1. Introduction

A parameter that is becoming increasingly studied with respect to predicting biopharmaceutical behaviour in formulation is the second virial coefficient (A_2, also known as B_{22}). Zetasizer Software Version 7.11 includes a macro which allows automatic determination of this value, as well as molecular weight, when employing the Zetasizer APS. For further information please refer to the Malvern Application note "Using A_2 and kD to assess protein interactions in formulations" accessed from the Resource Center.

This technical note details the practical aspects of the measurement procedure. In summary, the APS macro automatically measures a series of sample concentrations (highest to lowest), before measurement of the background solvent and scattering standard. This provides a value for A_2 and Mw.

2. Sample Preparation and Well-Plate Set-Up

2.1. Scattering Standard – 1M Arginine Hydrochloride

In SLS measurements, toluene is routinely used as the scattering standard due to its well-known optical properties however, this is not suitable for aqueous solutions. An alternative scattering standard has been identified, 1 M arginine hydrochloride, which acts as a scattering transfer standard with a Rayleigh Ratio of 2.79E-06 cm^{-1}.

2.2. Well-plate Set-Up

A SLS measurement involves preparation of a sample at a series of concentrations in selected buffer. For the APS SLS macro, the sample concentrations are analysed first (from highest to lowest concentration), then a blank buffer, then the scattering standard. The attenuator level is set by the initial sample and remains constant for all subsequent samples, including the solvent and scattering standard. For each concentration the intensity is recorded as the raw measurement data. This is converted into the KC/R factor using a combination of the values entered into the macro and measurements of the solvents and scattering standard intensities. The calculated KC/R value is plotted against concentration to produce a Debye plot for each concentration series, from which the molecular weight and A_2 values are derived.

The macro measures each concentration series by column. Therefore all samples in a series are loaded into the same column on the well plate. The highest concentration is in the first row to ensure the correct attenuation level is set. Remaining samples are loaded in order of decreasing concentration, followed by the blank buffer, and finally, the scattering standard. On a wellplate, numbers correspond to columns and letters to rows; so for two samples, X and Y, with nominal concentrations of 5, 4, 3, 2, and 1 in buffer, the loading sequence would be:

	1	*2*	*3*
A	**X 5**	**Y 5**	
B	**X 4**	**Y 4**	
C	**X 3**	**Y 3**	
D	**X 2**	**Y 2**	
E	**X 1**	**Y 1**	
F	**Sample Buffer**	**Sample Buffer**	
G	**Scattering Standard**	**Scattering Standard**	
H			

Figure 1. Sequence of loading on the well plate

Standard 96 or 384 wellplates can be used. It is recommended to remove any dust contaminants from the wells using an air duster before sampling loading. As many concentrations as can fit onto a single column (allowing for buffer and standard) can be used, as long as they remain in concentration order with the blank and scattering standard at the bottom. Each concentration series requires a separate column, therefore the number of concentration series that can be measured in a single run is limited to the number of columns in the wellplate (i.e. 8 series for 96 wellplate; 24 series for 384 wellplate). For a particular run, each concentration series needs to have the same *number* of sample concentrations, although the *value* of these concentrations can differ.

Please note that well A1 represents the top left corner of the plate as read by the APS macro, with letters corresponding to rows and numbers corresponding to column.

During the molecular weight measurement, 20 µl of sample is used to pre-flush the system and prevent dilution of the sample. An additional 20 µl aliquot is then used for the SLS measurement. The recommended volume of sample to load into the wellplate is therefore an additional 20 µl to that recommended for a size measurement. For a 96 wellplate, 70 µl is recommended for each well; for 384 wellplates, 50 µl of sample is recommended. Once all samples are loaded, the plate should be covered with a thin film of plastic to prevent evaporation or dust contamination.

2.3. APS SLS Macro Set-Up

The macro can be launched from the Zetasizer Software (Version 7.11 onwards) - Tools>Macros>APS Auto SLS. This will cause the plate holder of the APS to eject and the dialog box in Figure 2 to appear onscreen. Please note that the default values for the Standard (circled in Figure 2) need to be altered to reflect those for 1M arginine hydrochloride. Further details regarding each parameter of the macro dialog box can be seen in Table 1.

