Protocol for Batch *dn/dc*

Overview

dn/dc – differential refractive index increment (the change in refractive index per the change in concentration) is used in the Rayleigh ratio equation to determine MW. The dn/dc is unique for each analyte and solvent combination.
Batch – No column, direct injections to the Optilab RI detector

HPLC Pump WISH injector Optilab RI Waste

***The outlet of the WISH will go directly to the inlet of the Optilab.** Solvent flow must never be reversed through the Optilab.

Flow from the HPLC will bypass the autosampler and UV detector. Use a hand-tight fitting, 0.17 mm (green) stainless steel capillary from the purge valve of HPLC to the Wyatt high-pressure injection system (WISH). **Always make wet connections to instruments to limit air bubbles**. Allow 0.1 mL/min flow to establish at the end of each capillary before plumbing to any instrument.

Materials/Supplies

PPE: Several sets of nitrile gloves and safety glasses to be worn at all times

Mobile phase: minimum 300 mL (Sample prep and blanks 100 mL, purging and analysis 100 mL, excess in bottle 100 mL), HPLC grade

Test tubes: 7 test tubes (10+ mL)

Syringes: 14 plastic Henke-Ject syringes with luer lock (53548-003) for duplicate analysis or a single 3 mL glass syringe (**20 gauge blunt needle only**)

Syringe filters: 14 PTFE filters for THF analysis; nylon filters are acceptable for most other solvents.

Sample: 55 mg estimated for 10 mL sample prep

System storage solvent: 50 mL of IPA (HPLC grade) to store instruments

System prep checklist:

- Rinse solvent bottle – Depending on bottle size, use a small fraction of total volume for each of the following rinses: 3x Millipore water, 3x methanol (HPLC grade), 3x acetone (HPLC grade), 3x mobile phase (HPLC grade). The last solvent before your mobile phase must be miscible with the mobile phase.

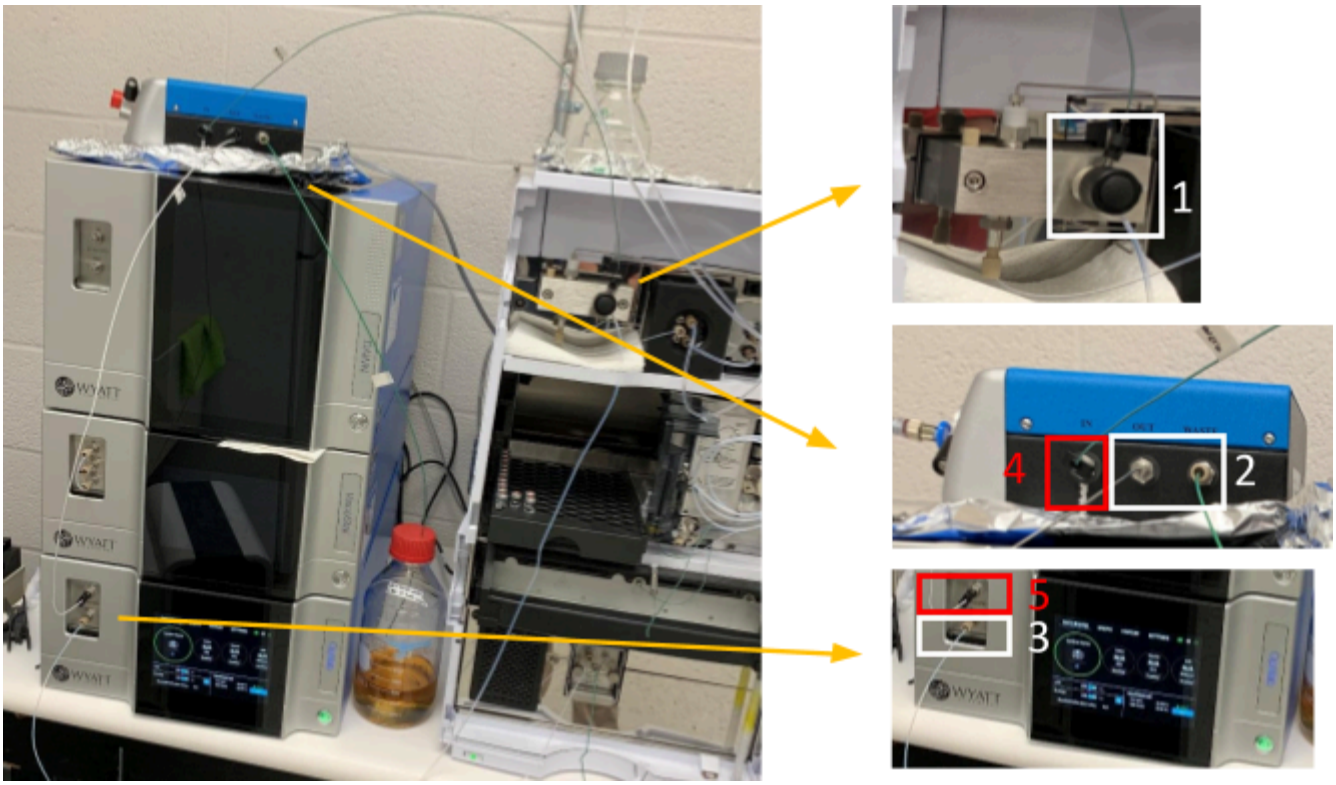
- Add solvent bottle to system – Be sure to set aside 100+ mL of mobile phase solvent for sample preparation. THF will always be run in line “b.” IPA or 50% organic solvent like ACN is the system storage solvent and will be in line “a.” Carefully remove the tubing and filter from the previous

