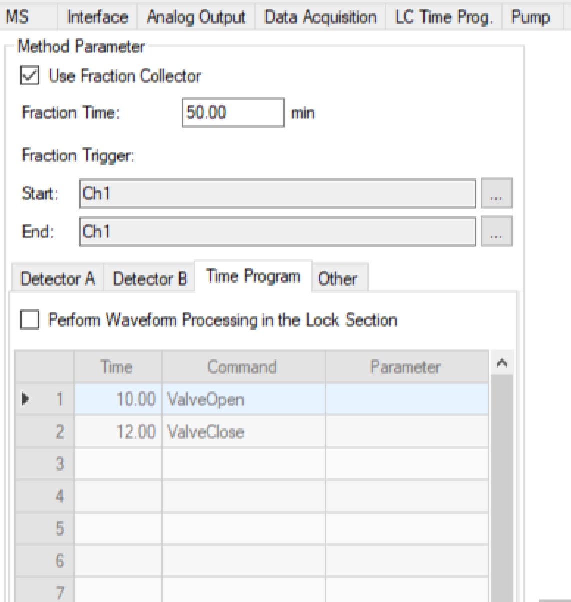
The fraction collector tab specifies the parameter settings for fraction collection, which can be based on three different triggers:

1. **Peak-based fractionation:** the fraction collector valve automatically opens/closes based the observation of a peak in the UV signal (Ch1), the MS signal of specified m/z (Ch2), or the UV + MS signal (Ch1 and Ch2).



The example above shows default values for fraction collection based on Ch1, the UV signal, with the "use" box enabled (checked) for Detector A. For programming fraction collection based on the MS signal, ask for staff assistance.

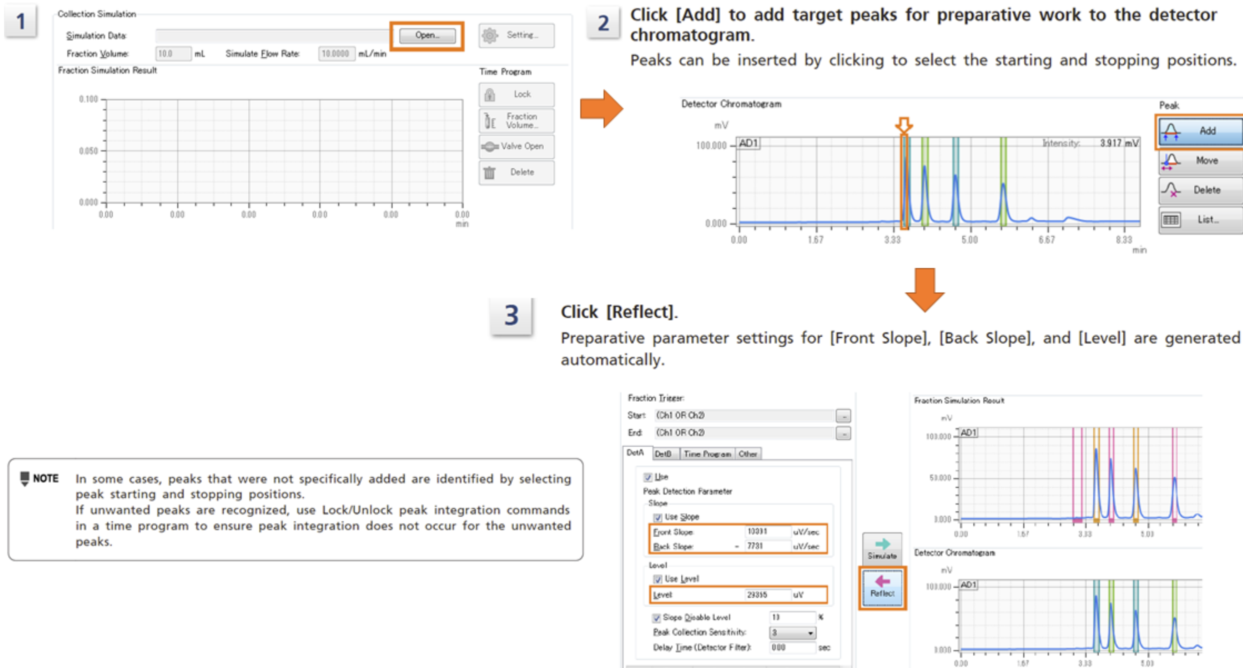
2. **Time-based fractionation**: the fraction collector valve is programmed to open and close at a specific retention time.



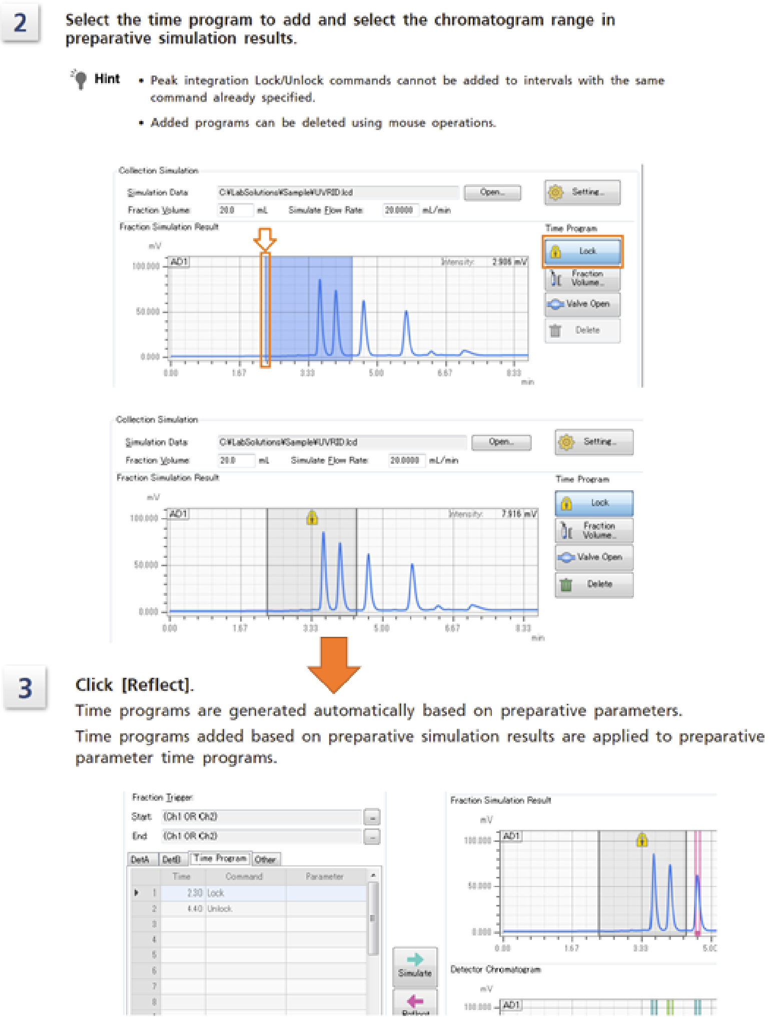
3. **Manual fractionation:** the user manually opens and closes the fraction collector valve based on the visual observation of a peak in the LC data output window, using these equivalent sets of buttons located at the top of software menu or within the control panel, respectively:

