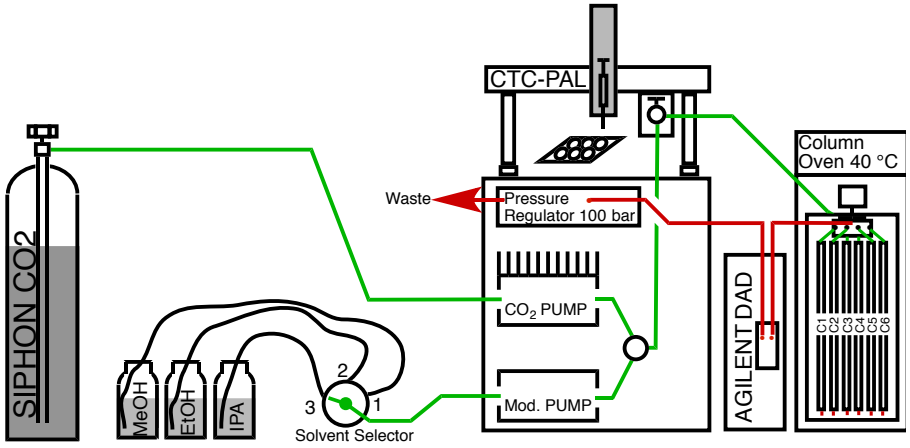
# SuperCritical Fluid Chromatography for Chiral Separation

-Scott Virgil, California Institute of Technology May, 2021

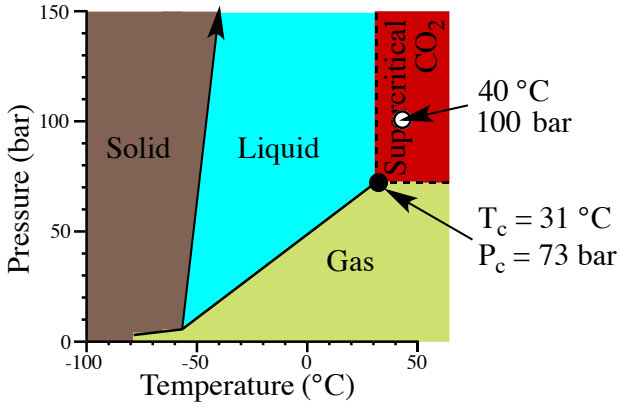
The use of supercritical $CO_2$ as a chromatography phase has greatly improved the speed and efficiency of many chromatographic analytical and preparative processes:

- Supercritical $CO_2$ has very low viscosity. So higher flow rates can be used while staying below 100 bar pressure across the column.
- Decrease in solvent costs and waste disposal costs.
- Enhanced peak shape and separation resulting primarily from the faster flow rates.




In the above diagram, the key features of the SFC system are shown:

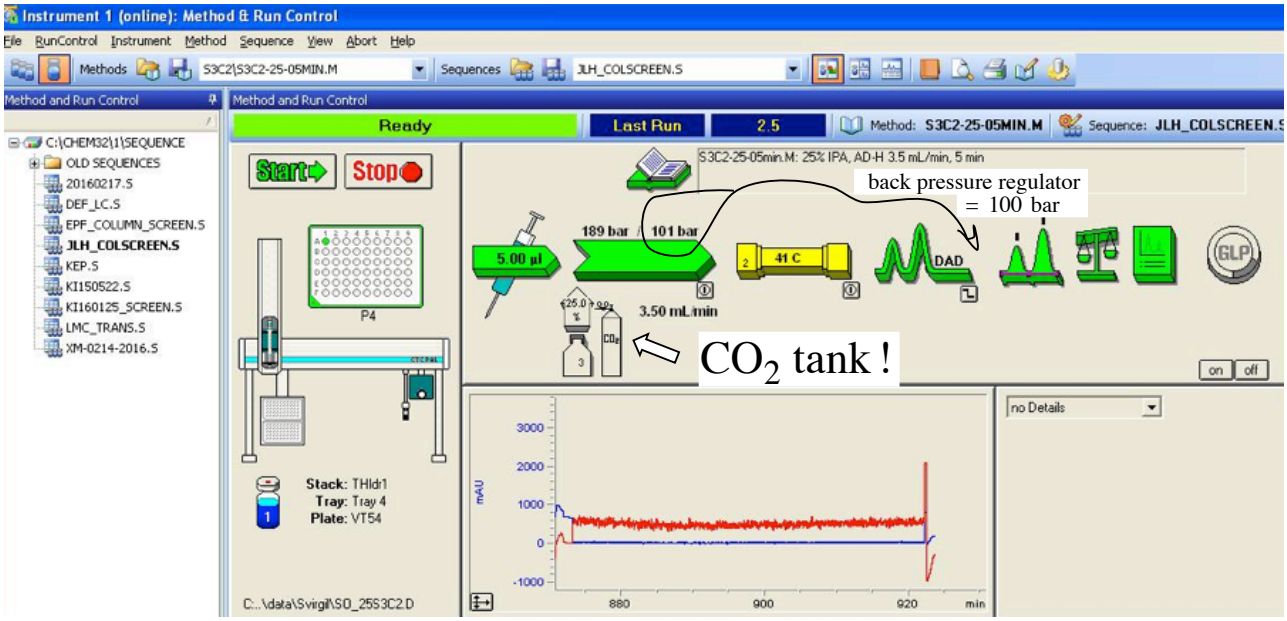
1. Liquid $CO_2$ at room temperature is drawn from the bottom of a **Siphon Cylinder** that has an internal **dip tube**. If you reorder $CO_2$, you must request a **Siphon Tank**. (The newest Agilent Aurora SFC system is able to use non-siphon tanks).
2. The $CO_2$ pump is cooled to 5 °C with a "Peltier" electronic cooler so that the liquid can be drawn without vaporizing (the heat exchanger for the Peltier can be seen in the photo).
3. The modifier pump delivers the cosolvent choice.
4. A CTC-PAL autosampler injects the sample.
5. The analyte components are separated on the choice of six columns in an oven at 40 °C.
6. After exiting the column the Diode-Array Detector returns the UV data to the computer.
7. The "**Back Pressure Regulator**" maintains the pressure at 100 bar before the fluid exits the system to waste.



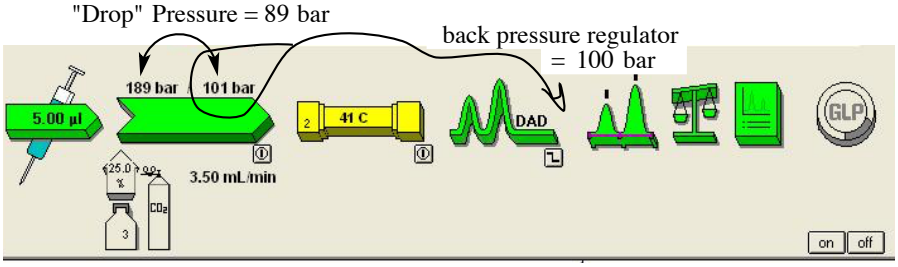

In the supercritical region, the viscosity of $CO_2$ is ~ ¼ the viscosity of hexane and allows flow rates of 3-5 mL/min through standard 4.6 x 250 mm analytical chiral columns even when mixed with co-solvents such as isopropanol.

# SFC System Operation with Agilent Chemstation

The SFC system used at Caltech was purchased from Thar SFC that is set up to run on Agilent Chemstation with add-on software to run the CTC autosampler and the Thar SFC components. As far as maintenance and operation are concerned, we can treat the system as if it was an Agilent HPLC with a $CO_2$ tank that can be seen in the online Chemstation screenshot.




In the online Chemstation window, it can be seen that two pressures appear above the pump icon. These two pressure values represent that the Fluid Control Module (Thar FCM2000) pumps the solvent mixture ***and*** manages the pressure of the system when the flow from the DAD is returned to the FCM on its way to waste. "**189 bar/101 bar**" indicates that the back-pressure regulator is holding the exit pressure at 100 bar (which is set by the method) and that the pressure that the pump is pushing with is 189 bar while flowing of 25% IPA in $CO_2$ at 3.5 mL/min. This is a typical pressure reading when everything is operating well. It indicates that the resistance of the column is such that moving 3.5 mL/min of fluid through it generates 89 bar of drop pressure.

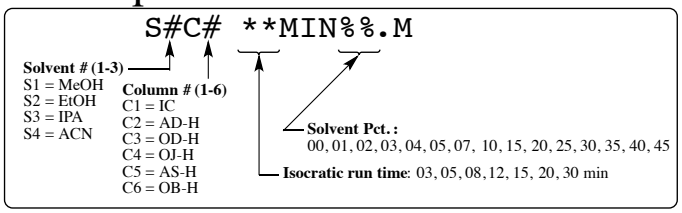



Two cases would indicate a problem:

1. If the fore-pressure is **way above 200 bar** (like 250-350 bar), there is likely some additional resistance. Either there is residual cosolvent that needs to be pushed through (which would take a minute or two) or somewhere there is clogging of the hplc lines. Extended running of the system at high pressures can cause problems with the pump and the columns.
2. If the fore-pressure is **below 150 bar** (like 120 bar), then it can be concluded that although the pump is moving the pistons at 3.5 mL/min, there is not a flow of liquid through the column at this flow rate. This would indicate that the $CO_2$ tank is empty. When either the $CO_2$ or the modifier solvent runs dry, the pump will make a squeaking sound.