solvent to not create any bubbles in the line, and gently wipe the tubing and blot the filter to remove excess solvent. Then add to your new solvent bottle and close the lid. Be sure the filter intake is completely submerged in the mobile phase.

- Power on – Press the power button on the Optilab and HPLC system in the lower right corner. Open HPLC Connect software from the PC's system icon tray. Once the software connects, open the HPLC dashboard by right-clicking the icon in the tray and turn on the pump in the software's "pump commands."

- Prime the HPLC pump – open the black knob on the HPLC system. Twist the knob several turns until it is loose. It is under the external cover and will need to be removed if it is on. In HPLC Connect, turn the flow to 5 mL/min and the gradient to 1 mL/min² and allow 5 min of purging to clear all the tubing of any air or alternative solvents. Make sure the correct solvent is selected by inputting 100% as line 1 or 2. With the purge valve open and the pump on, flow should be visible in the alternate path to waste under the black purge knob. After 5 minutes, turn the flow to 0. After reaching no flow, close the purge valve.

- Prepare tubing – connect the tubing as shown below, but create wet fittings, so make sure solvent is flowing out of each tubing before connecting it to the next instrument. Start by connecting the outlets only (labelled as 1, 2, 3). Starting with the HPLC pump at number 1 with the green stainless-steel tubing and hand-tight fittings. Do not plumb the end of tubing 1. Next move to number 2, the outlets from the WISH injector. Connect the long white stainless-steel tubing with hand tight fittings to the "out" and do not plumb the end of this tubing. The waste line should be a wide, peek tubing that either runs to an individual waste bottle as pictured below or to the main waste bottle under the HPLC instrument. Now number 3, the Optilab waste must be a wide tubing to limit back pressure and will run directly to the main waste under the instrument. Connections 4 and 5 begin in the next step.



- Plumb fittings – Turn the flow to 0.1 mL/min with a gradient of 0.1 mL/min[2]. The mobile phase will now flow through your tubing. With Kim wipe in hand, allow several seconds of flow to purge through tubing 1 and once no air bubbles are breaking the flow, plumb the tubing to the WISH inlet (4) (HPLC valve WISH inlet). Repeat this step as you plumb the outlet of WISH, number 2 (not the waste), to number 5 or the Optilab inlet. **Never reverse flow through the Optilab outlet**. Once the connections are completed, increase the flow to 0.3 mL/min for analysis. Monitor all connections for several minutes to check for any leaks. Use a Kimwipe at all connections to detect any solvent.