(Note there will be a time delay of ∼11 seconds between the click and opening of the valve due to the position of the fraction collector relative to the UV detector.)

The collection simulation window allows the user to confirm the appropriateness of the collection starting and stopping criteria. This is best used by opening a data file generated during a prep "test run" that involved only a small injection volume of 50–100 μL. Collection simulation can be used in conjunction with the signal and time-based triggers described above. To use collection simulation to tune the parameter settings for peak-based fractionation:



To use collection simulation to tune the parameter settings for time-based fractionation:



5.5.11 Download and Save the method

Once all the method tabs have been reviewed, click Download and Close to download the method to the system and exit the Method Editor. Save the method to your data folder by navigating to **File → Save Method File As...**

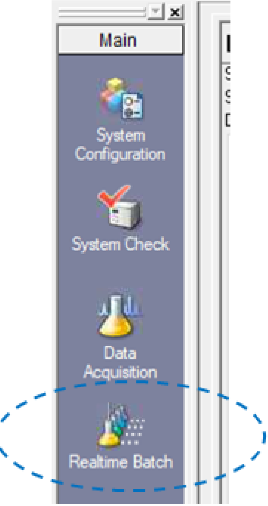
Create or locate your folder in the C:\LabSolutions\Data directory and name the method file such that the method is well-described. The filename "prep_5-95ACN_45min_col4.lcm" has a good naming structure because it indicates a prep method employing a 5–95% gradient of acetonitrile over 45 min using column 4.

5.6 Run a prep sample

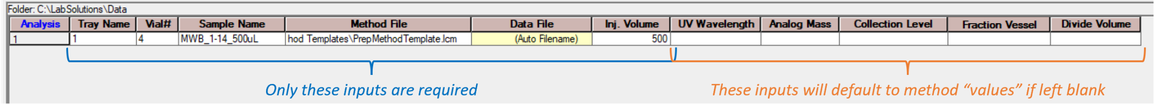
Ensure that all collection test tubes are clean, empty, and in place on the fraction collector.

5.6.1 Set up a batch

To run a sample, select "Real-time Batch" located under the main menu.

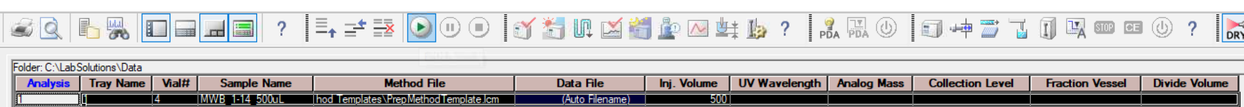


Create a new batch file, by clicking **File** → **New Batch File**. Select **Prep Batch Template**. Fill out the table with line entries for each injection/sample. To insert (or add) a row, highlight a line entry and right click. Select "Insert Row" (or "Add Row"). Input the following information for each sample/injection as defined below.



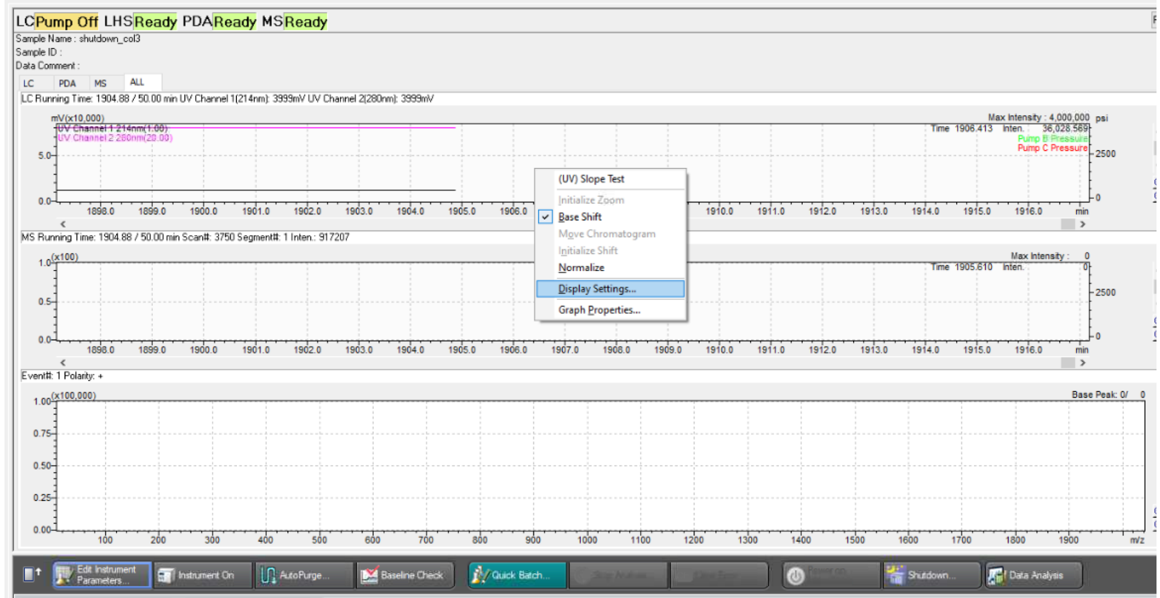
- **Tray Name and Vial#** = position of the sample vial in prep injector tray.
- **Sample Name** = name of generated data file. Sample ID (if shown) is not required.
- **Method File** = check that this path matches the name of the method created (or other method file you would like to use).
- **UV Wavelength** = the wavelength of absorbance used to trigger collection based on peak detection in signal (otherwise uses Ch1 specified in Detector A tab of method file).
- **Analog mass** = Only applicable for MS-triggered fraction collection. Specifies the m/z value for triggering collection based on MS peak detection.
- **Level** = specifies the level in used μV for peak detection in UV detector output.
- **Fraction Vessel** = sets the starting (or ending) rack or tube to be used in fraction collection.
- **Divide Volume** = volume used for each fraction collected. Setting range is 0-25 mL.