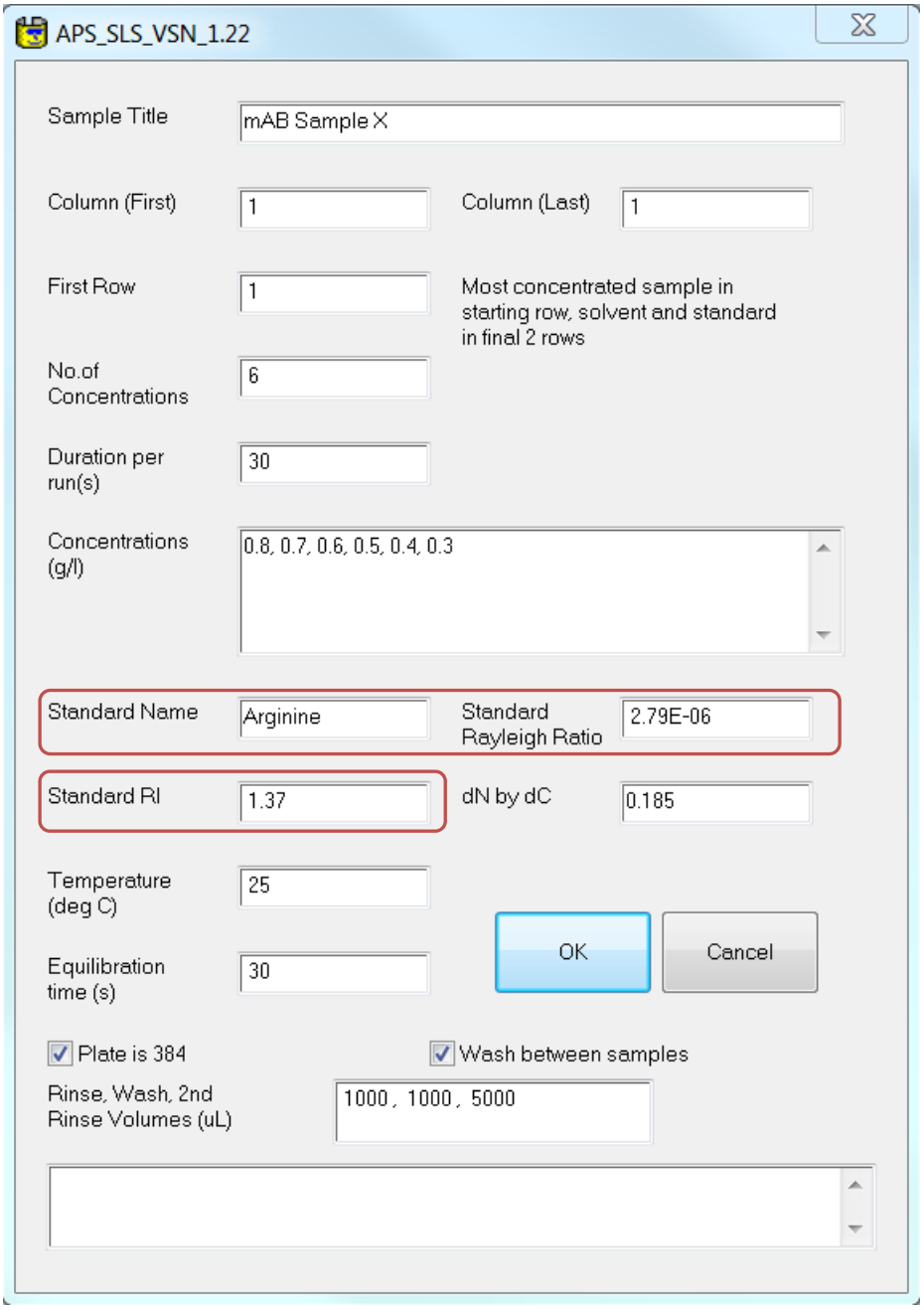


Figure 2 – The APS SLS Macro Dialog. Values for the scattering standard that require changing are circled in red.

Once the macro is set up, the 'OK' button can be clicked. The plate will retract into the APS and the measurements will begin. The macro dialog box will remain onscreen and the message box at the bottom informs the user of the current APS action. It is not currently possible to have the measurement window open concurrently.

The sample name on the record will be that typed into the 'Sample Title' box in the macro dialog and will be applied to all molecular weight records generated in each particular run. It is therefore recommended to keep a separate list of the order of the concentration series.

Parameter	Notes	Default Value	Recommended Value
Sample Title	This is the Sample Name that will be shown for all measurements in the Records View	New Sample	As relevant to your sample
Column (First) **Column (Last)**	The macro analyses each column of samples in turn, moving from left to right across the wellplate. As position A1 represents the top left of the plate, the first column to be analysed will have the smallest number	1 2	Dependent on order of loading onto the well-plate
First Row	This is the row that contains the highest concentration sample. The row letter needs to be converted to its corresponding number, so if the first row was D, the number 4 would be entered into the macro here	1	Dependent on order of loading onto the well-plate
No. of Concentrations	Enter the number of sample concentrations in each column here. For a single run all of the concentration series must have the same number of sample concentrations. This value does NOT include the scattering standard or blank solvent	6	Determined by the number of different concentrations in each series
Duration per run (s)	This is the time in which the intensity will be measured for each sample	30 s	30 s is a good starting point and can be increased or decreased if the sample is large of small, respectively
Concentrations (g/l)	Please enter your concentration values in this box, in mg/mL. If every set has the same concentration values they need only be entered once; if each set has different values all values will need to be entered	0.8, 0.7, 0.6, 0.5, 0.4, 0.3	Dependent on the samples concentrations you have made. This macro has been tested on protein concentrations from 2.0 to 30 g/L
Standard Rayleigh Ratio	This is the value obtained for 1M arginine hydrochloride	2.09E-06	2.79E-06 for 1 M arginine hydrochloride
dN by dC	The sample differential refractive index value	0.185	Commonly 0.185 for protein; this value will need to be measured for other substrates
Standard R.I.	This is the measured R.I. for 1 M arginine hydrochloride	1.35	1.37 for 1 M arginine hydrochloride
Temperature (deg C)	The temperature at which the measurement will take place	25	The temperature at which the Rayleigh Ratio for arginine hydrochloride was established was 23 °C
Equilibriation TIme	The time given for equilibration of sample in the measurement window before measurement	30 s	20 s is sufficient for samples kept at room temp. For cooled samples, 10 seconds per degree difference to the measurement temp is recommended.
"Plate is 384"	Tick if using a 384 well plate; untick if using a 96 wellplate	Box ticked	Dependent on wellplate
Wash controls (volume in µL)	Choose whether to wash between samples and other wash settings.	1000 Rinse), 1000 (Wash), 5000 (2nd rinse)	Default settings suitable for most globular proteins.