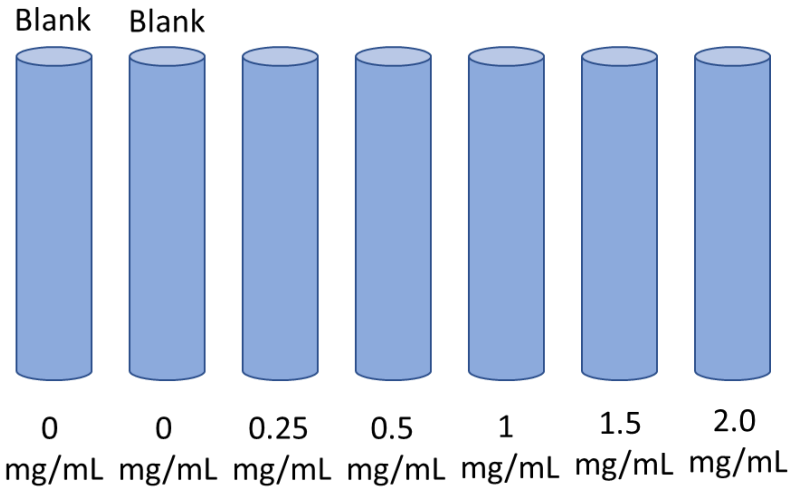
- Purge system – the Optilab instrument will be purged in roughly 1-2 hours, depending on the mobile phase. After all connections are established, on the front screen of the Optilab "Dashboard", turn on the purge while the solvent flows until the health indicator noise is green or close to 10^{-9} power.

Sample Preparation

- Prepare Samples – it is *extremely important to dissolve samples in the exact same solvent* as the mobile phase, so you must draw solvent for your sample prep directly from your mobile phase bottle if you did not set some aside earlier.

Many GPC solvents are harsh chemicals that can dissolve plastics and cause bodily injury or damage to instruments. Be sure to understand your chemicals and their compatibility and miscibility with solvents in the system. *Do not mix immiscible solvents inside the instruments!*

- Samples and solvents should be weighed, and the concentration should be calculated by density. If volatile solvents are used, a single sample (stock) can be prepared at a time, and each following sample can be a single-step dilution from the stock. Do not use serial dilution; this will introduce more errors.
- Each dn/dc plot should contain a minimum of **5 concentrations** with duplicate analysis at ~2-3 mL injections. Thus, 8-10 mL of each sample may be enough for two sets of analysis, and maybe an extra injection for any errors.
- Set aside a minimum of 70 mL of mobile phase to prepare your 5 samples as well as 2 blanks. Samples should be prepared in a range of 0.2-2 mg/mL.



Setup of Method in Software

The following section contains steps inside the ASTRA software to create a method for analysis

- Batch *dn/dc* – open Astra from the desktop. In ASTRA, open a new experiment from the method. Click File → New → Experiment from Method. Navigate to System → RI measurement and click on batch determine dn/dc
- Configuration – click on the configuration tab and open the "plus" sign, and click on the "Optilab" inside the software. Make sure the physical instrument matches the instrument in the drop-down menu. The instrument calibration constant may need to be added in this menu which can be found on the display screen of the Optilab under the "settings" tab. The wavelength needs to be changed to 785 nm. Be sure to hit "apply" in the bottom to save changes at each step.

- Solvent and Sample – These parameters can be adjusted after analysis, but can be added to the method now. Click the plus sign on the Optilab tab and input the solvent as the mobile phase. Then do the same for the sample. The sample can be named however you like, and you can adjust its dn/dc value in this tab

- Basic Collection – open the basic collection tab and adjust the collection time to at least 80 min or more. The collection will stop at this time if you are not done. Better to extend it longer and press stop when you are finished.