To start the batch, highlight all rows to be run, and click the green play button . Confirm the batch execution range and click Start.



Save the batch file in your folder using an informative filename such as the date.

During a run, the data can be monitored in the LC, MS, or All viewing windows/tabs. To change the top display in the All viewing window from PDA to LC (if necessary), right click on the display and select "Display settings".

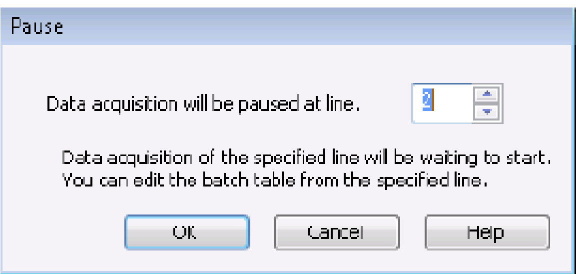


Remember to stop the pumps at the end of a batch by clicking Pump ON should they fail to turn off automatically!

The prep batch template is preconfigured to automatically shutdown the pump at the end of a run in order to save solvent. If necessary, click Power on Instruments to re-activate the pump.

5.6.2 Modifying a running batch

A running batch can be paused to edit the Batch Table. To pause a batch, click the (Edit table/Restart) icon. Set the row where the batch should pause and click OK.

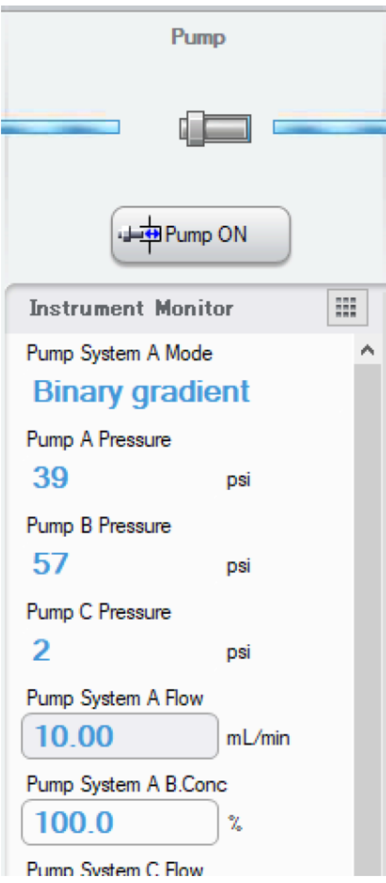


Data acquisition will stop at the selected row. Add, insert, or delete entries in the batch table by right-clicking on the row and selecting the row action. **Do not delete entries that have already been ran.** Click the save button on the toolbar. Click the (Edit table/Restart) icon to resume the batch.

5.6.3 Early termination of a run

Data acquisition can be stopped by at any time by selecting either stop icon Stop or Stop. Check both boxes and click OK to end the run immediately.

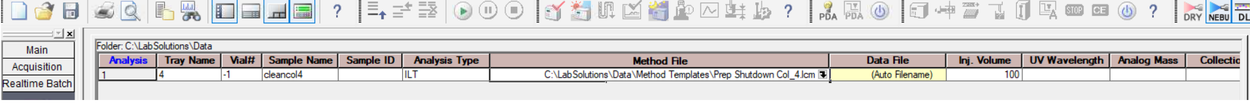
Remember to flush the column with 95–100% B to remove any residual material remaining on the column before continuing with subsequent injections. This can be done in the control panel area shown below:



5.7 Clean columns and shut down the instrument

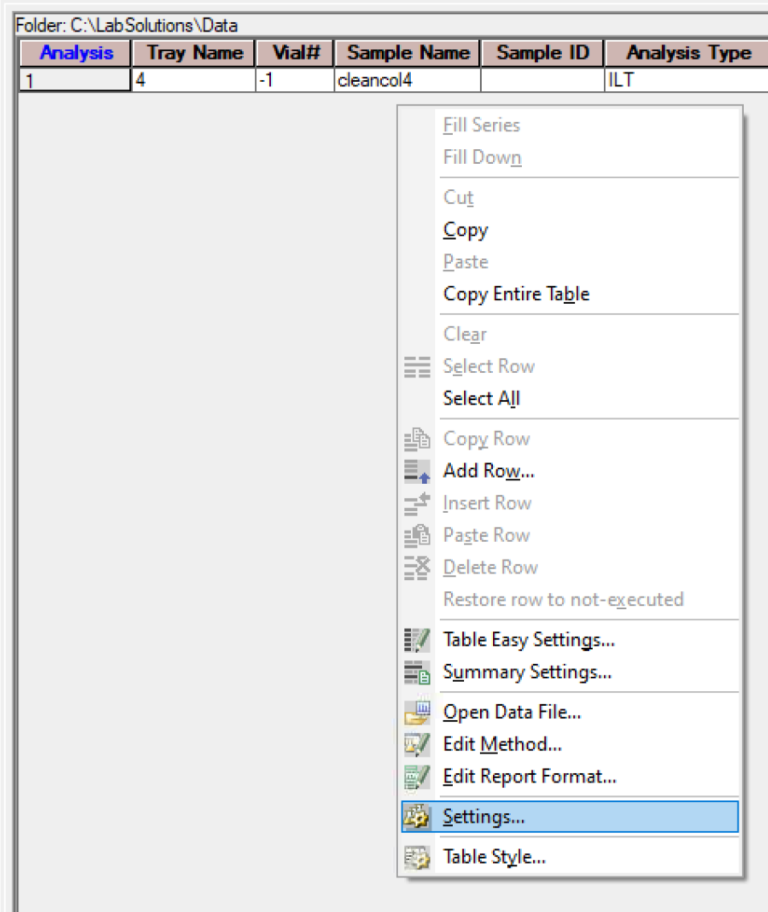
Each prep column used should be flushed with 10 column volumes of pure acetonitrile and stored without TFA. To automate the cleaning procedure, so-called shutdown methods have been created to facilitate cleaning of each column and shutdown of the instrument.

