**LAB: SEC-MALS Experiment**

### SEC-MALS Experiment Topics:

* Hardware and software required to use a DAWN or a miniDAWN with an HPLC system (most often, SEC or FFF) to obtain molar mass and radius distributions of fractionated polymer samples.
* Configurations and ASTRA methods
* Sample injections using an autosampler
* Connecting third party instruments
* Setting up ASTRA sequences (SEC-MALS with UV and RI, optional QELS, SEC-MALS-VIS)
* Collecting SEC/MALS/UV/RI data using ASTRA 6 software

### Goals:

To properly assemble a SEC/MALS/RI/UV system, to set Configuration parameters, and to collect and process ASTRA data to obtain molar mass and radius distributions.

### HPLC System Setup:

**Instruments and connections:**

* HPLC system (Waters, Dionex, Shimadzu, Agilent, Akta Micro or others)
* Solvent reservoir with stirred PBS buffer (50 mM sodium phosphate, 50 mM NaCl, 200 ppm NaN3, pH 6.8). Stirring the mobile phase helps obtaining stable RI baseline.
* Degasser
* Pump with Pulse dampener
* Inline filter kit between pump and injector (aqueous, organic, PTFE options)
* Autosampler
* Heated column compartment
* WTC-030S5 protein column and WTC guard column from Wyatt Technology
* UV detector @ 280 nm
* Connect UV analog out to AUX2 port of the HELEOS
* DAWN HELEOS
* Autoinject cable: HELEOS – connected to Autosampler Remote Port
* Optilab rEX (**Never** reverse inlet and outlet of the Optilab rEX!)
* Waste tubing

**Recommended tubing inner bore diameters:**

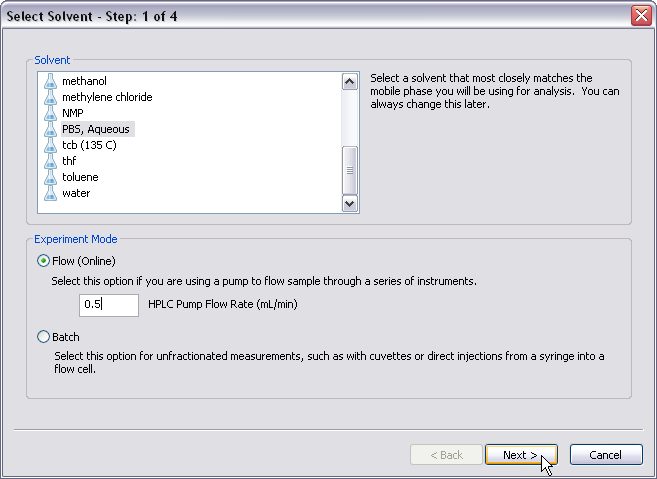
* 1/8” tubing before pump
* 0.02” after pump to injector (PEEK for aqueous, stainless steel or organic SEC work)
* 0.01” after autoinjector and between detectors
* 0.03” to waste or fraction collector

### Note:

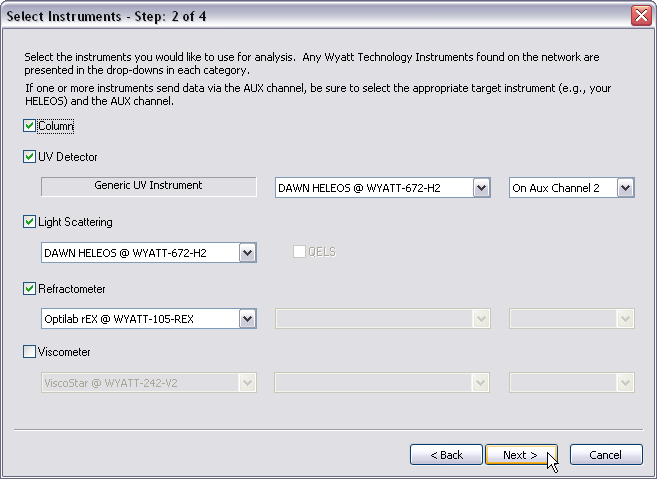
* A list with part numbers of supplies and equipment used in this session can be found in Section 14 of your LSU binder.