# SFC System Operation with Sequence Table

SFC Methods can be individually run in Chemstation in **Single Sample mode** or assembled into a Sequence Table and run in **Sequence mode**.



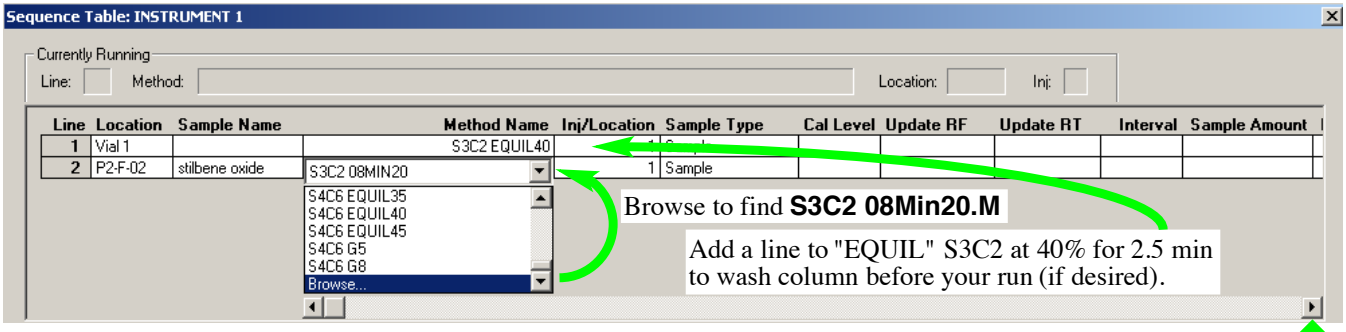

To run your sample(s) using a sequence table:

**Step 1:** Click on the icon to change to sequence mode.

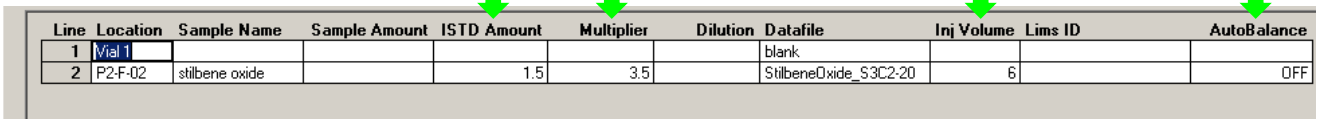
**Step 2:** Open the sequence table and set up the table as shown below.

For the sample to be run, Browse… to find the desired method (in this case S3C2 08Min20) and enter the vial location (Plate#-Row-Column). If you want to insert an equilibration run before your sample (to wash the column with 40%), choose the method (S3C2 EQUIL40) and enter "1" (= Vial 1) for the vial position which will be ignored by the Equilibration run (no injection will occur). The sample name is not the data file name and is an optional entry.

Scroll over to enter the Datafile name. Also, you may enter an injection volume other than the 5 uL default. In addition, three features have been added with a macro: **preEquil time (min)** [entered in the column ISTDAmount], **flowrate** [entered in the column Multiplier], and **Autobalance** [will occur for all sample runs unless "OFF" is entered].

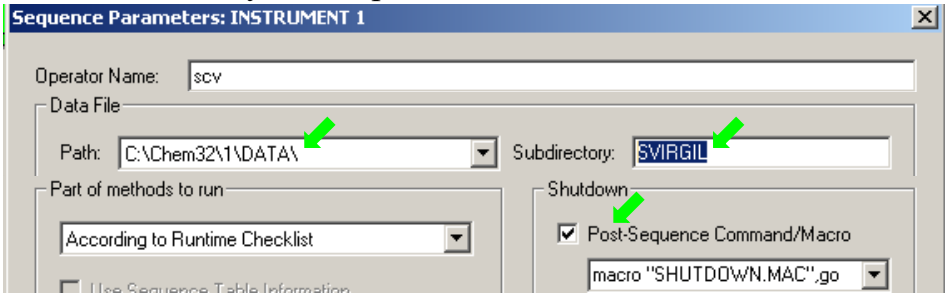




Then scroll over to get these additional options:
ISTD Amount - This column is used to delay for # minutes before injection (pre-equilibration at 20%).
Multiplier - This column is used to change the flowrate (mL/min, default = 2.5 mL/min).
Inj. Volume - can be set to 1-20 uL (default = 5 uL).
Autobalance - A prerun autobalance will be performed on non-EQUIL runs unless Autobalance = "OFF".



| Line | Location | Sample Name | Sample Amount | ISTD Amount | Multiplier | Dilution | Datafile | Inj Volume | Lims ID | AutoBalance |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Vial 1 | | | | | | blank | | | |
| 2 | P2-F-02 | stilbene oxide | | 1.5 | 3.5 | | StilbeneOxide_S3C2-20 | 6 | | OFF |

**Step 3:** Finally, Enter the folder name and check Shutdown in **SequenceParameters**, turn the SFC on and click START to run your Sequence.

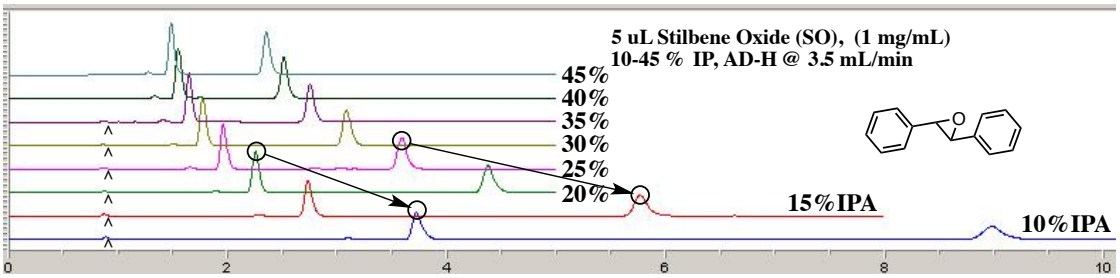


# SFC Optimization Strategies

## 1. The simple approach

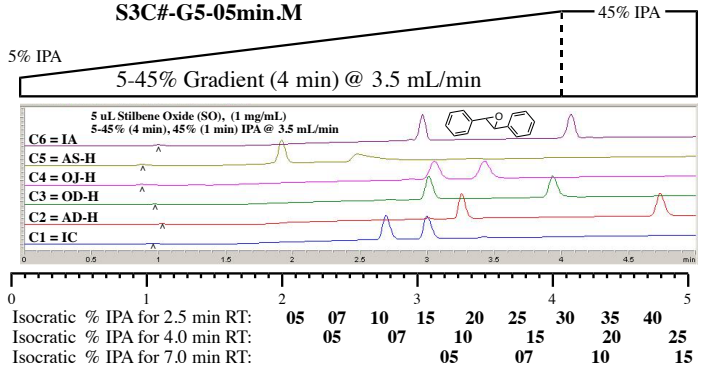
When searching for a suitable solvent and column, it is often best (and fastest) to start with a high enough percentage of cosolvent so that the sample can be confidently expected to elute from the column and then to drop the percentage to see if the separation is acceptable. Once a suitable percentage is found for one or two columns, the rest of the Daicel columns will usually give similar behavior for a given percentage of cosolvent.

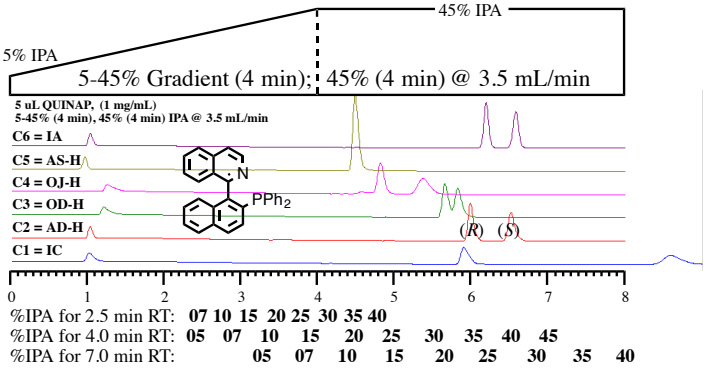
With *trans*-stilbene oxide, a stack of runs at increasing IPA percentage is shown below. In these runs, the solvent spike (^) is at ~ 1 min retention time at 3.5 mL/min. If we have a run where an analyte peak elutes with a retention time ($RT_{old}$) and we want to lower the solvent percentage to get a longer retention time ($RT_{new}$) such that $(RT_{new}-1)/(RT_{old}-1) = 2$, this generally is achieved by dropping the solvent percentage by 10-15% as shown below.




## 2. Using Gradient Methods to screen multiple column/solvent combinations

Another way to find optimum solvent percentage for a new compound is by first running a gradient method. Each solvent-column combination has a 5-min gradient method, **S#C#-G5-05.M** and an 8-min gradient method **S#C#-G8-08.M** for the purpose of rapidly screening solvents and column combinations for optimum conditions. Based on the results below, we could conclude that column 2 (AD-H) gives the best separation for Stilbene Oxide and that S3C2-25-05min.M would be a good choice for a 5-minute isocratic run or S3C2-40-03min.M would probably be a good 3 min run. Also, QUINAP would separate well on AD-H with S3C2-40-05min.M.