To start the shutdown procedure, switch the solvent line of prep pump B back to the bottle of pure acetonitrile. Ensure there is sufficient solvent for the cleaning procedure (~400 mL per column). Then create a new batch file using the prep batch template. Add one entry line for each column that needs to be cleaned. The example below shows an entry for cleaning prep column 4:

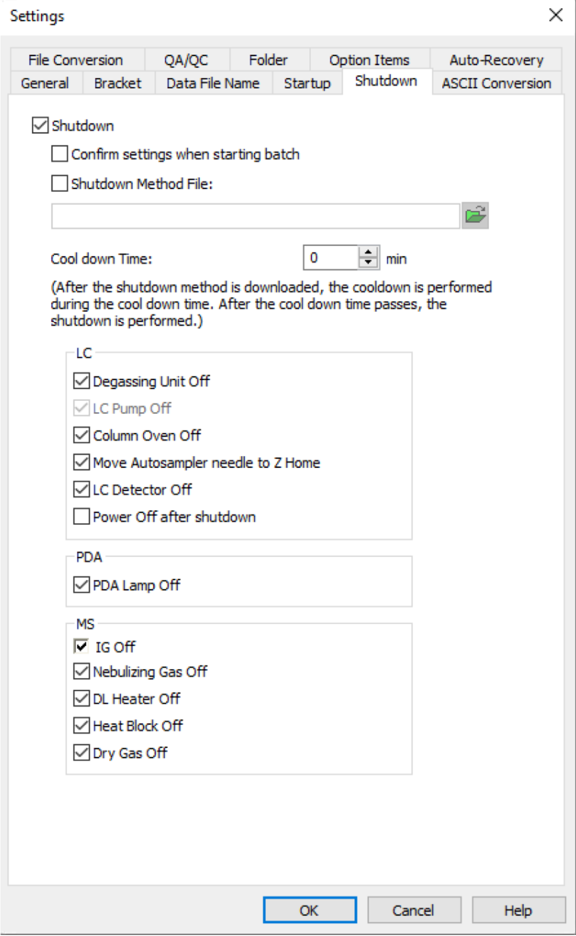


The tray name does not matter. The vial# must be -1 so that no injection occurs. The method file selected corresponds to the column that will be cleaned. Shutdown method files are located in the method templates folder.

Next, right-click within the batch window and navigate to settings.



Within the shutdown tab, fill-out the boxes as shown below and click OK.



Save and start the batch. The system will now proceed to clean the column ($\sim$ 20 min) and then shutdown automatically.

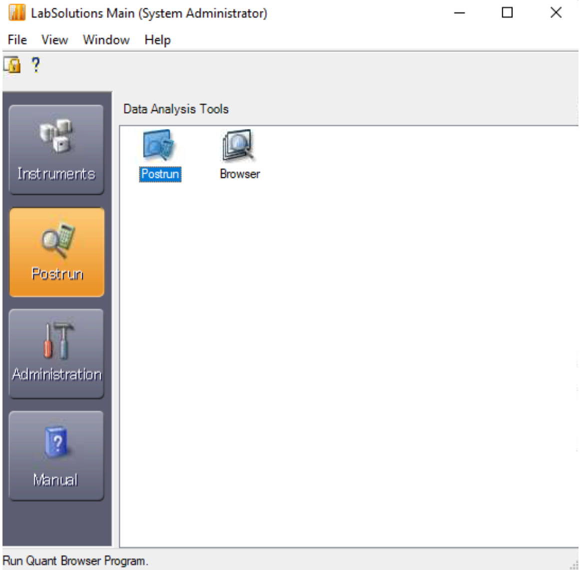
6 Analyzing the data

Quantitation uses chromatographic data to determine the amount of a given component in a mixture. There are several types of quantitation methods commonly used. This section covers the very basics of data analysis, utilizing peak area percent obtained from an integrated UV or PDA chromatogram as a rough estimate of the relative amounts of analytes present. This method assumes that the wavelength absorbance properties of all compounds observed are equivalent, which is likely not a valid assumption. The MS data is used only for mass confirmation of the observed analyte.

Other more in depth methods of quantitation require the use of internal and external standards and are not commonly employed in the routine analysis of newly synthesized materials. For more information on these types of analysis, refer to the manufacture manual entitled "Operators Guide (LCMS Edition)".

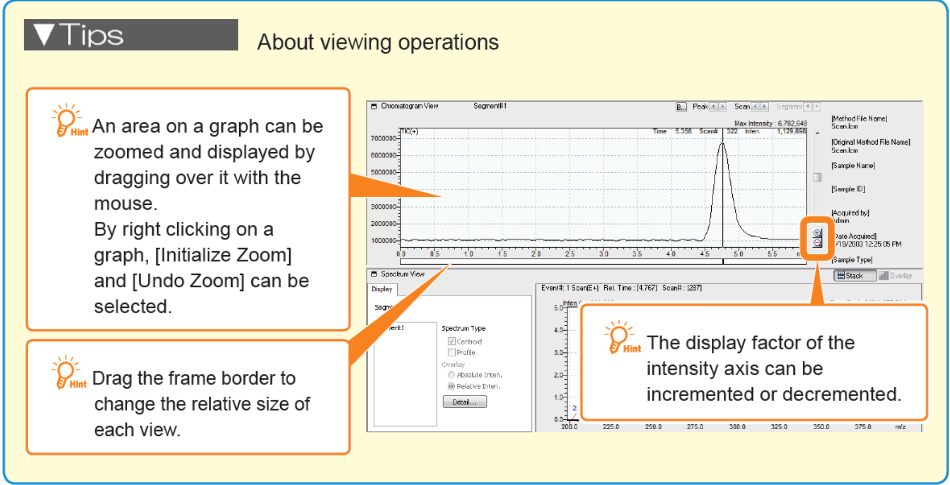
6.1 Postrun program for analysis

Select “Postrun” from the LabSolutions Main window.



