**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 Templates
* Sample injections using an autosampler
* Connecting third party instruments
* Setting up ASTRA sample sets (SEC-MALS with UV and RI, optional QELS, SEC-MALS-VIS)
* Collecting SEC/MALS/UV/RI data using ASTRA 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:**

* Agilent 1200 HPLC system
* 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
* Agilent VWD or DAD UV detector @ 280 nm
* Connect UV analog out to AUX2 port of the HELEOS
* DAWN HELEOS
* Autoinject cable: HELEOS – connected to Agilent Autosampler Remote Port (9-pin Sub D connector)
* Optilab rEX (**Never** reverse inlet and outlet of the Optilab rEX!)
* Waste tubing

**Recommended tubing inner bore diameters:**

* 1/8” tubing before pump
* PEEK 0.02” after pump to injector
* PEEK 0.01” after autoinjector and between detectors
* PEEK 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 first ASTRA template for SEC-MALS with UV and RI as concentration detectors for a new system setup (from the System Templates folder)

Open ASTRA V (current version):

**File**

**New**

**Experiment from Template**

**System Templates**

**Light Scattering**

**Online**

Or: **with QELS**

**Online**

Add UV:

(right-click) **Manage**

**Configuration**

**Replace**

**Example Configuration**

**HELEOS (or TREOS, EOS, etc.)**

**With Optilab rEX**

**UV + LS + RI online**

Or: **with QELS**:

**UV + QELS + LS + RI online**

**Changes to be made to the Configuration:**

* Generic Pump – change default flow rate from 1.00 to 0.5 mL/min
* Solvent (default is water) – change to PBS aqueous
* Injector – injection volume 100 μL
* Sample: Pierce BSA, concentration 2 mg/ml, dn/dc 0.185 mL/g, UV extinction coefficient 667 mL/(g x cm)
* Generic UV – Agilent 1200, 280 nm wavelength, cell length 1 cm, response factor 1AU/V
* DAWN HELEOS – attach Physical Instrument, Calibration Constant (if with QELS, check the QELS box and select QELS detector, the default is #12; attach QELS physical instrument)
* Optilab rEX, attach Physical Instrument
* Aux channel Connection – verify that the UV Aux channel is 2
* Auto Inject Connection – defaults to Light Scattering Detector

**Changes to be made to the procedure:**

Basic Collection:

* Trigger on Auto Inject, ✓
* Duration – 1 min
* LS instrument interval 0.5 sec
* Details: waste/recycle settings can be changed, laser saver mode option, Comet option, Injector delay option.

Save as TEMPLATE! (in the My Templates folder)

### Notes:

* The duration of the experiment is set to 1 min to demonstrate setting up Sample Sets. 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 *Template* 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 sample set 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 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:

εmass [mL/(g\*cm)] = εmolar [L/(cm\*mol)] / Mw [g/mol] \* 1000

☺ Detailed Information on how to connect your Wyatt detectors to your HPLC system (e.g. Agilent, AKTA, Waters, Shimadzu,…) can be found on your USB memory key in: **LSU Training Binder\08 - SEC-MALS Lab\HPLC Equipment Interface.** ☺

☺ Processed example data files for BSA and Polystyrene standards can be found on your USB memory key in: **LSU Training Binder\08 – SEC-MALS Lab**. ☺

### Creating a first Sample Set

File

New

Blank Sample Set

**Configuration Tab:**

* Description (e.g. LSU sample set)
* Select Default Experimental Template

Click on “**…**”

**My Templates**

LSU folder

Select template created in Part I

* Number of Samples: **2**

Click **Apply** when done.

**Samples Tab:**

* Enter Well number (informational only)
* Enable
* Name (enter PBS and BSA)
* Enter Description (optional)
* Number of Injections – enter **1** for each
* 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)
* Template (default template can be changed)
* Can change other parameters if desired
* Additional samples can be added to the sample set during the run
* Utility templates (**Light Scattering** → **Utilities**) can be added to the sample set, e.g., COMET, Orbit, Laser on/off. Do not forget to select your Physical Instrument in those templates and save in the **My Templates** folder.

Click **Apply** when done.

**Collection Tab:**

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

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

### Notes:

* The sample set can also be saved as a Template. This Template can be opened by selecting **File** → **New** → **Sample** **set 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 sample set is running.
* The ASTRA file name convention for samples sets for single injections of the same sample: **name[sample set name]**, e.g. **BSA[set1]**.
* For multiple injections of the same sample: **name(injection#)[sample set name]**, e.g. **BSA(001)[set1]**.

