Calibration and Normalization of MALS Detectors

Summary

This technical note describes the calibration and normalization procedures necessary to performing multi-angle light scattering (MALS) measurements. This note will cover why each procedure is necessary and when to perform them.

Related Technical Notes and References

M1000	ASTRA 6 User's Guide
M3000	miniDAWN TREOS User's Guide
M3200	DAWN HELEOS II User's Guide
M3500	µDAWN User's Guide
TN1007	ASTRA 6 Quick Guide
TN1010	Correcting for Absorbance at the Laser Wavelength
TN3001	Batch Light Scattering Measurements (Zimm Plots)
TN3002	Batch Light Scattering Measurements (Debye Plots)
TN3003	Measuring Molar Mass for Fluorescing Samples

Contents

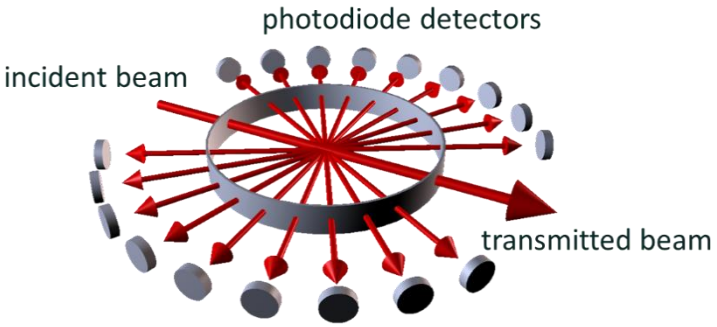

Figure 1: Schematic of the Multi-Angle Light Scattering experimental setup.

Background

Calibration

The Rayleigh ratio R_θ is the ratio of the scattered to incident light intensity, corrected for the size of the scattering volume and the distance of the detector from the scattering volume.

$$R_\theta = \frac{I_\theta r^2}{I_0 V} \quad (1)$$

Where I_θ is the scattered intensity at angle θ, I_0 is the incident intensity, r is the distance from the scattering volume to the detector, V is the illuminated volume of the scattering medium from which the detector at θ collects light. The schematic of the light scattering setup inside the Wyatt HELEOS and TREOS MALS detectors is shown in Figure 1.

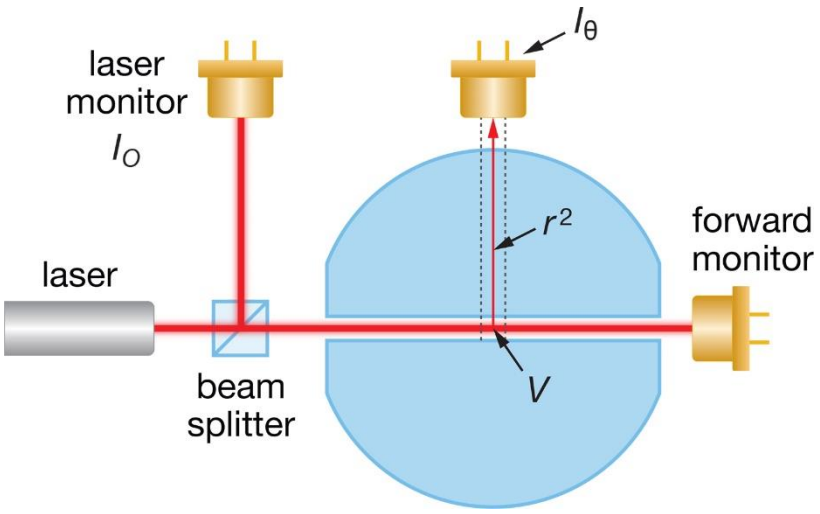

Figure 2: Schematic of the flow cell inside the TREOS and HELEOS MALS detectors. The laser's incident intensity is determined by the laser monitor before the beam enters the flow cell. The laser beam then progorgates through the flow cell bore. The scattered light detected in the horizontal plane propogates through the flow cell glass to the photodetectors. The remaining light that is unscattered reaches the forward monitor. The forward monitor reading is only used for diagnostic purposes or in the case of samples that absorb at the laser wavelength.