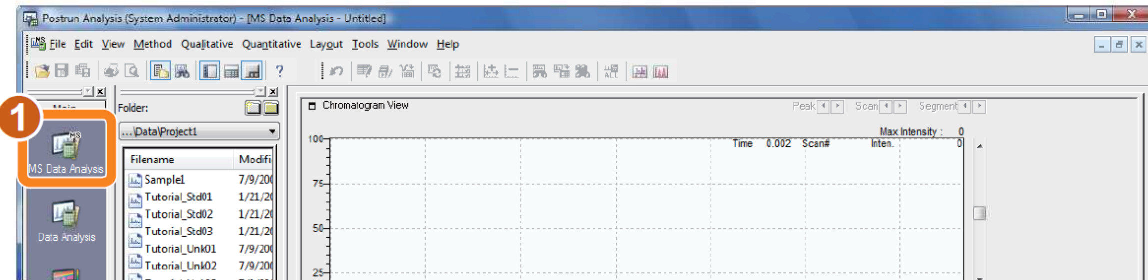
Drag-and-drop a data file onto the Data analysis window from the Data Explorer sub-window.

6.1.1 Viewing operation tips

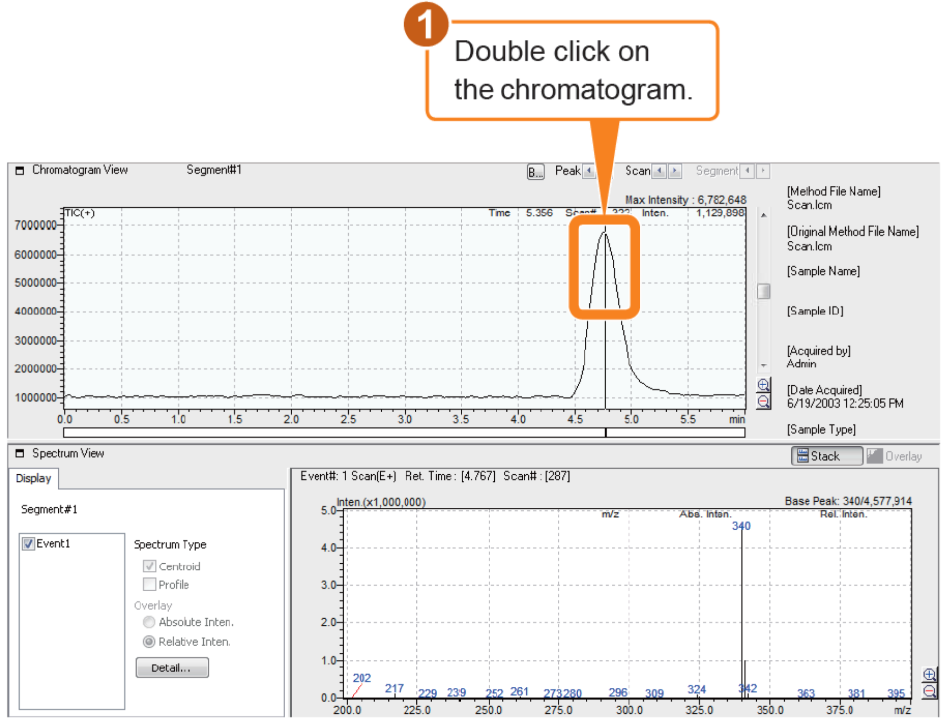


6.1.2 View MS chromatogram and spectra

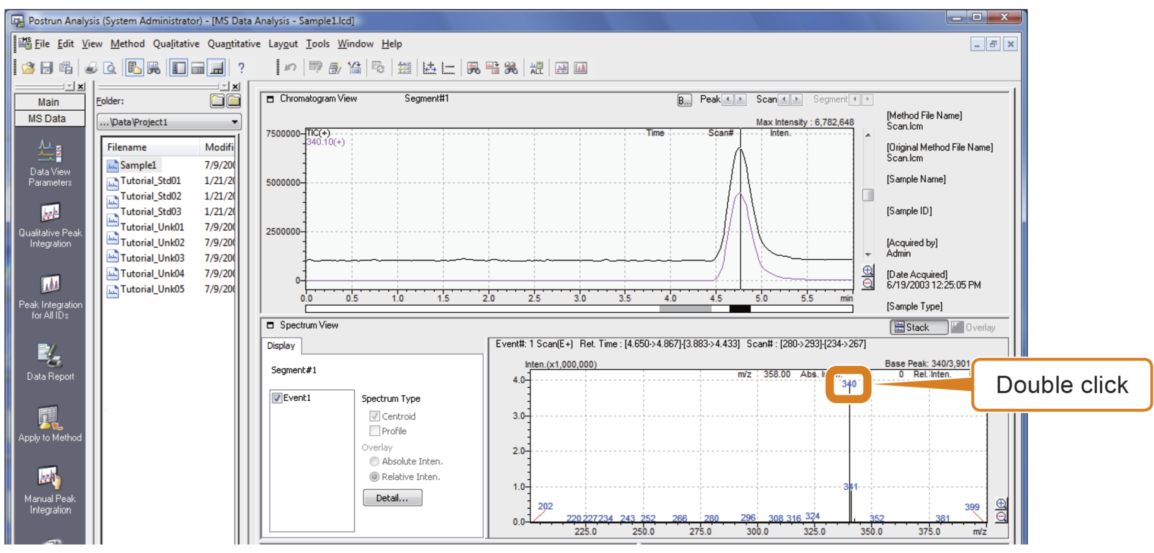
For analyzing MS data, click [MS Data Analysis] under the [Main] window.