### Generate a Method for SEC-MALS with UV and RI as concentration detectors

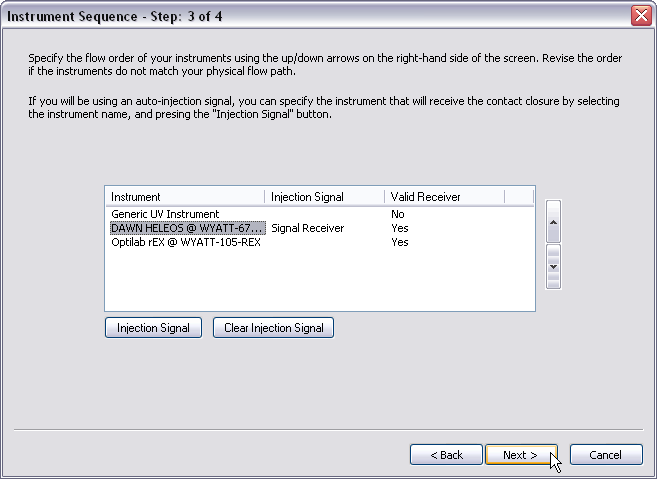
***Open*** ASTRA 6 (current version) and select **System** → **Configuration Wizard**. In the **Select** **Solvent** Dialog, select your **Solvent** (PBS, aqueous) and set the **Experiment Mode** to **Flow (Online)**. Set the HPLC Pump Flow Rate to 0.5 mL/min.



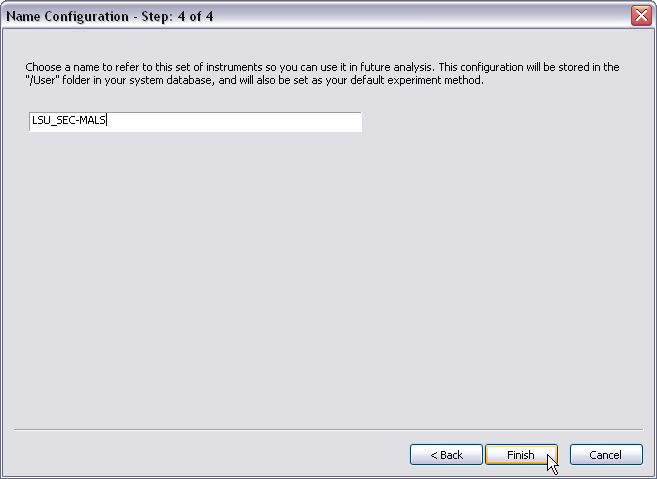
Click on **Next** to go to the **Select Instruments** Dialog. Select your **Light Scattering Instrument** (e.g. Heleos or Treos). If your instrument has a QELS unit, the QELS checkbox will be automatically checked. Also select **UV Detector** and corresponding **Aux channel**. Select your **Refractomer** and **Viscometer** (if connected). Selecting a **Column** is optional unless you want to utilize the *conventional* and *universal column calibration* methods in ASTRA.



Click on **Next** to go to the **Instrument Sequence** Dialog. You can change the instrument that will receive the injection signal from your autoinjector if necessary (it defaults to your LS instrument). You can also change the order of your instruments as they appear in the Configuration.



Click on **Next** to go the **Name Configuration** Dialog. Choose a name for this configuration, e.g. LSU\_SEC-MALS. Click on **Finish**. You have now created a **Method** that you can run.



In ASTRA, go to **File** → **New** → **Experiment from Default**. This will open a new file called **Experiment 1** using the **Configuration** that you’ve just created. We will now make a few changes to our Configuration and Procedures before we can start data collection.

Note that you can start a data collection immediately and change all parameters post acquisition, but it’s just better practice to set everything up correctly before starting a run.