Photodetectors used in MALS instruments generate electrical voltages that are proportional to the intensity of light scattered from the sample. In order to relate the electrical signal to the Rayleigh ratio, a calibration constant must be determined for the detectors. The simplest and most robust calibration procedure utilizes scattering from a known scatterer at 90° to the incident light beam. The proportionality between the Rayleigh ratio R_{90} and the detector voltage is described in Eq. (2) and is used to derive a calibration constant.

$$R_{90^\circ} = C_c \frac{V_{90^\circ} - V_{90^\circ,dark}}{V_{laser} - V_{laser,dark}} \quad (2)$$

where C_c is a calibration constant, V_{90} is the voltage reading of the 90° detector, V_{laser} is the voltage reading of the laser monitor, $V_{90,dark}$ is the voltage of the 90° detector when the laser power is off, $V_{laser,dark}$ is the voltage of the laser monitor when the laser power is off.

Several common solvents have been thoroughly studied, and their Rayleigh ratios are well known. This allows us to use a pure solvent as the calibration standard. Using pure solvent as the scattering standard makes the calibration completely independent of any particular sample. Toluene has the highest Rayleigh ratio of any of the common solvents. In addition, high-purity, HPLC-grade toluene is readily available. This makes toluene both suitable and desirable for use as a calibration standard for MALS instruments. Once the sample cell is filled with toluene, the ASTRA software can measure the voltage of the 90˚ detector with the laser on and off and can calculate a calibration constant based on the known Rayleigh ratio for toluene.

Normalization

Each detector has its own geometrical factors, scattering volume, and angular sensitivity to measured light intensity. We must correct for these factors to measure the true angular dependence of intensity for a given sample. Furthermore, these effects vary from solvent to solvent. The refractive index of the solvent changes the scattering angles due to refraction at the glass-solvent interface and the geometrical factors for each detector.

To account for these effects, normalization coefficients must be calculated to relate the scattering at each of the detectors to the 90˚ detector. These coefficients must be determined using the same temperature and the same solvent that are used for the sample measurement. To determine the normalization coefficients, the detector voltages are measured for an isotropic scatterer (sample with RMS radius <10 nm) prepared in the same solvent as the sample. Isotropically scattering molecules scatter equally in all directions, therefore, variations in the scattered intensity between detectors are due to the geometry of the flow cell. However, the Rayleigh ratio is the same at all angles for an isotropic scatterer. Since the Rayleigh ratio has been calculated for the 90˚ detector, it is therefore known at all other angles and a normalization coefficient relating the Rayleigh ratio and the voltage detector at that angle is calculated. The normalization coefficient at 90˚ is set to 1 and a normalization coefficient can be calculated for all other angles as given by Eq (3). Equation 3 describes the excess Rayleigh scattering in which scattering from the solvent is subtracted from the total scattered intensity to obtain the scattering from the sample itself.

$$R_{90^\circ} = R_\theta = N_\theta C_c \frac{V_\theta - V_{\theta,baseline}}{V_{laser} - V_{laser,dark}} \quad (3)$$

Where N_θ is the normalization coefficient at angle θ, V_θ is the voltage reading of the normalization standard at the θ angle detector, $V_{\theta,baseline}$ is the voltage reading of the solvent at the θ angle detector, V_{laser} is the voltage reading of the laser monitor, $V_{laser,dark}$ is the voltage of the laser monitor when the laser power is off.

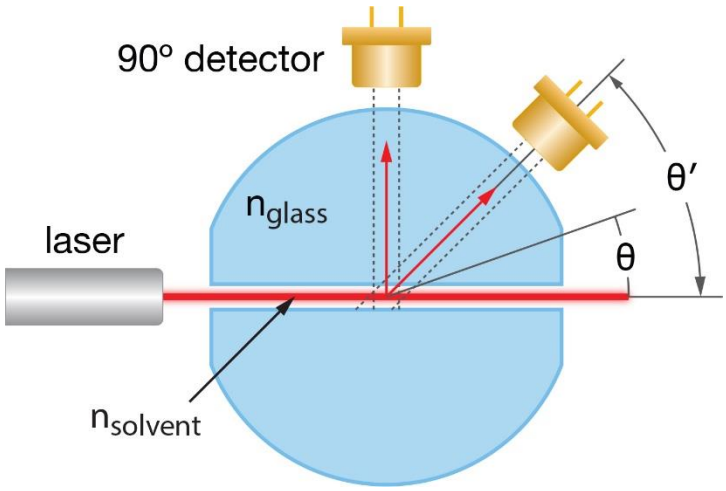

Figure 3: Schematic of flow cell in the TREOS and HELEOS MALS detectors. Normalization relates each of the detectors to the 90° detector by correcting for the different scattering volumes and the refraction at the glass-solvent interface. The light reaching the detector positioned at angle θ′ is scattered at angle θ but due to refraction at the solvent-glass interface is collected at angle θ′.