To generate a spectrum, double-click the time point in the chromatogram. Click the arrows for "peak" or "scan" to move the spectrum extraction line to the next peak time or by the next preset scan unit.

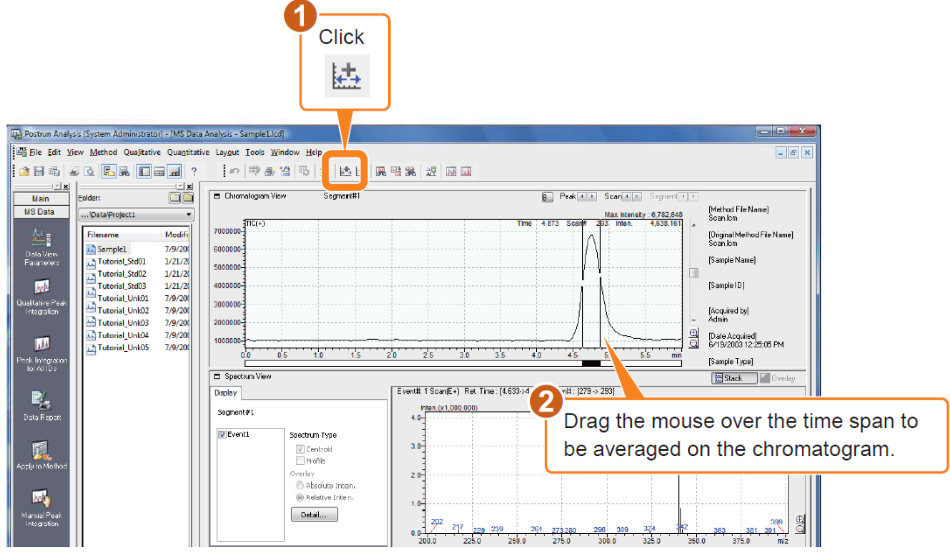


Double-click the m/z in [spectrum view] to extract as a chromatogram. The chromatogram of that m/z is added to the [Chromatogram View].

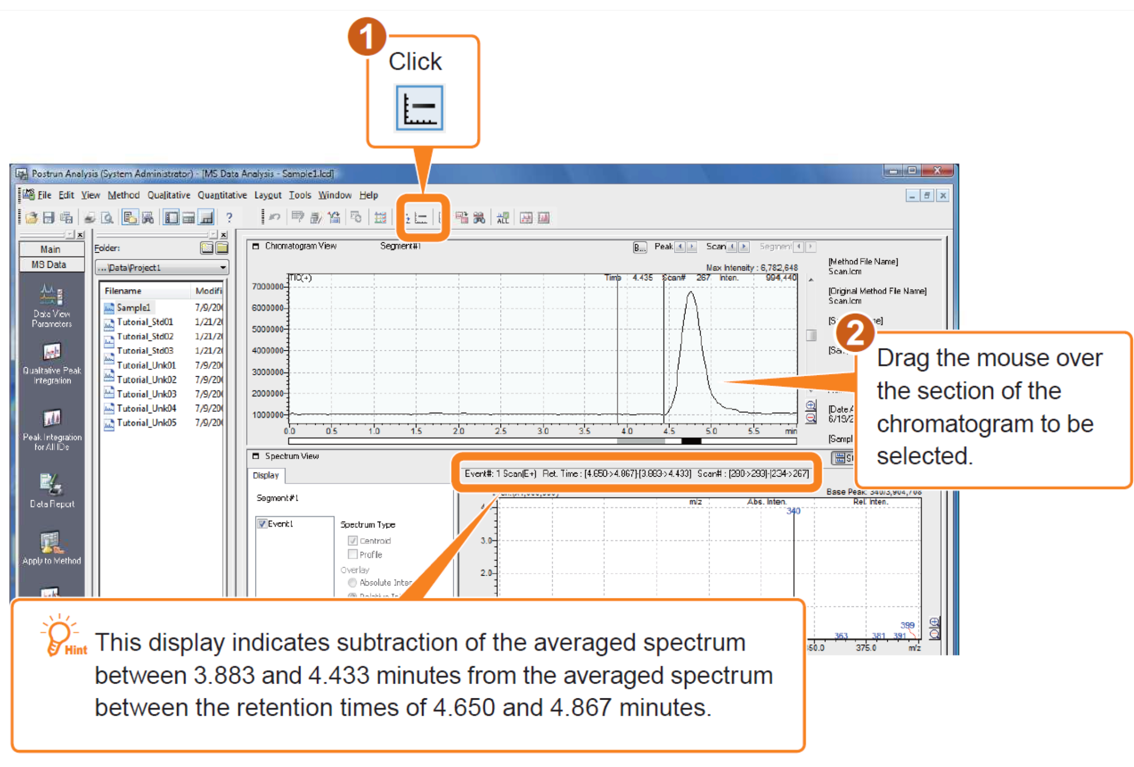


Alternatively, to add extracted chromatograms of other m/z values to the chromatogram display, edit the MS Data view parameters sub-window under the MS Data tab as shown.