**Configuration:**

* Double-click on **Configuration**. You can add a **Description** or **Notes** there. You can also select **Abscissa Units** (e.g. **min** or **mL**) and change the **Concentration Source** from **RI** to **UV**.
* Expand the “+” sign next to **Configuration**. You can verify that the HPLC pump **flow rate** is **0.5 mL/min** and that your solvent is set to **PBS, aqueous**.
* Injector: set the **Injected Volume** to **100 uL**.
* Sample: Set the **Name** to **Pierce BSA**, set **Concentration** to **2 mg/ml**, **UV extinction coefficient** to **0.667 mL/(mg x cm)**. The value for **dn/dc** already defaults to **0.185 mL/g**.
* Generic UV: Set **Cell Length** to **1 cm**, **wavelength** of **Channel 1** to **280 nm** and **UV response** (here: **1 AU/V**).
* DAWN HELEOS: Select the correct **Sample Cell** (**Fused Silica** in newer instruments, **K5** in older instruments) and **Calibration Constant.**  If your LS instrument has a QELS unit, verify that the QELS box is checked and select the correct Replaced Detector, the default is 12.
* Aux channel Connection: Verify that the UV **Aux channel** is set to **2** and the **Destination** **Device** is **DAWN HELEOS**.
* Auto Inject Connection: Defaults to DAWN HELEOS.

**Procedures:**

Basic Collection:

* Confirm that **Trigger on Auto Inject** is checked
* Confirm that **Collection Interval** is set to **0.5 sec**
* If you have a QELS unit, set the **QELS interval** to **2 sec**
* Set the **Duration** to **1 min**
* Details: waste/recycle settings can be changed, laser saver mode option, Comet option, Injector delay options can be set.

Save the file as METHOD in the **User** → **Methods** folder in the ASTRA database. You can overwrite the LSU\_SEC-MALS method in this folder if you wish to.

### Notes:

* The duration of the experiment is set to 1 min to demonstrate setting up a **Sequence**. In real chromatographer’s life, one would set the **Duration** to 30 min (or whatever time or volume is appropriate to elute the sample), run and process the sample to determine Normalization Coefficients, Interdetector Delay Volume and Band Broadening. *A quick processing guide can be found in Section 7 of your LSU Course Manual.***After determining the system parameters above one would save the data file as a data file and also as a *Method* from which then all new experiments are created.** We just don’t want to sit here and watch paint dry (or rather, peaks elute) for the rest of the class!
* The second group of today’s lab session will run a sequence consisting of a buffer injection and BSA (available in your LS Detector Hardware Kit), which will be processed jointly in the afternoon.
* For organic solvents, a monodisperse 30 kDa PS standard (in your LS Detector Hardware Kit) will work well to determine Normalization, Interdetector Delays and Band Broadening parameters.
* The green vertical bar on the front panel display of your HELEOS or TREOS indicates that the autoinject signal was received by the instrument.
* Starting a run in ASTRA 6 (and ASTRA 5.3.4) will automatically:
  + Set the Optilab rEX purge valve to OFF
  + Set the Orbit recycle valve to WASTE
* UV detector and extinction coefficients: ASTRA requires that the extinction coefficient is entered in mass concentration units.

Conversion from molar extinction coefficients (ASTRA 6):

εmass [mL/(mg\*cm)] = εmolar [L/(cm\*mol)] / Mw [g/mol]

* Detailed Information on how to connect your Wyatt detectors to your HPLC system (e.g. Shimadzu, Waters, Agilent, AKTA, …) can be found on your USB drive in: **LSU Training Binder\08 - SEC-MALS Lab\HPLC Equipment Interface.**
* Processed example data files (both ASTRA 6 and ASTRA 5 file formats) for BSA and Polystyrene standards can be found on your USB drive in:   
  **LSU Training Binder\08 – SEC-MALS Lab**.
* Instructions on how to set up a Method (ASTRA 5: Template) and Sequences (ASTRA 5: Sample Sets) in ASTRA 5 can be found in:   
  **LSU Training Binder\08 - SEC-MALS Lab\08 - SEC-MALS Lab 2011 - ASTRA 5.**
* **For QELS and DynaPro users:** Custom aqueous solvent viscosity calculator “SEDNTERP” freeware is available at www.jphilo.mailway.com.   
  Download & install SDTR0601.exe. A copy of the program is available on your USB drive in: **\Other Resources.**

### Creating a Sequence

**Configuration**

* Select **File** → **New** → **Blank Sequence**
* Click on the **Sequence** tab at the bottom of the Workspace and double-click on **Configuration** of **Sequence1**.
* Enter **2** for the **Number of Samples**
* Select **LSU\_SEC-MALS** as the **Default Method** by clicking on “…” and navigating to your method.
* Click **Apply** when done.