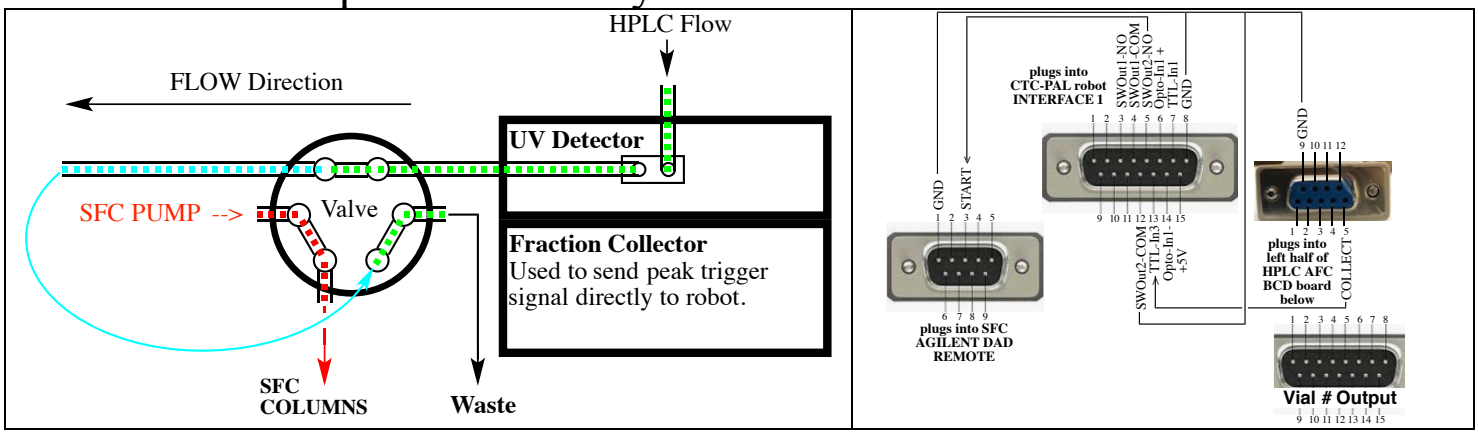


# HPLC-SFC Relay for Chiral Catalysis Screening

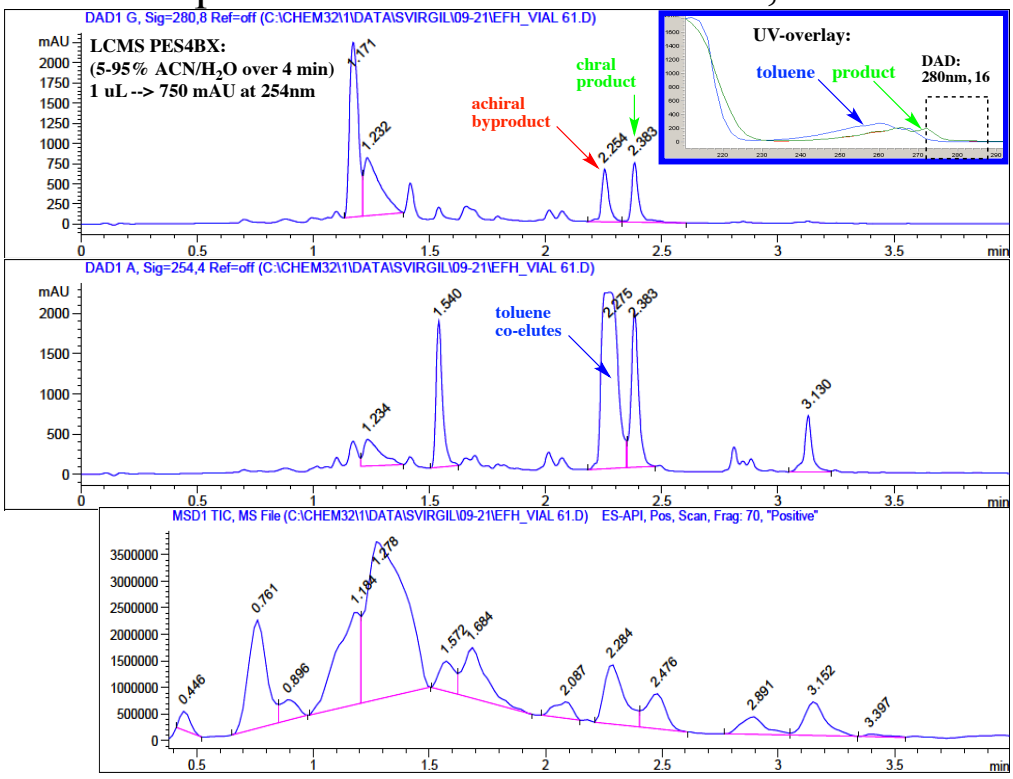
-Scott Virgil, California Institute of Technology September, 2021

Reversed-phase HPLC can handle samples with an abundance of polar contminants and solvents without column degradation. Using this system, a chosen peak may be relayed directly to an SFC Chromatography system for chiral analysis. The relay of the peak is achieved as the HPLC flows through the DAD detector to a valve containing a transfer loop. When the fraction collector "triggers" to collect a peak, the signal is sent to switch the valve after a prescribed delay time.

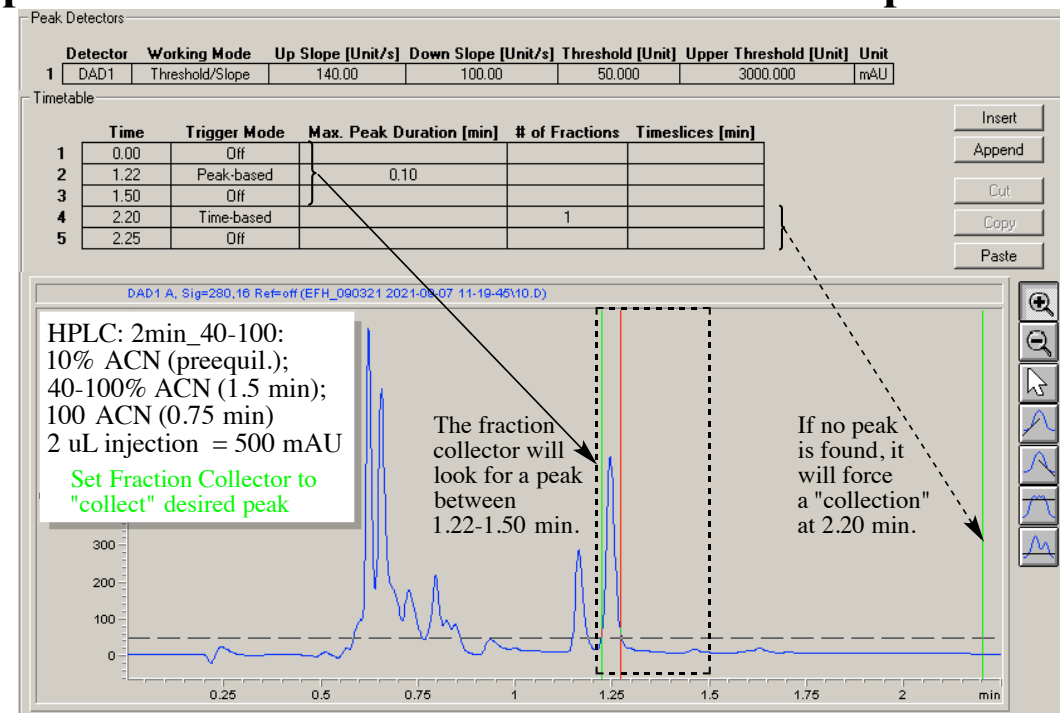



## Selecting wavelength for an easily "collected" peak:

In the HPLC chromatogram, the choice of the wavelength is made to give an easily "collected" peak for the fraction collector, in this case 280nm.