To average MS spectra over a specified time span:

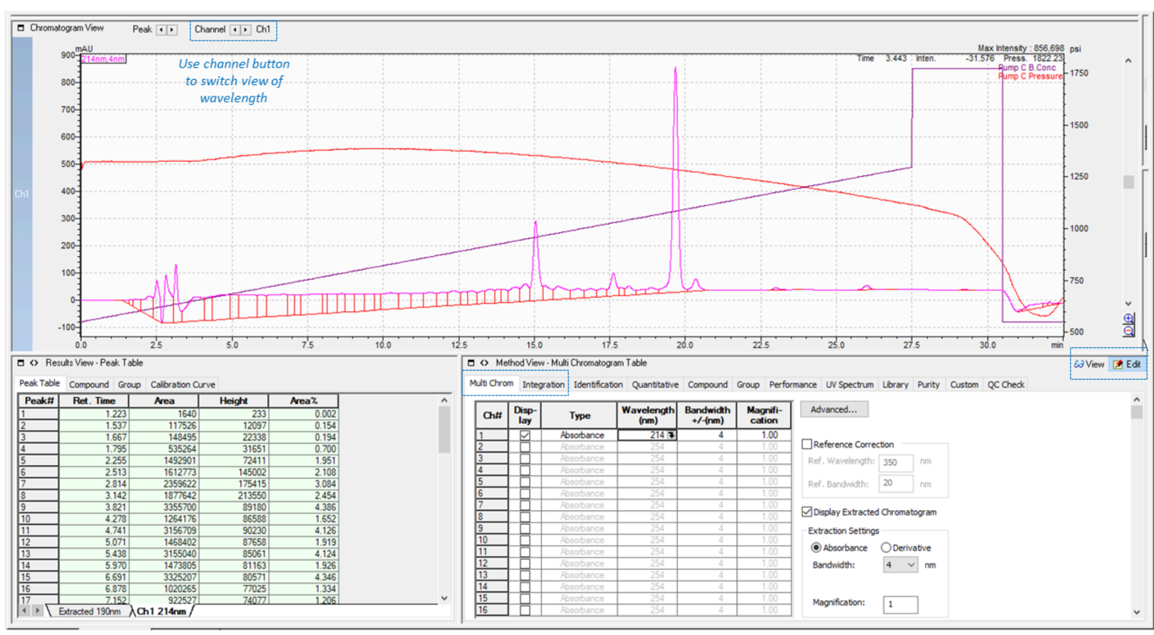


The spectrum display can be improved by subtracting the background MS spectrum from the averaged spectrum:



6.1.3 View the PDA data (Analytical)

To view the analytical PDA in post-run, click "PDA data analysis" icon located under the main sub-menu on the left. The data should appear in a simplified form showing only the chromatogram view, peak table, and method view. If there are more windows shown, under the Layout menu, select the PDA layout, or drag the frame borders so that only these windows are shown for simplicity.



Select the Edit button to modify the Chromatogram, and then return to View mode to reflect any changes.

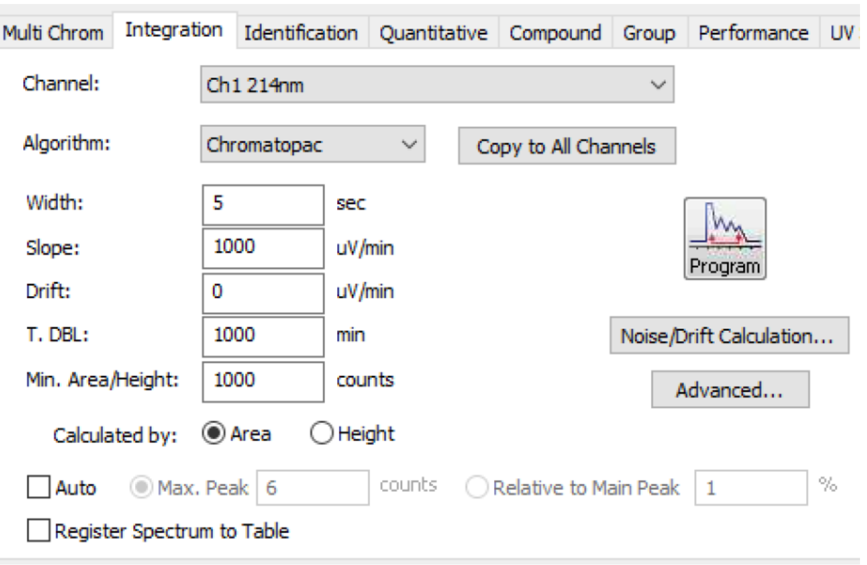
In edit mode, under the "Multi Chrom" tab, change the Ch1 wavelength of absorbance to the wavelength of interest (e.g., 214 nm for peptoids and peptides).

6.1.4 View the UV data (Prep)

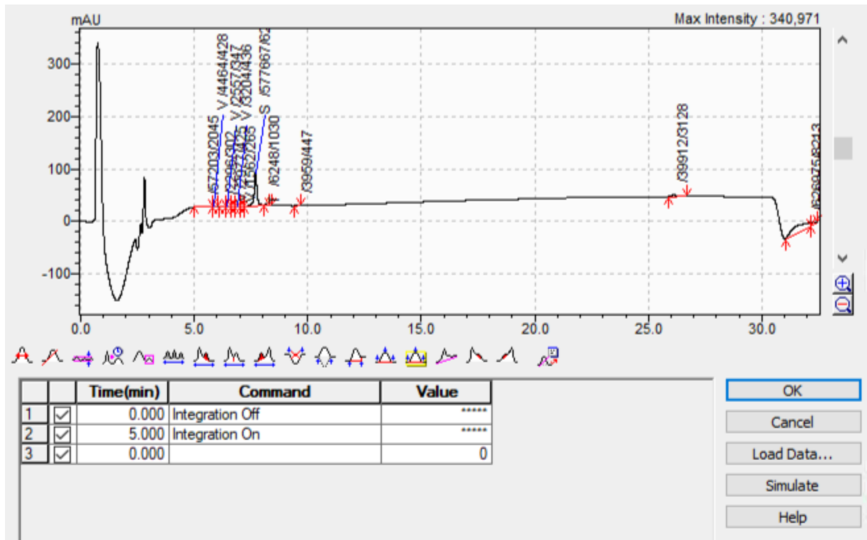
To view the prep UV chromatogram in post-run, click "Data Analysis" icon Data Analysis located in the main sub-menu on the left. Select the Edit button to modify the Chromatogram, and then return to View mode to reflect any changes.

6.1.5 Modify the integration parameters

In edit mode, under the integration tab, select the channel of interest. If necessary, reduce the number of peaks included due to noise in the data by increasing the [Slope] or [Min. Area/Height] by a factor of 10.