- Run – After adjusting parameters as you like, you can right-click the experiment name and save this as a method for future use, or you can press run to begin analysis. After pressing run, it will ask if it is okay to begin or fail if it cannot establish connections to instruments.

Injections (measurements)

- Needle – specific needles are required for the WISH injector with a blunt tip. Do not use a regular syringe with a sharp needle or without a luer lock tip

- Syringe – 3 mL syringes work well and can be glass or Henke-Ject syringes without any rubber plunger (53548-003)

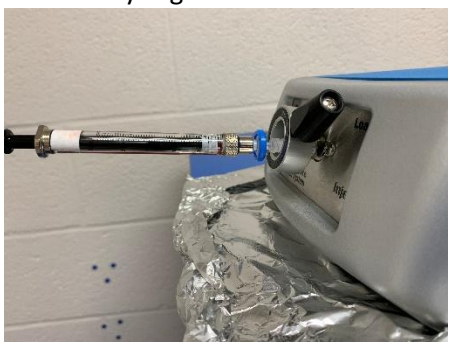
- Filters – Syringe filters are required for each injection and should be between 0.02-0.45 µm pore size. THF requires PTFE filters, while other solvents like DMF and DMSO are compatible with nylon filters.

- Injection – after the system has been under stable flow and the Optilab is purged with green health indicators, injection can take place. Be sure the WISH injector is in load position and fill the sample loop with a full syringe of mobile phase. This is to clean the sample loop. Now, refill a syringe with mobile phase and fill the loop again to begin a blank injection and switch WISH to inject. The blank injection will be followed by low to high concentrations of samples and a repeat of a blank injection at the end.

Note: Air bubbles will diminish results and need to be minimized at all costs.

- **STEPS TO INJECTION**
    1. Fill the syringe with ~2.5 mL and then fill with an excess of air. Remove the needle and add a syringe filter. Replace the needle and purge all air out of the syringe, forcing any bubbles out of the needle.
    2. After removing all air, carefully push the needle of the syringe into the WISH injection port until it stops naturally. (Be sure the correct blunt needle is attached)
    3. Slowly begin to depress the plunger at a consistent flow rate, but not creating too much back pressure. As the plunger approaches completion, stop short of completing the full injection to prevent any air into the system.

4. Let the syringe rest in the system and do not remove it until the sample loop is cleared, as seen below. After filling the sample loop, let the solution equilibrate for about 15 seconds and switch the WISH injector to “inject” position while holding the top of the injector down to not jostle the system. Any air bubble at the base of the plunger should be left in the barrel of the syringe.



5. Once a plateau is seen in the detector, the WISH can be switched back to load position and the syringe can be removed. The next sample can then be prepared. Wait for the detector to return to baseline before injecting the next sample.

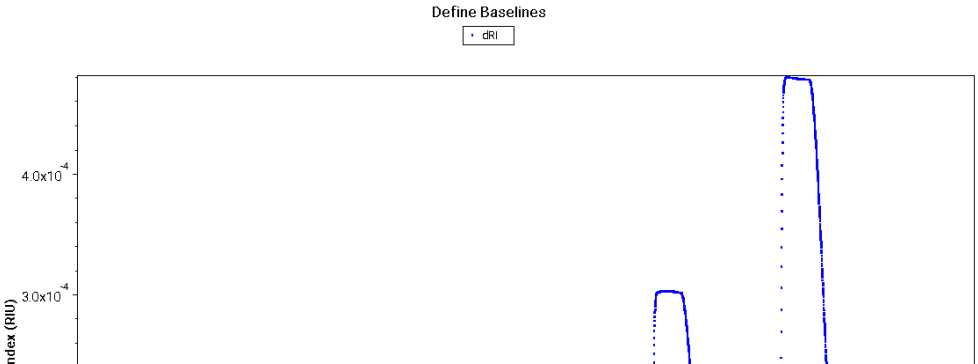
- Analysis

1. Injection order should be blank, followed by samples from low to high concentration, followed by another blank.
2. Once a plateau is generated by your sample or blank flowing through the system (a couple of minutes), the WISH injector can be switched back to “load” and the syringe can be removed from the system.
3. The syringe filter should be removed to waste, and the glass syringe can be rinsed. Repeat the injection steps for the rest of the samples.
4. A blank needs to be injected again after all samples have been injected.