Note: The µDAWN flow cell design is such that the laser beam is aligned perpendicular to the flow cell bore and the sample flow direction. The scattering angle does not depend on the solvent refractive index; the scattering angles are fixed at the detector angle positions.

When to calibrate or normalize the MALS detector

Event	Should I calibrate the MALS detector?	Should I normalize the MALS detector?	Notes
I've used my detector for 12 months.	✓ Yes	✓ Yes	• Verify new calibration constants. • Run a validation standard.
I've cleaned (disassembled) my flow cell.	✓ Yes	✓ Yes	
I've changed my mobile phase.	× No	✓ Yes	Check the solvent RI value, then re-normalize (& obtain new dn/dc) only if a significant change in RI is seen.
My sample absorbs light at the MALS laser wavelength.	× No	× No	Set "Divide by Monitor" to "Forward Monitor" in the MALS configuration window to correct for absorption.
My sample fluoresces light at the MALS laser wavelength.	Maybe*	Maybe*	*Adding Interference filters may reduce the effect of emitted fluorescent light, but also might require re-calibration and/or re-normalization.
I've moved my detector across the lab.	× No	× No	
I've changed or replaced tubing between detectors.	× No	× No	
I've replaced my SEC column or FFF membrane.	× No	× No	

Calibration and Normalization in ASTRA

Calibration

Different sample cells such as a microcuvette, scintillation vial or a flow cell have different calibration constants. In calculating the calibration constant, ASTRA takes into account the type of sample cell used. Ensure that the correct sample cell is selected in the MALS detector configuration window (Figure 4) when setting up a calibration experiment.

Note: Please consult the *ASTRA User's Guide Editing Procedures Chapter* and the *TREOS, HELEOS II, or µDAWN User's Guide* on how to set up a calibration experiment in ASTRA and prepare the sample cell in toluene for a calibration measurement. For batch MALS measurements using the microcuvette or scintillation vial, consult the *DAWN HELEOS II User's Guide Off-Line Measurements Chapter* for information about the calibration procedure.

If your sample cell does not contain a solvent miscible with toluene, it is critical to flush with miscible solvents until toluene is present. Typically, this involves flushing in the following order: **Aqueous mobile phase** > **filtered water** > **100% filtered alcohol** > **filtered toluene**.

The calibration experiment will record the voltages of the 90˚ detector for 30 seconds with the laser set to 100% power and then will record the detector voltages for 30 seconds with the laser power set to 0% to determine the dark counts (the voltage of the detector when no light is reaching the detector). Once the collection has been completed, to view the measured calibration constant, double click on the **LS Calibration** procedure (Figure 5) or the **Report (summary)** (Figure 6).

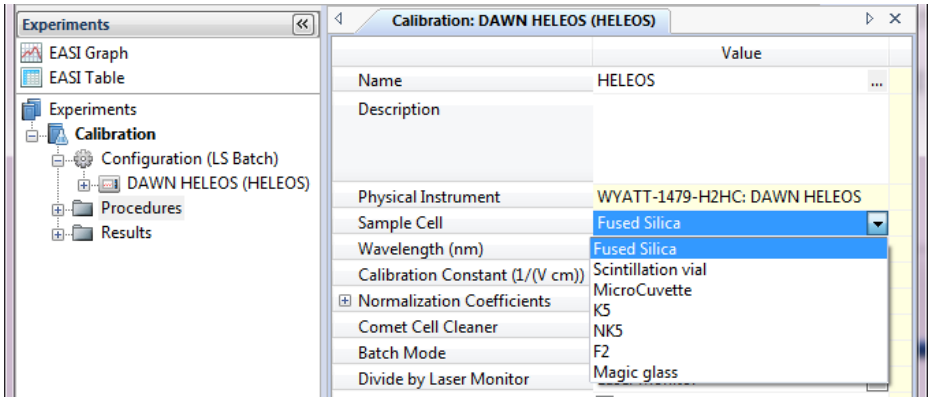

Figure 4: Screenshot of the MALS configuration window in ASTRA. Select the correct Sample Cell when calibrating.