**Samples**

* Enter **Well** number (informational only)
* Check the **Enable** box
* **Name**: enter PBS and BSA
* Enter Description (optional)
* **Inj** – enter **1** for the number of injections per Well
* Make ASTRA Collection time 1 min shorter than the Collection time of your HPLC system (give ASTRA time to save the data before the next autoinject signal)
* **Method**: If necessary, you can change the default method here
* Change other parameters if desired, such as **Duration**, **UV Ext**, **dn/dc**, **Vol**, etc.
* Additional samples can be added to the sequence during the run by right-clicking into the table and selecting **Add** or **Insert**. Samples can also be **deleted**.
* Utility methods (**Light Scattering** → **Utilities**) can be added to the sequence, e.g., COMET, Orbit, Laser on/off.   
  ***ASTRA 6 users***: Your Physical instrument will be automatically selected in these utility methods.  
  ***ASTRA 5 users***: Do not forget to select your Physical Instrument in those methods and save in the **User** → **Methods** folder.
* Click **Apply** when done.

**Collection Tab:**

* Validate (optional)
* Save As (give your Sequence a name) or: Will be asked which folder to save the data into, and give it a name (e.g. LSU). The sequence name will be appended to the file name.
* Create Sequence in your HPLC software
* Start ASTRA Run (click on Run icon)
* ASTRA screen shows : Waiting for auto-inject signal
* Start the Sequence in your HPLC software…

***…and wait for the data collection to start!***

### Notes:

* The sequence can also be saved as a Template. This Template can be opened by selecting **File** → **New** → **Sequence from Template.**
* After a single experiment run has been completed, it can be opened as a regular ASTRA data file and be processed while the rest of the sequence is running.
* The ASTRA file name convention for sequences for single injections of a sample: **name[sequence name]**, e.g. **BSA[set1]**.
* For multiple injections of the same sample: **name(injection#)[sequence name]**, e.g. **BSA(001)[set1]**.

***When using a Viscometer and QELS:***

When using QELS to determine the hydrodynamic radius Rh, the Method: **Light Scattering** **→ With QELS → Online** needs to be applied to the experiment. Rh can either be determined from QELS or Viscometry, but not simultaneously, i.e. in the same data file. To compare, save the experiment under a different filename and use EASI graph.

### Using Conventional and Universal Calibration:

To use the conventional or universal calibration feature of ASTRA 6, you will need to apply the appropriate methods:

* To set up a *conventional calibration* curve, apply the **Conventional calibration curve** method in **System** → **Methods** → **RI Measurement**.
* To perform a molar mass measurement using *conventional calibration*, apply the **Conventional calibration analysis** method in **System** → **Methods** → **RI Measurement**.
* To set up a *universal calibration* curve, apply the **Universal calibration curve** method in **System** → **Methods** → **Viscometry**.
* To perform a molar mass measurement using *universal calibration*, apply the Universal calibration analysis method in **System** → **Methods** → **Viscometry**.

***Note:***

Check out the self-guided Tutorial “The ASTRA Challenge”, Universal and Conventional Calibration, for more details on how to use the calibration features and to practice!

**Using a Laser Pointer to Qualitatively Assess Mobile Phase Contamination**

|  |  |
| --- | --- |
| The photo to the right shows a laser pointer beam shining through contaminated mobile phase. The photo is taken with the laser shining from the back of the sample bottle and the camera in front at a shallow angle to the beam. This gives the best viewing conditions to observe the beam shining through the mobile phase. The beam is visible throughout the contaminated mobile phase to the right, because the light is preferentially scattered forward from the particles suspended in the mobile phase. | **Contaminated mobile phase**  laser pointer and mobile phase 009 |
| Fresh mobile phase as shown to the right may show one or two particles but will not show the beam shining through the liquid Only the entry and exit point of the laser beam is visible (scattering from the glass interface).  **Be careful not to look directly in the laser beam!** | **Fresh mobile phase**  laser pointer and mobile phase 007 |

**Guidelines for Product Qualification of an SEC-MALS System**

These guidelines are in Q/A format. They are not Product Qualification (PQ), but to provide guidance on establishing PQ and/or Standard Operation Procedures.