Table 1. List of configurable parameters in the APS SLS Macro Dialog Box

The settings for the macro are not saved within the software or recoverable from the Measurement Records. It is therefore recommended to make a separate note of the settings used for future reference before beginning the macro.

2.4. Data Analysis

During the course of the macro, a molecular weight measurement record will be generated for each concentration series, containing all parameters associated with a standard molecular weight measurement (e.g calculated molecular weight and A_2, Kc/R for each concentration, raw intensities measured, buffer and standard measurements). All of this data can be exported to excel for further manipulation as required.

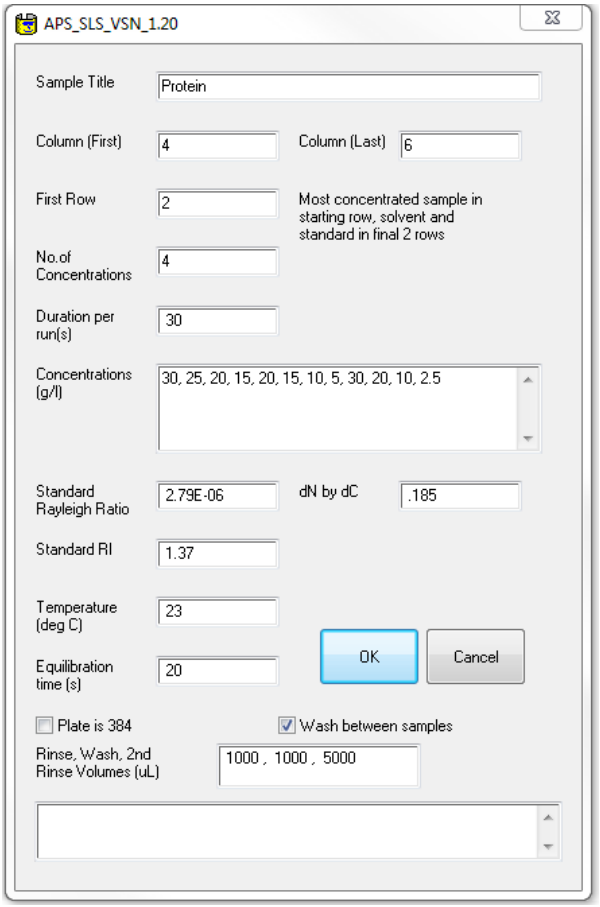
3. Example Macro Set-Up

The molecular weight and A_2 of a protein was measured in three different buffers, with four concentrations each. A 96 well-plate was used. Four concentrations were measured for each buffer.
Buffer 1 had concentrations of 30, 25, 20, and 15 mg/mL.
Buffer 2 had concentrations 20, 15, 10, 5 mg/mL.
Buffer 3 had concentrations 30, 20, 10, and 2.5 mg/mL.

Example of a correct wellplate set up (concentrations entered in cell):

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B				30	20	30						
C				25	15	20						
D				20	10	10						
E				15	5	2.5						
F				Buf 1	Buf 2	Buf 3						
G				Scat Std	Scat Std	Scat Std						
H												

Correct Macro Set-Up for wellplate above:


APS_SLS_VSN_1.20
Sample Title
Protein
Column (First)
4
Column (Last)
6
First Row
2
Most concentrated sample in starting row, solvent and standard in final 2 rows
No.of Concentrations
4
Duration per run(s)
30
Concentrations (g/l)
30, 25, 20, 15, 20, 15, 10, 5, 30, 20, 10, 2.5
Standard Rayleigh Ratio
2.79E-06
dN by dC
.185
Standard RI
1.37
Temperature (deg C)
23
Equilibration time (s)
20
OK
Cancel
Plate is 384
Wash between samples
Rinse, Wash, 2nd Rinse Volumes (uL)
1000 , 1000 , 5000