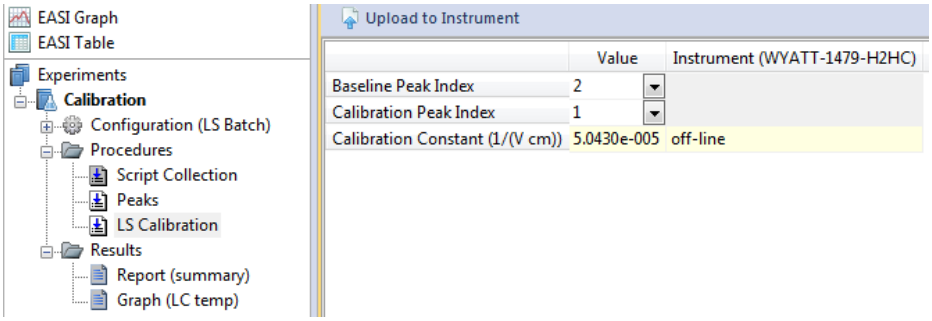

Figure 5: Screenshot of LS Calibration procedure in ASTRA.

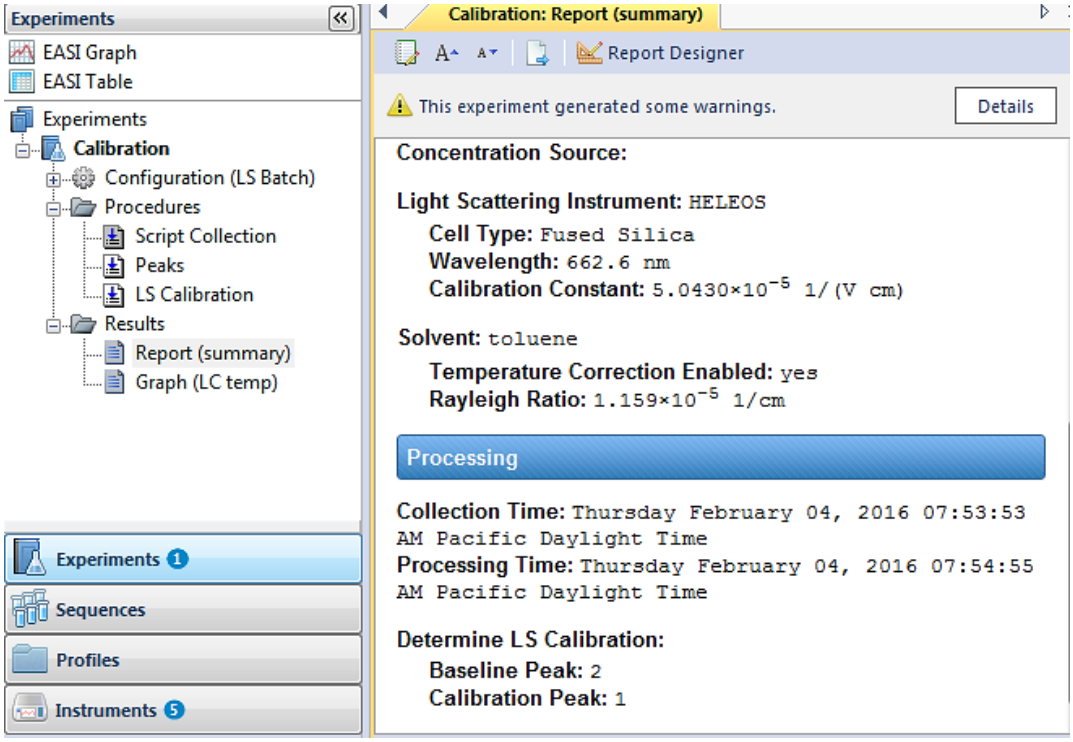

Figure 6: Screenshot of the Results Report with the measured calibration constant.