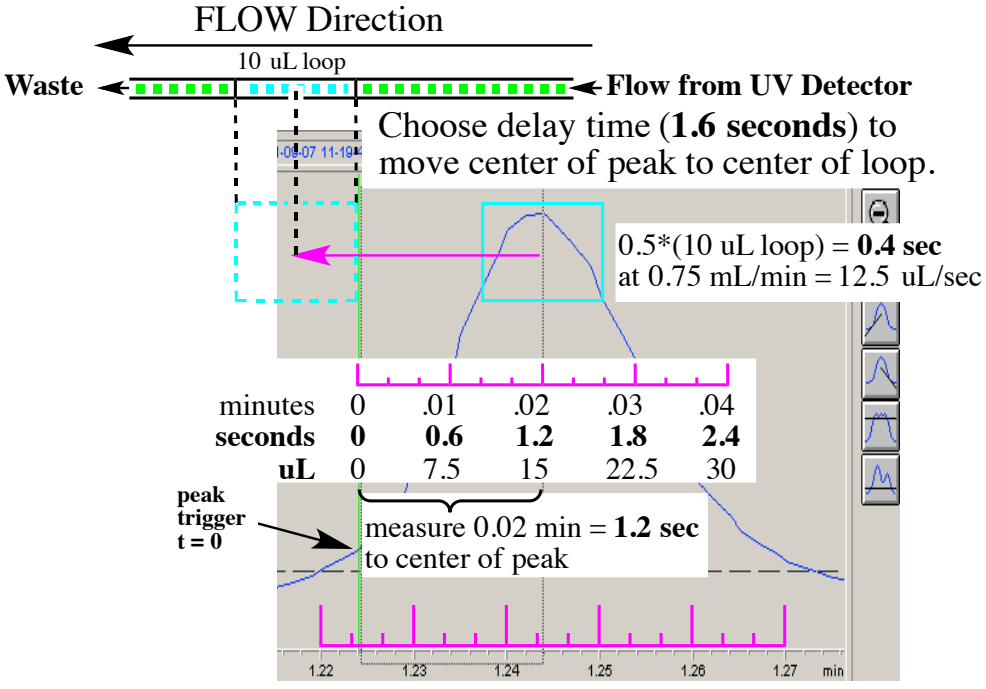


## Setting up fraction collector to “collect” the desired peak:



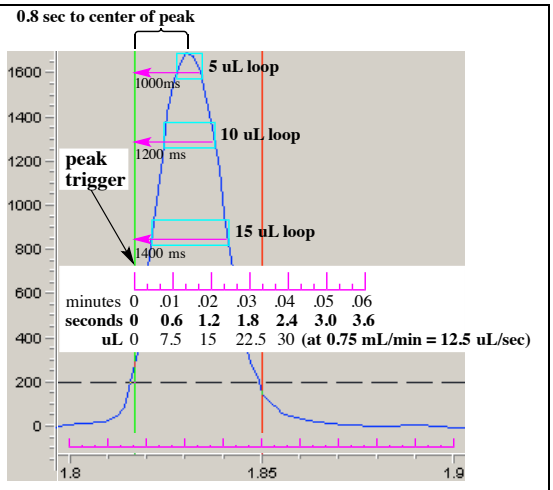

## Measuring delay time to bring the center of the peak into the loop:

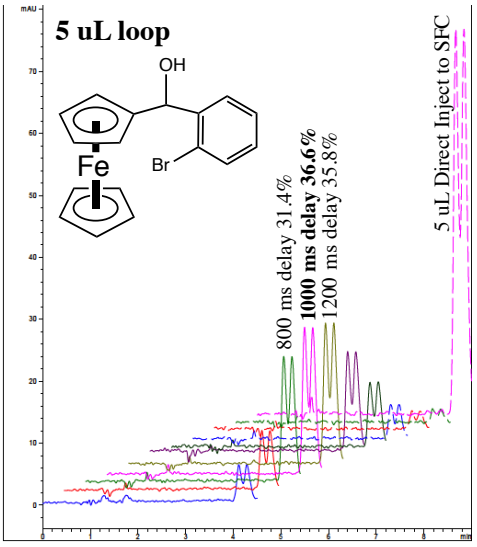
If one imagines the HPLC flow moving right-to-left and arriving at the 10 uL loop almost instantaneously, switching the valve with a delay time of t = 0 sec would place the peak trigger poing at the entrance of the loop. In order to move the center of the peak to the center of the loop, the necessary delay time = (the measured time from the trigger point to the center of the peak) + (½ of the volume of the loop/flow rate). This generally gives optimum delay times of 1-2 sec.

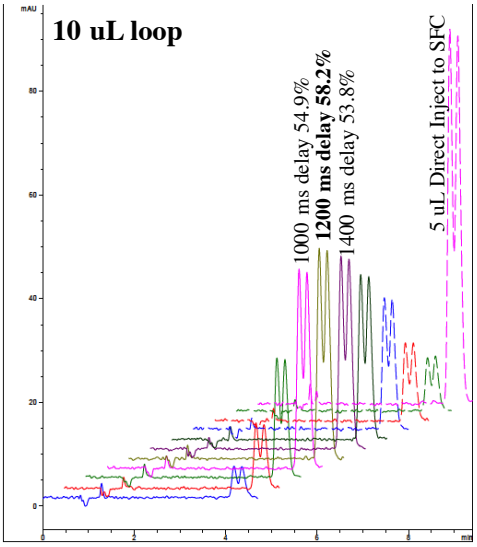


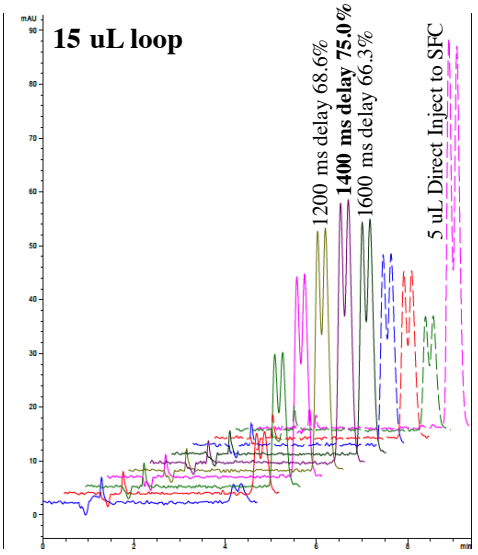
## Comparison of loop size with delay times:

The ferrocene compound was run with a series of delay times on three different loops (5 uL, 10 uL and 15 uL). Although the precise setting of the delay time gives the strongest peak and 35-75% of the peak is relayed to the SFC, settings that are off by 0.2-0.4 seconds still give enough signal to provide useable data.