***How often do I need to calibrate the light scattering and concentration (e.g. refractive index) detectors?***

Calibration of each detector is performed during IQ, or after receiving the detectors from the manufacturer. Re-calibration of these detectors is not necessary unless the molar mass of the system-check standard is out of specification or a calibration has not been performed within the last 12 months. In fact, it is sometime advantageous to avoid unnecessary calibration, since calibration may involve solvents and conditions different from the SEC conditions, which can cause unnecessary down time. Instead, the entire SEC-MALS system can be validated by checking the molar mass (and RMS radius when greater than 10 nm) of a standard. The same standard may be used for normalization or even delay volume determination (dextran and pullulan are polydisperse thus cannot be used for the latter). Since the analysis of the standard is under the exact same conditions as for your samples, you may perform a system check often.

***Which standard will you recommend?***

*Aqueous Mobile Phase:* If your columns can separate bovine serum albumin (BSA) monomer from its aggregates, BSA or any other protein monomer is a good candidate. If your columns cannot provide baseline-resolution between BSA monomer and its aggregates, dextran or pullulan in the similar molar mass range of your own samples can be used as standards.

*Organic Mobile Phase:* 30 kD polystyrene is the perfect candidate for normalization, delay volume determination, and system check. If the mobile phase will not dissolve polystyrene, consider a PMMA standard with polydispersity less than 1.05.

***Any suggestions on setting the acceptable molar mass range for the standards?***

Besides the calibration constants, the molar mass calculated by light scattering detector also depends on the *dn/dc* value. You can find the *dn/dc* value in the literature, measure it yourself off-line, or estimate it on-line assuming 100% mass recovery. Once you decide on the *dn/dc* value, you need to fix it. The acceptable molar mass range can be established based on the results from multiple injections under all possible SEC conditions (different flow rates, injected amount, etc.) that will be used for your samples.

***Why should I not choose “assume 100% mass recovery”?***

The assumption of 100% mass recovery may be used initially to estimate a *dn/dc* value on-line. However, once *dn/dc* value is decided, 100% mass recovery should not be used routinely in obtaining molar masses for a system check or comparing samples from different batches or lots. The *dn/dc* value is an intrinsic and constant property of a polymer in a particular solvent; however, the “100% mass recovery” approach involves assumptions on known injected amount and total mass recovery from the column (the latter is often a bad assumption for aqueous SEC systems). Nevertheless, mass recovery can provide useful information about the sample and your system. You may record it but it is wise not to use it as a specification or give it a loose specification.

***Which molar mass should I report?***

The Mw molar mass is directly measured by MALS detection and does not depend on the column resolution. If only one value is reported (which is the case for monodisperse samples such as proteins), Mw should be used.

Other molar mass averages, such as *Mn* and *Mz* depend on the separation quality. For a polydisperse sample, it may be necessary to report both weight- (*Mw*) and number-average (*Mn*). A specification may be set for *Mn* as well to help evaluate the column performance if polydispersity of your sample is of interest as well.

***Any other things I need to pay attention to when I develop my SOP or PQ?***

* *Baseline noise or signal-to-noise ratio*

High baseline noise or low signal-to-noise ratio increases uncertainty of the measurement.

* *Normalization*

Normalization can be performed during the system check provided the standard has a RMS radius less than 10 nm.

* *Peak region selection*
* *Uncertainty of the results*

One standard deviation (or relative standard deviation) of each parameter is reported in ASTRA software and is recommended to be included as part of the results or specification.

* *Cumulative percentages*

Cumulative percentages in ASTRA report may facilitate differentiation among samples.