Normalization

The normalization coefficients are calculated from the LS signal measured from an isotropic standard that is less than 10 nm in RMS radius. The normalization standard should be prepared in the same solvent as your mobile phase. For batch LS measurements, typically a 10 kDa dextran sample is used for aqueous solvents and a 30 kDa polystyrene sample is used for organic solvents. To set the alignment and band broadening parameters for SEC-MALS experiments as well as the normalization coefficients, the standard should also be monodisperse. Typically, a BSA monomer peak is the standard used for an aqueous system and a 30kDa polystyrene is the standard used for an organic system.

Note: Refer to *TN3001 Batch Light Scattering Measurements (Zimm Plots)* and *TN3002 Batch Light Scattering Measurements (Debye Plots)* for more information about batch MALS experiments and *TN1007 ASTRA 6 Quick Guide* for processing SEC-MALS experiments.

There are two procedures to setting the normalization coefficients in an ASTRA experiment:

1. Navigate to **Experiment** → **Configuration** → **Normalize**. Select the peak that corresponds to the normalization standard and click **Normalize**.

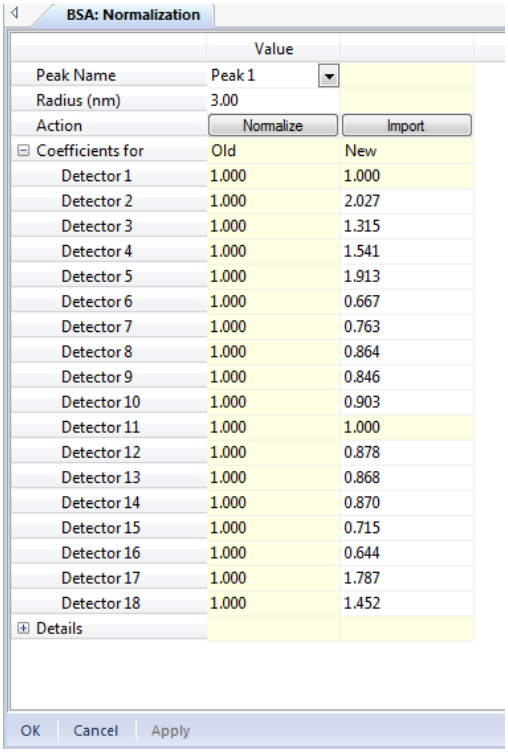

Figure 7: The normalization coefficients are set for all detectors using the selected peak (detectors from a HELEOS are shown in this figure). Detector 1 is unavailable when the flow cell is used for measurements. Detector 11 is the 90˚ detector and therefore the normalization coefficient is always set to 1.

2. Alternatively, normalization coefficients can be imported post-collection from another experiment. The imported normalization coefficients must have been determined using a standard prepared in the same solvent as the current sample of interest. To import the normalization coefficients:
    a. Open the experiment from which the normalization coefficients will be imported and the experiment with the newly collected sample data.
    b. Navigate to **Experiment → Configuration → Normalize**. Click on the **Import** button.
    c. In the **Import Normalization From** window, select the desired file and click **Import**.
    d. The imported values will populate the **New** column. Click **Apply**.

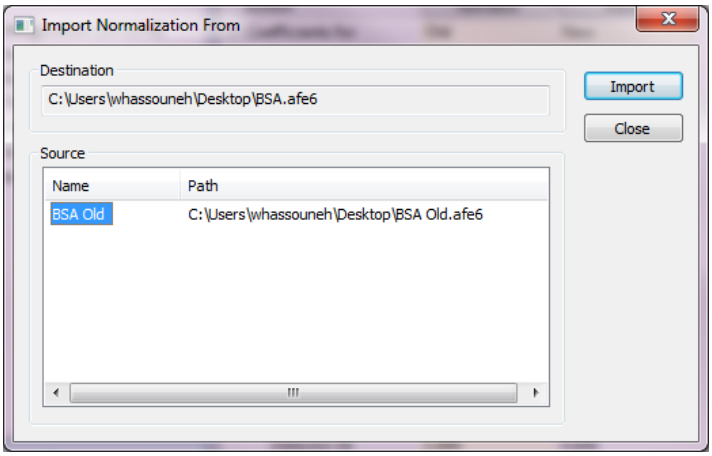

Figure 8: Screenshot of the Import Normalization From window in ASTRA which allows users to import the normalization coeffieicients from one experiment to the other.