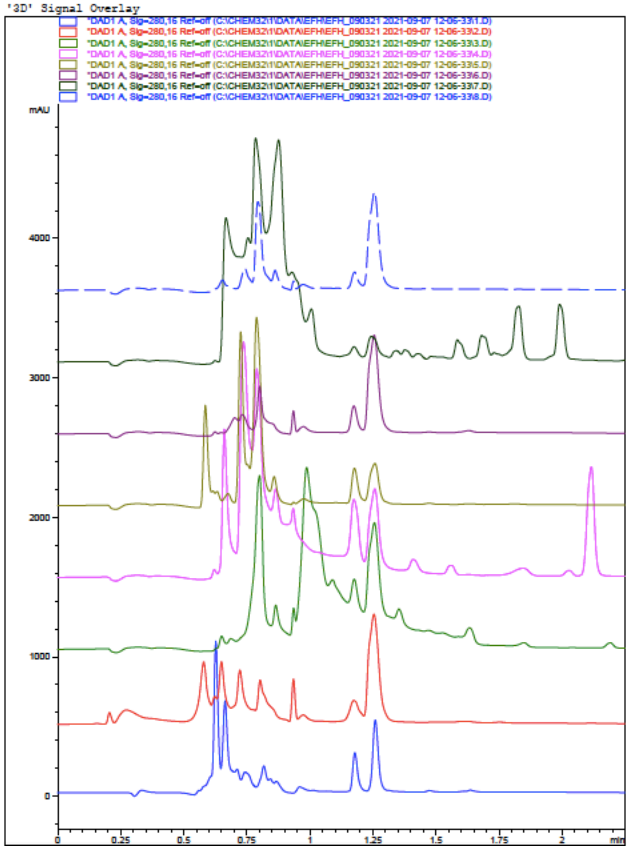


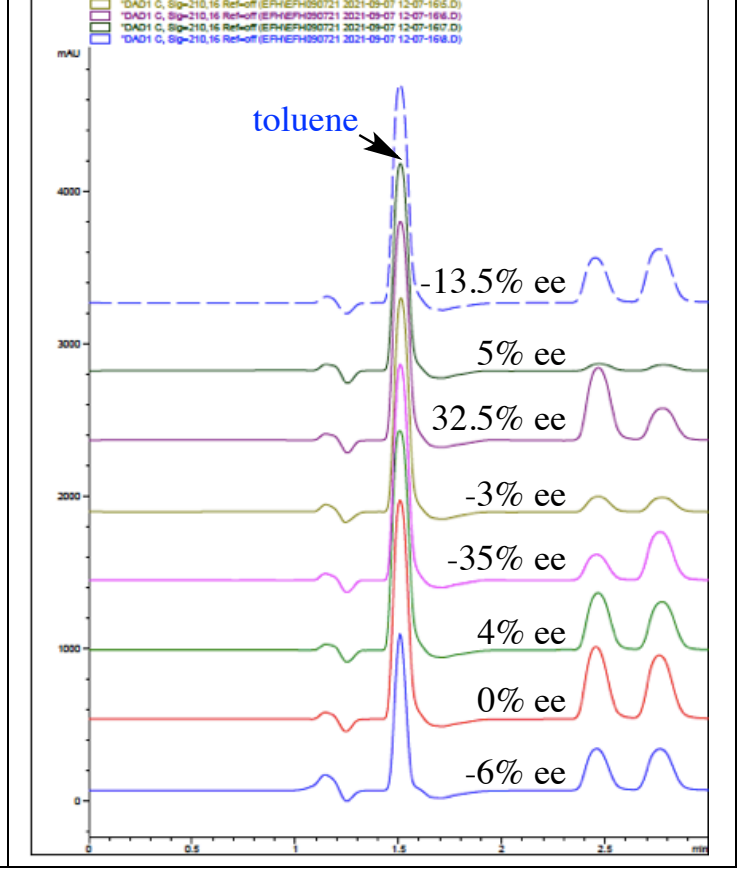



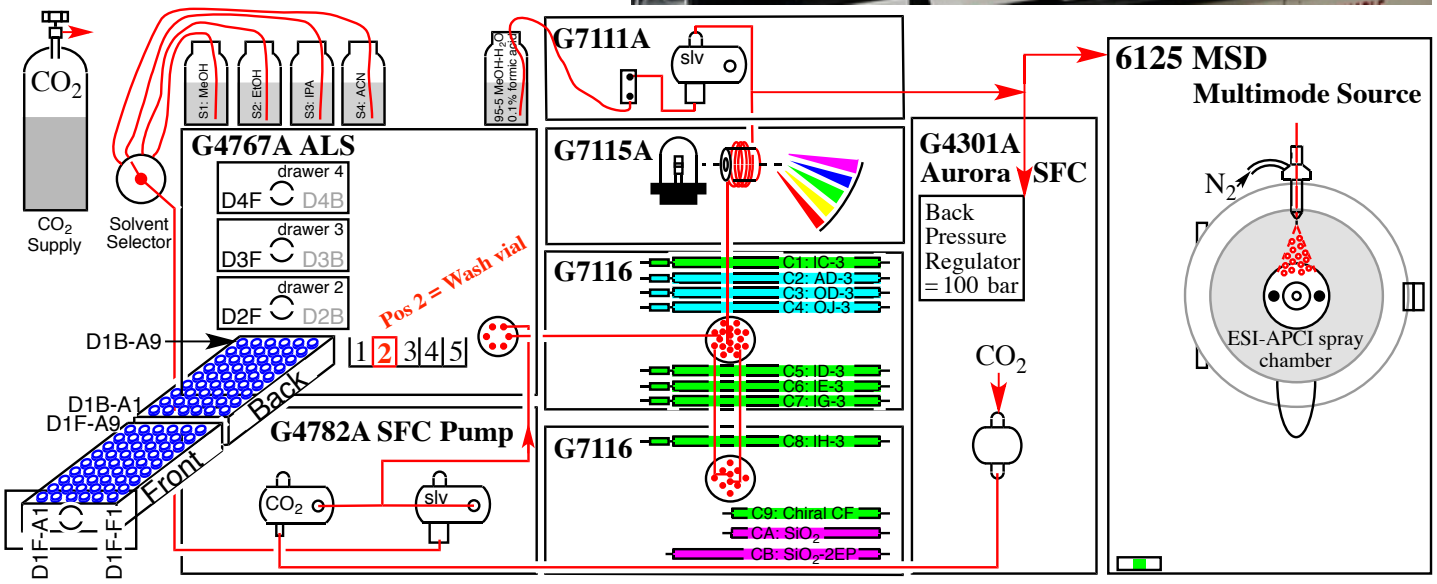
# Example Data from Pd Screen:



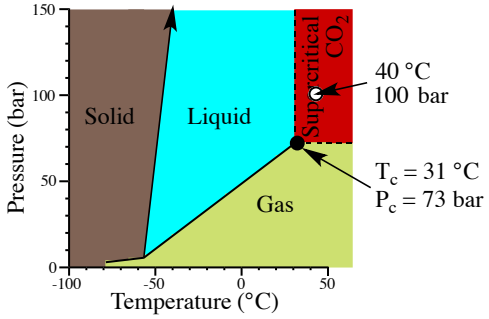
# Agilent 1260 Infinity II SFC-MS

-Scott Virgil,
California Institute of Technology 2024




SFC greatly improves the speed and efficiency of chromatographic analytical and preparative processes by using $CO_2$ under supercritical conditions (40°C, 100 bar):

- Higher flow rates due to the low viscosity of $CO_2$
- Decrease in solvent costs and waste disposal costs.
- Miscibility of $CO_2$ with MeOH, ACN
- Enhanced peak shape, resulting from properties of $CO_2$




The Agilent 1260 Infinity II SFC-MS System incorporates several features to enhance the performance of analytical SFC and includes a 6125 MS detector with an ESI/APCI source:

- SFC fluid delivery system uses gaseous $CO_2$ → improved baseline, reproducibility of RTs
- Analytical UHPLC connections and high pressure DAD flow cell → narrowest peak shapes
- 3 micron DAICEL columns (4.6 x 250 mm) → highest resolution for chiral analysis

This manual describe the operation of this SFC-MS under a variety of analysis conditions:

- Chiral analysis without MS using (IC3, AD3, OD3, OJ3, ID3, IF3, IG3, IH3, or Chiral-CF)
- Chiral analysis with MS detection by ESI or APCI scanning mode (or ES+APCI mixed MM)
- Chiral analysis with MS detection in Selected Ion Mode (SIM) with up to 11 ions
- Achiral analysis as above with $SiO_2$ or $SiO_2$-2-Ethylpyridine column.

# SFC System Operation with Agilent Chemstation

## Care of Chiral Columns:

Chiral columns 1-8 of this system are 4.6 x 250mm Daicel columns with 3 um particle size and purchased from Chiral Technologies. Maintaining the exceptional peak shapes delivered by these columns can be aided by:

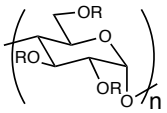
(1) avoid unnecessary injection of crude samples containing strongly retained material;

(2) avoid large injection volumes of samples for which data can obtained by e.g., 1-2 uL or less;

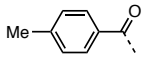
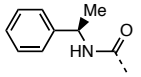
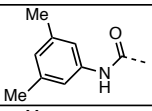
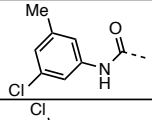
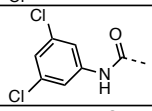
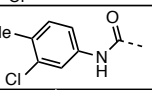
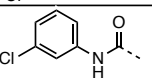
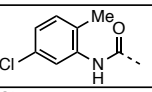
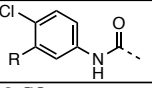
(3) don't use strong solvents like $CH_2Cl_2$ and $CHCl_3$ for sample prep on non-immobilized clumns;

(4) avoid high pressures that can accompany startup with high concentrations of cosolvent;

**DAICEL Columns:**
**(Chiral Technologies)**



**Amylose ("A" columns)** **Cellulose ("O" columns)**

| | | |
|---|---|---|
|  | | **OJ-3** (Column 4)<br>IJ-3 |
|  | **IH-3** (Column 8)<br>AS-H | |
|  | **AD-3** (Column 2)<br>IA (5 micron) | **OD-3** (Column 3)<br>IB (5 micron) |
|  | **IG-3** (Column 7) | |
|  | IE-3 | **IC-3** (Column 1) |
|  | **IF-3** (Column 6)<br>AZ-H | IM-3<br>OZ-H |
|  | **ID-3** (Column 5) | |
|  | AY-H (5 micron) | |
|  | | OX (R = Me)<br>OF (R = H) |
| MeCO---<br>PhCO---<br>PhNHCO---<br>pTolNHCO---<br>PhCH=CHCO--- | | OA-H (5 micron)<br>OB-H (5 micron)<br>OC-H (5 micron)<br>OG-H (5 micron)<br>OK-H (5 micron) |

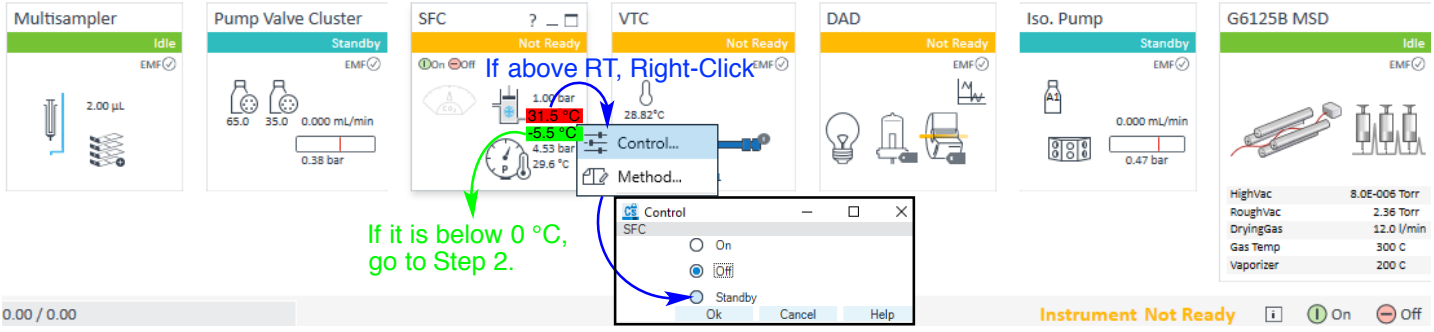
Column 9: 4.6 x 150 mm Agilent Chiral-CF (cyclofructose-6)
Column A: 4.6 x 150 mm Agilent Zorbax $SiO_2$
Column B: 4.6 x 250 mm 5 um Waters $SiO_2$-2-Ethylpyridine