To prevent integration of extraneous peaks at early or late times in the run, click , and fill-out the table to turn off integration for specific regions:



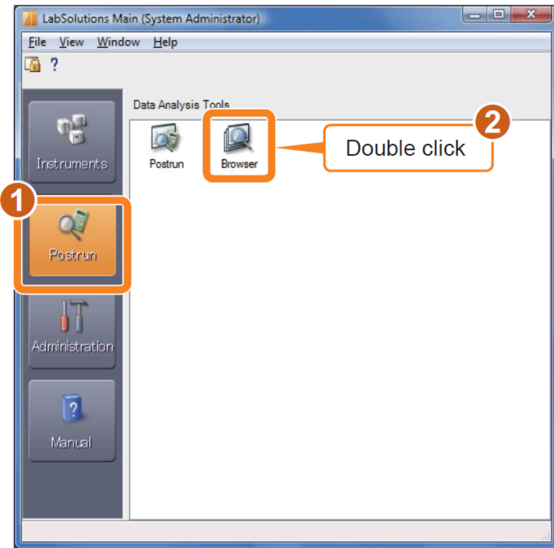
These steps are generally sufficient for processing. Press the help button to learn more about peak integration commands if desired.

6.1.6 Export the data

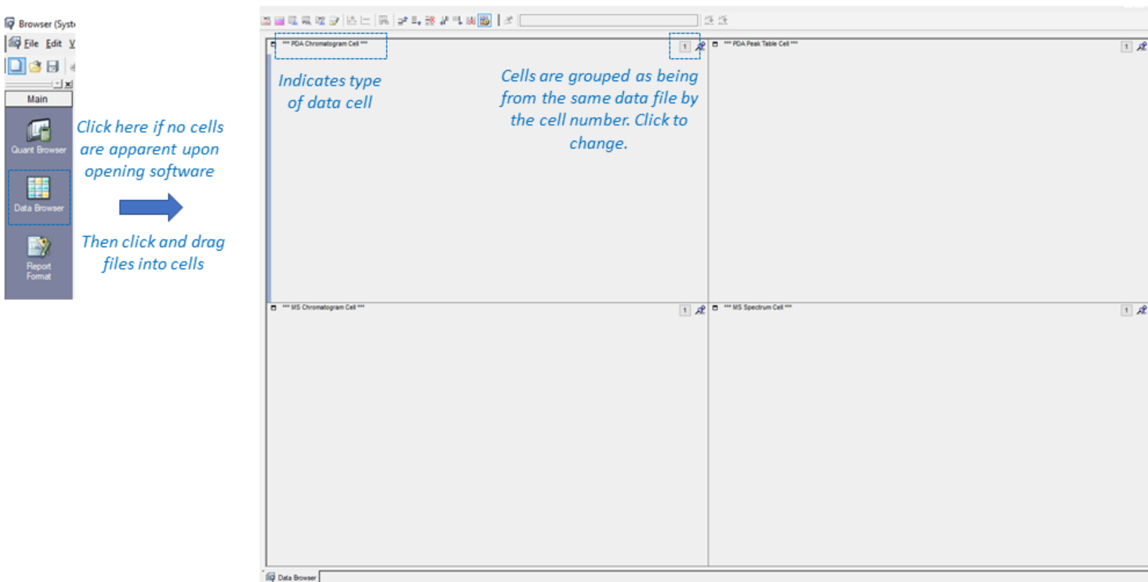
To export the peak tables, chromatograms (PDA and UV), MS Chromatogram TIC, and MS Spectrum, go to **File** → **Export Data** → **Export Data (ASCII Conversion)**. Select the Output file option and check the boxes for the desired output options (peak table, chromatogram, etc.) to save the data as a tab-delimited .txt.

6.2 Data Browser

Open Data Browser to display chromatograms and spectra from multiple data files for comparison or to overlay data from different detectors.



6.2.1 Comparing multiple files



Within Data Browser, data files are opened by drag-and-drop from the data explorer window to the display cells on the right. To change the type of data displayed in each cell, right-click the cell and select "Change data type".

Use the layout property icon to change the row x column arrangement of cells. To add a row or column to an existing layout, use the icons on the top panel.

Cells are linked by their cell number shown in the upper right-hand-corner of each cell. For example, cells labeled with "1" will correspond to the same opened file, and cells labeled "2" will correspond to a second open file. Click on the cell number to change it. Drag-and-drop files to the different sets of cell numbers to view multiple files simultaneously.

6.2.2 Overlay PDA and MS data (analytical)

This section describes how to use a layout template to overlay PDA and MS data for a single analytical data file:

1. Go to the menu **Layout** → **Open Layout File**.

2. Select the layout **Analytical.lyt** from the Browser Layout Templates folder located on the Desktop. Drag and drop a file from the data explorer window to the display cells on the right.

3. Click the Data Processing Parameters icon Data Processing Parameters in the data browser menu.

4. On the "Multi Chrom" tab, enter the absorbance wavelength of interest and uncheck "Display Extracted Chromatogram". Click OK.

5. Double-clicking within the PDA chromatogram will generate the mass spectrum for that time point. MS spectra can also be averaged or background subtracted using the same icons described in section 6.1.1.

6. The auto-integration parameters can be adjusted (if not previously performed in post-run analysis), in the Integration tab of the Data Processing parameters window.

7. To overlay the PDA and MS data within the same window. Right-click on the MS chromatogram and open "Display settings". Navigate to the "LC Settings" tab and check the Disp. box. Click OK. The MS and PDA chromatograms are overlaid.

6.2.3 Layout Templates for comparing multiple files

Three additional layout templates have been pre-configured to assist in comparing of multiple data files:

1. **Analytical_Comparison.lyt** compares the chromatograms and spectra of two analytical data files.

2. **Prep_Comparison.lyt** compares the chromatograms and spectra two prep data files.

3. **Analytical_Prep.lyt** compares the chromatograms and spectra of one analytical and one prep data file.

Click on the menu **Layout** → **Open Layout File**. Select the layout from the Browser Layout Templates folder located on the Desktop. Drag and drop files from the data explorer window to separate cell windows labeled "1" and "2".