***Generate first ASTRA template for SEC-MALS with Viscostar (from the Systems Template folder)***

Open ASTRA V (current version):

**File**

**New**

**Experiment from Template**

**System Templates**

**Viscometry**

**With Light Scattering**

**Online**

To add QELS or UV:

(right-click) **Manage**

**Configuration**

**Replace**

**Example Configurations**

**Viscometer**

**Viscostar**

**With Optilab rEX**

**With Heleos (or TREOS, EOS,…)**

with QELS:

**QELS + LS +VS + RI online**

With UV:

**UV + LS + VS + RI online**

***Note:***

When using QELS to determine the hydrodynamic radius Rh, the Template: **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.

***Generate first ASTRA template for SEC-MALS with Universal Calibration (from the Systems template folder)***

To determine the universal calibration parameters (column profile, **column + LS +VS + RI**):

**File**

**New**

**Experiment from Template**

**System Templates**

**Viscometry**

**Calibration**

**Determine UC column profile**

To run a sample using universal calibration once the column profile has been determined:

**File**

**New**

**Experiment from Template**

**System Templates**

**Viscometry**

**Universal calibration**

***Generate ASTRA template for SEC-MALS with Conventional Calibration***

To determine the conventional calibration parameters (column profile, **column + LS + RI**):

**File**

**New**

**Experiment from Template**

**System Templates**

**RI measurement**

**Determine CC column profile**

To run a sample using universal calibration once the column profile has been determined:

**File**

**New**

**Experiment from Template**

**System Templates**

**RI measurement**

**RI calibration**

***Note:***

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

**SEC-MALS Analysis Start-Up Guide**

|  |  |  |
| --- | --- | --- |
| **Action** | **References** | **Notes** |
| 1. Confirm that your instruments are connected to your computer. | LSU Manual, Chapter 3 | Use the Diagnostic manager to view your instruments in ASTRA or start a short data acquisition to test. |
| 1. Confirm that all fluid connections between your HPLC, column and instruments are set up properly and auxiliary connections and autoinject connections are made. | LSU Manual, Chapter 8 | Keep fluid connections as short as possible. Auxiliary signals can be viewed on the front panel of your Wyatt instrument. |
| 1. Check that your MALS calibration constant has been determined within the last 12 months. | LSU manual, Chapter 3, Astra user’s guide p 8-15. | The validity of the calibration constant can also be checked by confirming that the correct molar mass is obtained for the validation standard. |
| 1. Ensure that your HPLC system is clean:   Check baseline levels and noise on your 90° LS detector at flow rate with the column connected: *Aqueous (PBS):* baseline 0.009-0.02V , noise less than 100 μV, ideally less than 50 μV. *Organic (THF):* baseline 0.025-0.029V , noise less than 50 μV, ideally less than 20 μV. | LSU manual, Chapter 2 | Flush a new column overnight before connecting to your MALS setup.  If your baseline noise is too high, try to locate the source of the noise: column, mobile phase, HPLC contamination, dirty LS cell by isolating the corresponding components or replacing buffer, filters and tubing, if necessary. |
| 1. Perform a blank (buffer or solvent) injection. |  | This will identify any “ghost” peaks or contamination of your column. |
| 1. Inject validation standard:  * BSA for aqueous protein work * BSA or aqueous polymer for aqueous polymer work * 30kDa Polystyrene for organic mobile phase | LSU manual, Chapter 7 |  |
| 1. Check system constants, such as Normalization, Alignment, Band Broadening. | LSU manual, Chapter 7 |  |

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| 1. Check molar mass from your concentration detector. Use molar mass from both RI and UV, if present. |  | If the molar masses are correct, your system is ready for analysis. |
| 1. Check mass recovery |  | Prepare your validation standard in the exact concentration. Pierce BSA ampoules have a concentration of 2 mg/mL and the mass recovery across all peaks should be > 95%. |
| 1. Save your validation experiment as a template. Use this template for all future data acquisitions. | LSU manual, chapter 7, ASTRA user’s guide, Chapter 6. | Your template will contain all the correct system constants. If your template includes processing parameters, make sure to check baselines and peaks settings and adjust values for dn/dc, UV extinction coefficients, concentration and other sample parameters, if necessary! |
| 1. Periodically check your HPLC-MALS performance with your validation standard. |  | A weekly interval is recommended for medium throughput labs, more checks may be appropriate for higher use.  A check is also recommended if anything in the hardware setup was changed or the system has not been in use for a while. |

**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.