## SFC Startup

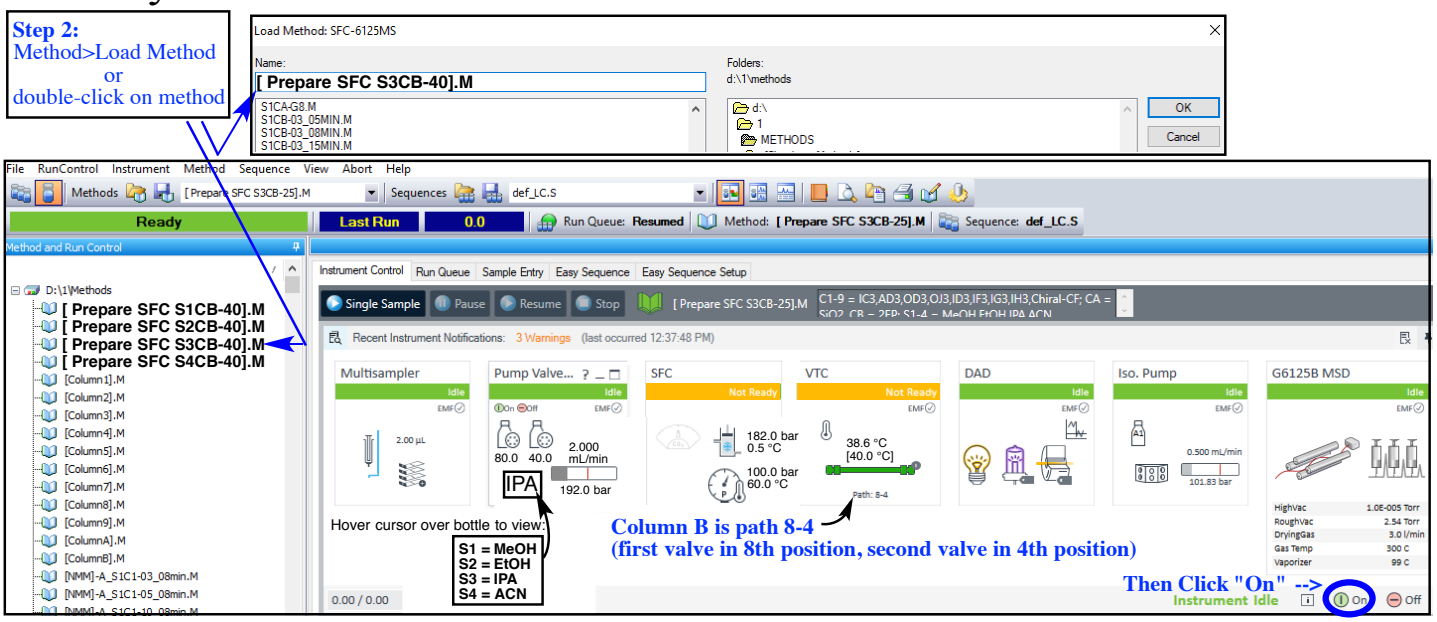
Steps 1 & 2 are described to help avoid over-pressurization at Startup.

### Step 1 Check SFC temperature

Make sure that the SFC chiller temperature is ready for Startup (below 0 °C). If the chiller temp. is room temperature or above, you can right-click on the SFC icon and put the chiller in "Standby" and give it a few minutes to get below 5 °C before all other components are turned on.



**Step 2** **Load one of the four methods** [ Prepare SFC S#CB-40].M of the solvent you will use. Then click "On" to start all modules.

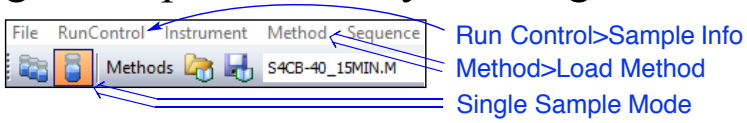



This will reduce the flow and use the achiral column to minimize pressure fluctuations during startup. All modules will turn green in ~3 min when:

- SFC back pressure at 100 bar and fore-pressure close to pump pressure
- Column compartment temperatures at 40.0 °C

## Single-Sample Step 3 Load desired Method and Submit Sample

Load the desired method and set the mode to "Single Sample Mode" by clicking on the icon.




PAPCI-A_S#C#-%%_**MIN.M

**Solvent # (1-4)**
S1 = MeOH
S2 = EtOH
S3 = IPA
S4 = ACN

**Column 1-9,A,B**
C1 = IC-3
C2 = AD-3
C3 = OD-3
C4 = OJ-3
C5 = ID-3
C6 = IF-3
C7 = IG-3
C8 = IH-3
C9 = Chiral-CF, CA=$SiO_2$, CB=$SiO_2$-2EP

**Isocratic run time:**
05, 08, 15 min

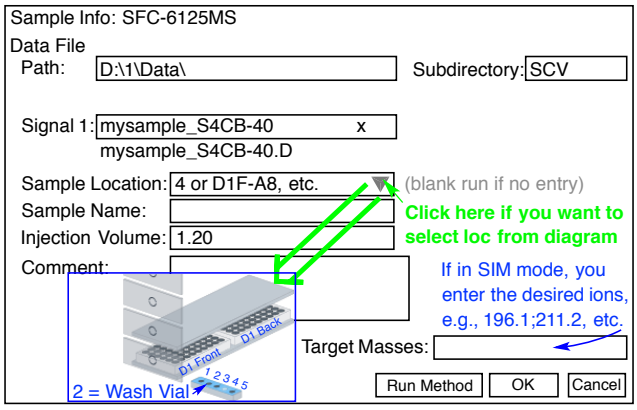
**Solvent Pct. :**
01,03,05,10,15,20,25,30,35,40

**MS modes:**
no prefix: no MS
PES-A_: pos. electrospray
PAPCI-A_: pos. APCI
SIM-PAPCI_:+ SIM mode
zNAPCI-A_: neg. ES+APCI
zSIM-NAPCI_:- SIM mode

Enter the Sample Info:

- Data Path should be D:\1\Data\
- Subdirectory = your folder name
- Data Name
- Sample Location
  D1F-A8 = Drawer 1 Front,row A,col 8
  ***or*** position 1,3,4 or 5 in Ref. Vial Rack
- Inj. Volume (default = 2.0 uL)

***optional:*** Sample Name, Comment




***optional:*** SIM ions - e.g., 239.2 (single ion) or 197.1;253.1 (up to 11 ions)

# Sequence Step 3 Prepare Sequence Table, Enter Sequence Parameters

- Note: A change of solvents requires and extended 4 minute flush at 35%.
- Note also a 1.5 minute pre-equilibration delay after a column flush.

Sequence>New Sequence

Sequence Table: SFC-6125MS

| Line | Sample Location | Sample Name | Method Name | Injection Source | Injection Vol... | Inj/Loc | Sample Type | ISTD8 Amo... | Data File | Target Mass |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | |

Flush col 1 with S3
Append line
(empty Location = Blank)

| Line | Sample Location | Sample Name | Method Name | Injection Source | Injection Vol... | Inj/Loc | Sample Type | ISTD8 Amo... | Data File | Target Mass |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | S3C1-35_02minFLUSH | As Method | | 1 | Sample | | | |

Append Lines (Ctrl+Shift+Insert)

First sample run:
need Location, Method
Inj. vol., data file
Also need 1.5 min delay (ISTD8) to delay for column equilibration from 35% to 20%.

| Line | Sample Location | Sample Name | Method Name | Injection Source | Injection Vol... | Inj/Loc | Sample Type | ISTD8 Amo... | Data File | Target Mass |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | S3C1-35_02minFLUSH | As Method | | 1 | Sample | | | |
| 2 | D1F-A1 | | S3C1-20_05min | As Method | 1.5 | 1 | Sample | 1.5 | SO_S3C1-20 | |
| 3 | | | | | | | | | | |

Add run with MeOH

(can right-click
to copy/paste lines)

| Line | Sample Location | Sample Name | Method Name | Injection Source | Injection Vol... | Inj/Loc | Sample Type | ISTD8 Amo... | Data File | Target Mass |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | S3C1-35_02minFLUSH | As Method | | 1 | Sample | | | |
| 2 | D1F-A1 | | S3C1-20_05min | As Method | 1.5 | 1 | Sample | 1.5 | SO_S3C1-20 | |
| 3 | | | PAPCI-A_S1C1-35_02minFL... | As Method | | 1 | Sample | 2 | | |
| 4 | D1F-A1 | | PAPCI-A_S1C1-15_05min | As Method | 1 | 1 | Sample | 1.5 | SO_S1C1-15_APCI | |

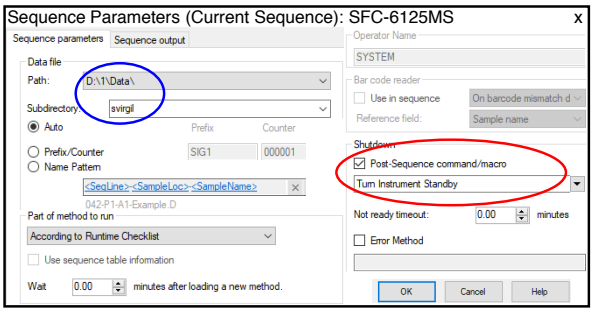
Need 2+2 = 4 min total to change from IPA to MeOH @ 35%. Then enter method with 1.5 min pre-equil.

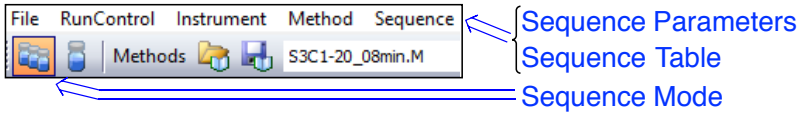
Now changing to col 2
for SIM mode run.