To demonstrate the effect of normalization on the LS signals, Figure 9 shows the Rayleigh ratios calculated for two samples, an isotropic and an anisotropic scatterer, before and after normalization. Before normalization, the Rayleigh ratios measured for both the isotropic scatterer and anisotropic samples display an irregular angular dependence. After normalization, the effects of the scattering volume and flow cell geometry are accounted for. For the isotropic sample, the Rayleigh ratios at all angles are equal and no angular dependence is observed. For an anisotropic scatterer, the Rayleigh ratio decreases with increasing angle. The change in the Rayleigh ratio for the anisotropic sample after the correction is due to properties of the sample only and the RMS radius can be calculated accurately from the angular dependence of the scattered intensity.

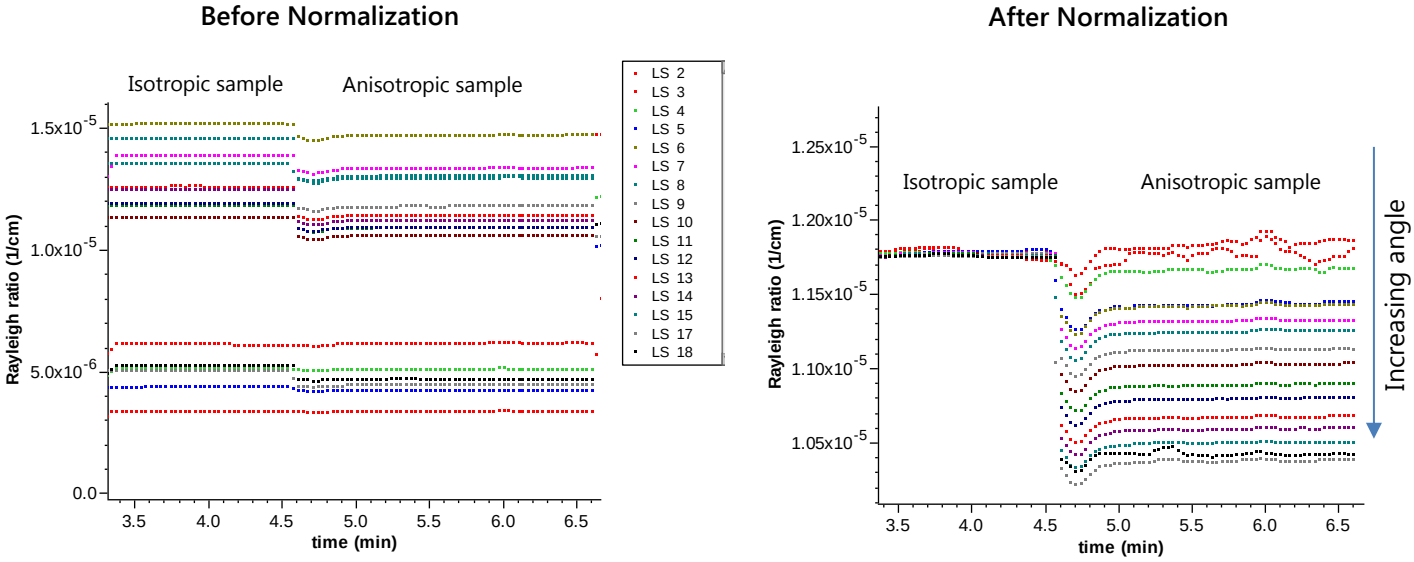

Figure 9: Plot of the Rayleigh ratio at multiple scattering angles before normalization (left) and after normalization (right) for two samples, an isotropic scatterer and an anisotropic scatterer (detectors from a HELEOS instrument are shown in this figure).

Summary

Calibration and normalization are two processes necessary for static light scattering measurements. The calibration procedure calculates a calibration constant that relates the measured voltages by the photodetectors to the Rayleigh scattering ratio. A calibration constant is calculated based on the voltage of the detector at 90˚ using toluene as a standard. The normalization procedure relates the detectors at all other angles to the 90˚ detector. Normalization coefficients are calculated from the scattering of an isotropic scatterer (<10 nm in RMS radius). The normalization coefficients account for the difference in the scattering volumes that the detectors see and the effect of the flow cell geometry. Once the signal from the detectors is normalized, the observed angular dependence of the scattered intensity is a result of the sample properties only allowing for the calculation of the RMS radius.