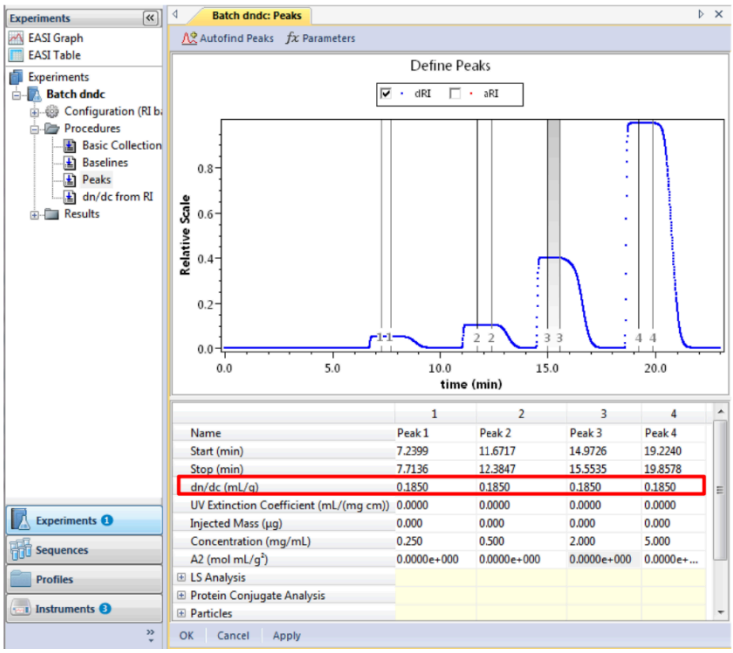
- Stop – after all injections have been completed, press stop in the top toolbar. If analysis is done for the day and will continue tomorrow, you can lower the flow rate to 0.03 mL/min or begin purging the system with the storage solvent IPA.

ASTRA Data Analysis

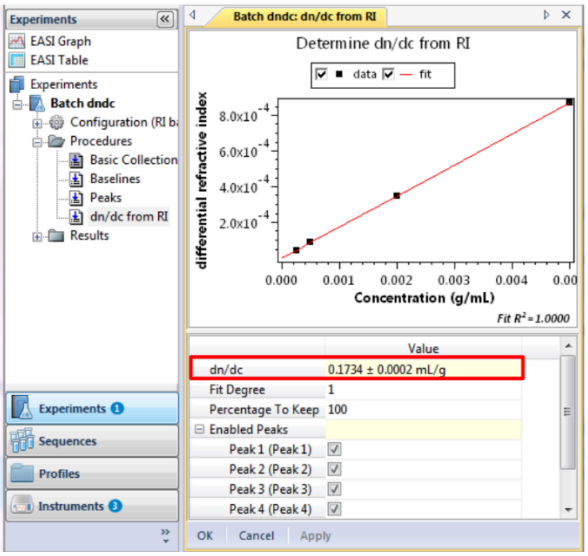
- Baseline – open the baseline tab in the experiment and click and drag between the two blank injections to set the baseline to include all of your peaks, and press apply in the bottom left.



- Peaks – Highlight the plateaus of your samples. In the table below, be sure to add the correct concentrations of each peak highlighted.



- *dn/dc* from RI – Double click on the dn/dc from RI procedure to display the differential refractive index versus concentration plot. The table below the graph displays the dn/dc value. If you want to eliminate a peak from the dn/dc calculation, it can be deselected by clicking the "+" sign next to Enabled Peaks and unchecking the unwanted peak.



- Results – the results tab will generate a report with all determined values and the concentrations used for each peak.