(target masses entered)

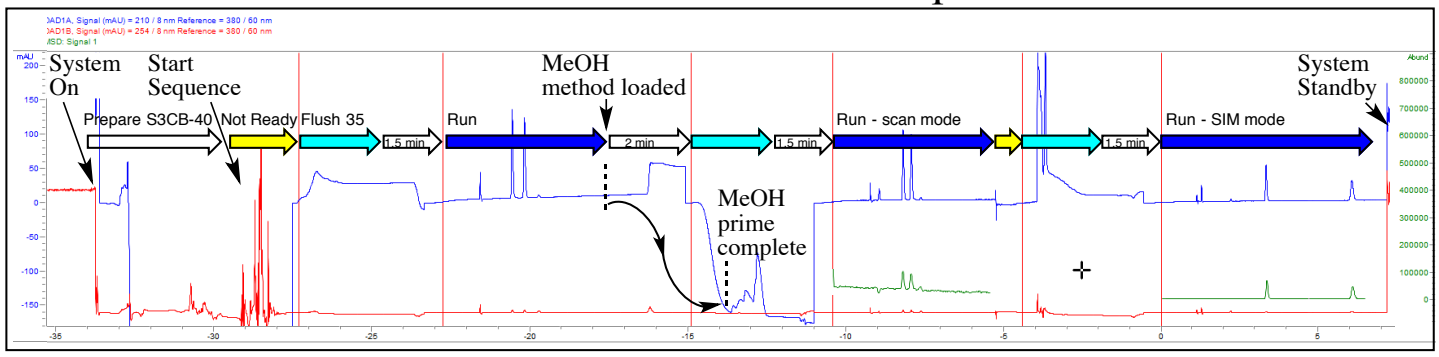
| Line | Sample Location | Sample Name | Method Name | Injection Source | Injection Vol... | Inj/Loc | Sample Type | ISTD8 Amo... | Data File | Target Mass |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | clean column 1 w IPA | S3C1-35_02minFLUSH | As Method | | 1 | Sample | | | |
| 2 | D1F-A1 | run without MS | S3C1-20_05min | As Method | 1.5 | 1 | Sample | 1.5 | SO_S3C1-20 | |
| 3 | | change to MeOH, need 4 min total | PAPCI-A_S1C1-35_02minFL... | As Method | | 1 | Sample | 2 | | |
| 4 | D1F-A1 | run with MS scan mode | PAPCI-A_S1C1-15_05min | As Method | 1 | 1 | Sample | 1.5 | SO_S1C1-15_APCI | |
| 5 | | change to col 2, clean it with 40% | SIM-PAPCI_S1C2-40_02MINFL | As Method | | 1 | Sample | | | |
| 6 | D1F-A1 | run at 30% in SIM mode | SIM-PAPCI_S1C2-30_05MIN | As Method | 0.5 | 1 | Sample | 1.5 | SO_S1C2-30_SIM | 197.1;211.1 |
| 7 | | | | | | | | | | |

→ Set the mode to "Sequence Mode"
→ Enter Sequence Parameters.
* Check "Turn Instrument Standby"
- or else the instrument will run all night
→ Run Sequence

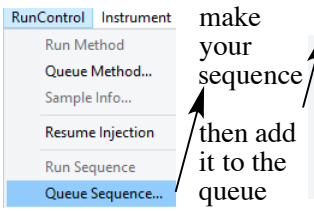



The Online Plot below shows the runs of this sequence table:

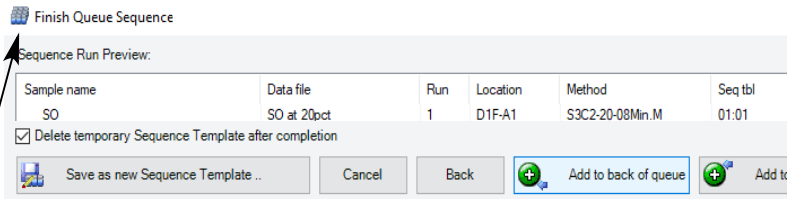


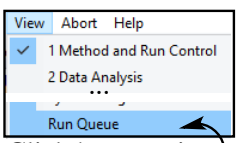

The Agilent C.01.09 Chemstation allows Queueing of new sequences while others are running:




make your sequence

then add it to the queue

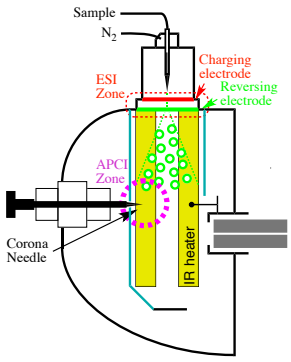



Click here to view the Seq. Queue

## Considerations for Choosing Methods:

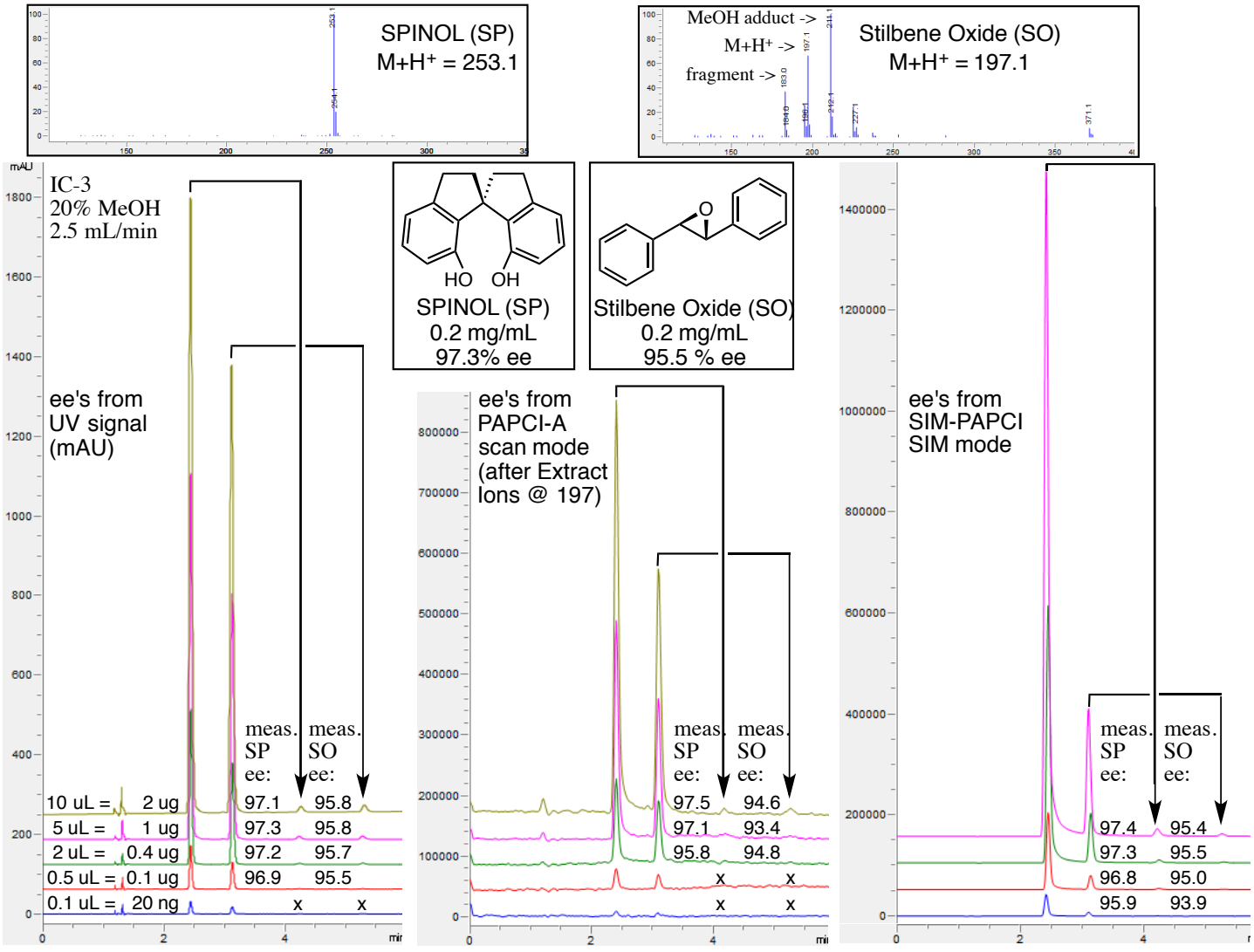
***Choice of MSD Mode – will usually be APCI:*** The multimode source* may be used in ESI, APCI or mixed multimode (ESI+APCI) ionization. For normal phase chromatography (SFC), APCI will ionize many of the non-polar analytes that are unaffected by ESI. For non-polar analytes consistent with SFC separations, there will be very few cases that ionize by ESI, but not by APCI.




* https//www.agilent.com/cs/library/technicaloverviews/public/5989-2935EN.pdf

***Considerations for MS Quantitation of ee*** *(example below)**:***

1. Choice of solvent, ionization mode and selected ion can be important.
2. UV signal provides sharpest peaks, but fails to detect below 5 ng.
3. In scan mode, "Extract Ions" is used to provide ee ratios of $M+H^+$ ions.
4. In SIM mode, sensitivity is improved for accurate *ee* measurements.



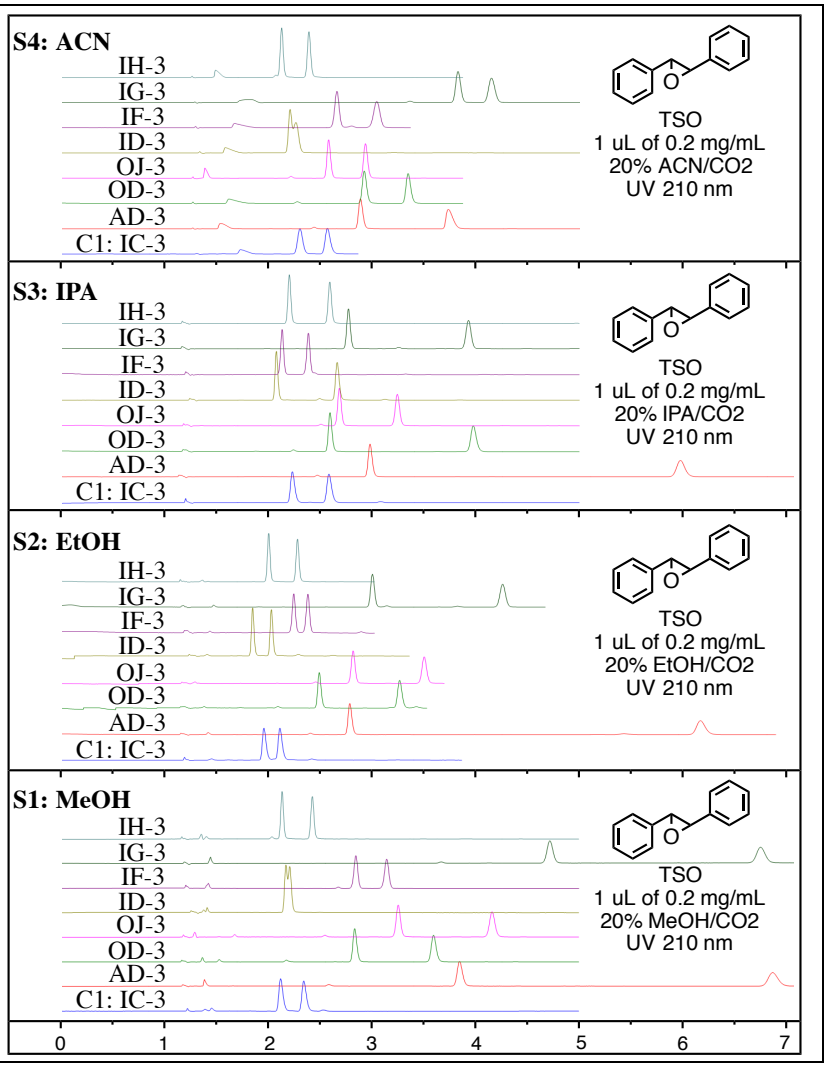
# SFC System Solvent and Column Screening

**Chiral Technologies Columns:**

The Amylose and Cellulose-based columns from Chiral Technologies often times afford similar retention times for a given analyte. They are usually used with alcohol cosolvents MeOH/EtOH/IPA, but can also be used with acetonitrile.

Most of the columns are derivitized aromatic urethanes contaning Me- or Cl-groups. IH-3 (the crosslinked version of AS) is the only column bearing chiral centers on the urethane.

With highly non-polar analytes, there can be difficulty when the columns simply do not retain the analyte enough to separate enantiomers. OJ-3 contains ester groups and is sometimes best for non-polar analytes. Also, IC-3 (bearing two chlorine atoms) can give longer retention times for some non-polar analytes.

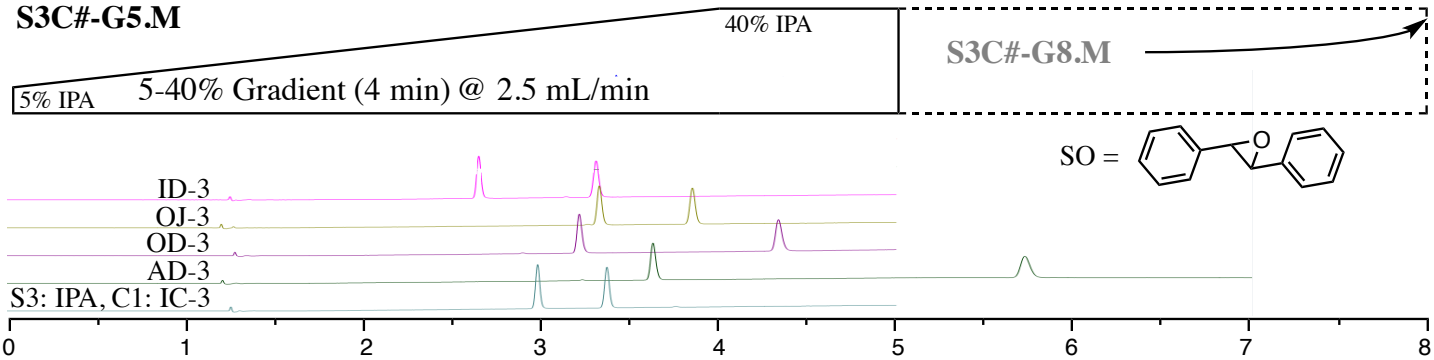



## GRADIENT METHODS:

**_S#C#-G5.M** 5-40 gradient (4 min) then 1 min at 40%

**_S#C#-G8.M** 5-40 gradient (4 min) then 4 min @ 40%

There are some gradient methods that can be used for screening analytes across several columns for which the retention times may vary considerably. Based on the results below, we could conclude that column 2 (AD-3) gives the best separation with IPA and that S3C2-20-08min.M would be a good choice for an 8-minute isocratic run.



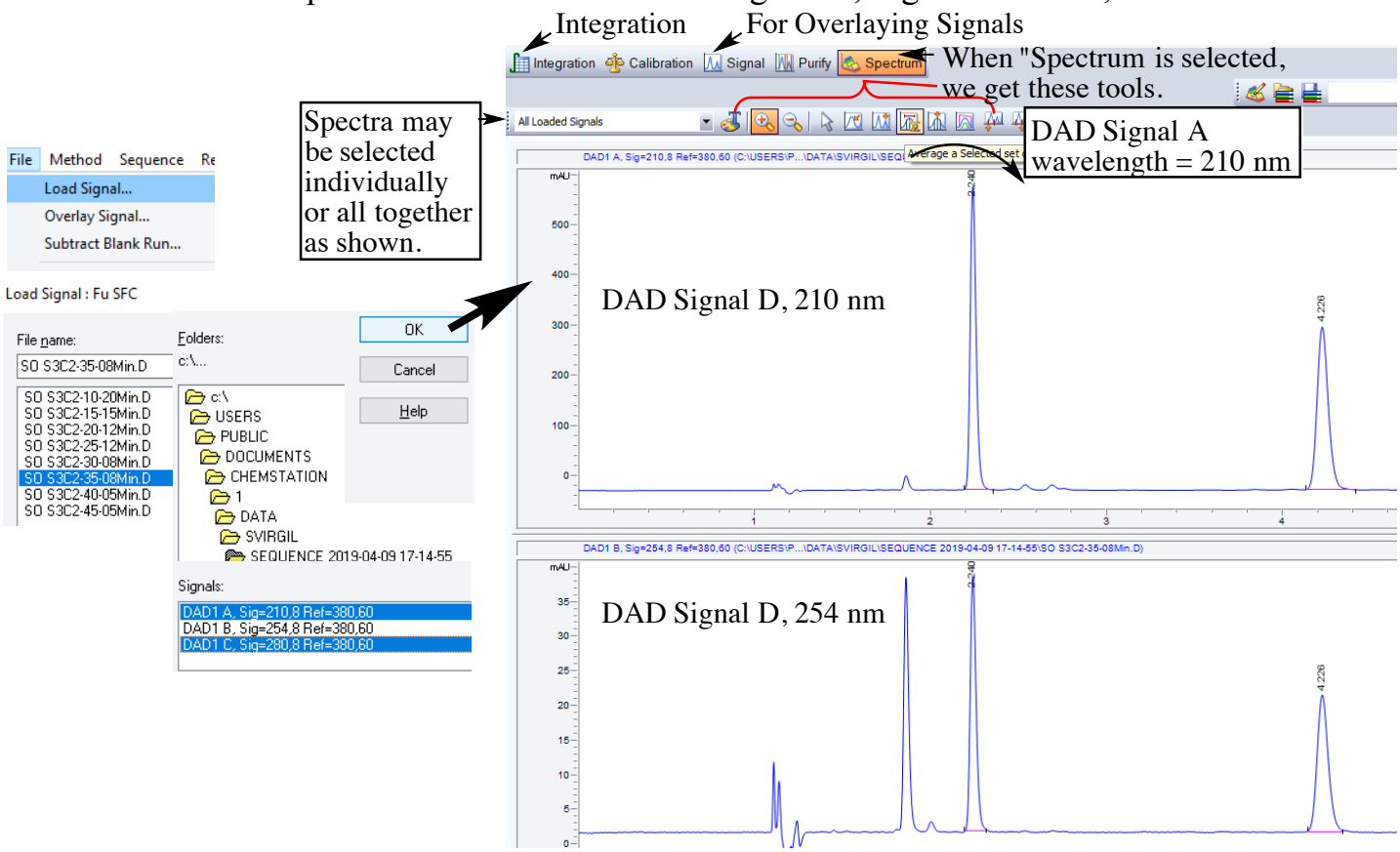

Isocratic % IPA for 2.5 min RT: **10 15 20 25 30 35 40 45**

Isocratic % IPA for 4.0 min RT: **05 07 10 15 20 25 30 35 40 45**

Isocratic % IPA for 7.0 min RT: **05 07 10 15 20 25**

# Tools For Data Analysis

When a data file is loaded, it will usually consist of up to four DAD chromatograms. They can be viewed individually or all together by selecting "All Loaded Signals". In addition, five buttons above the data provide different tools for Integration, Signal selection, etc.




The "Spectrum" button allows one to view the UV spectrum of a selected region of the chromatogram (dragging with Σ sign tool), or multiple UV signals may be examined overlaid (by dragging with "apex" tool) to confirm the enantiomer relationship.