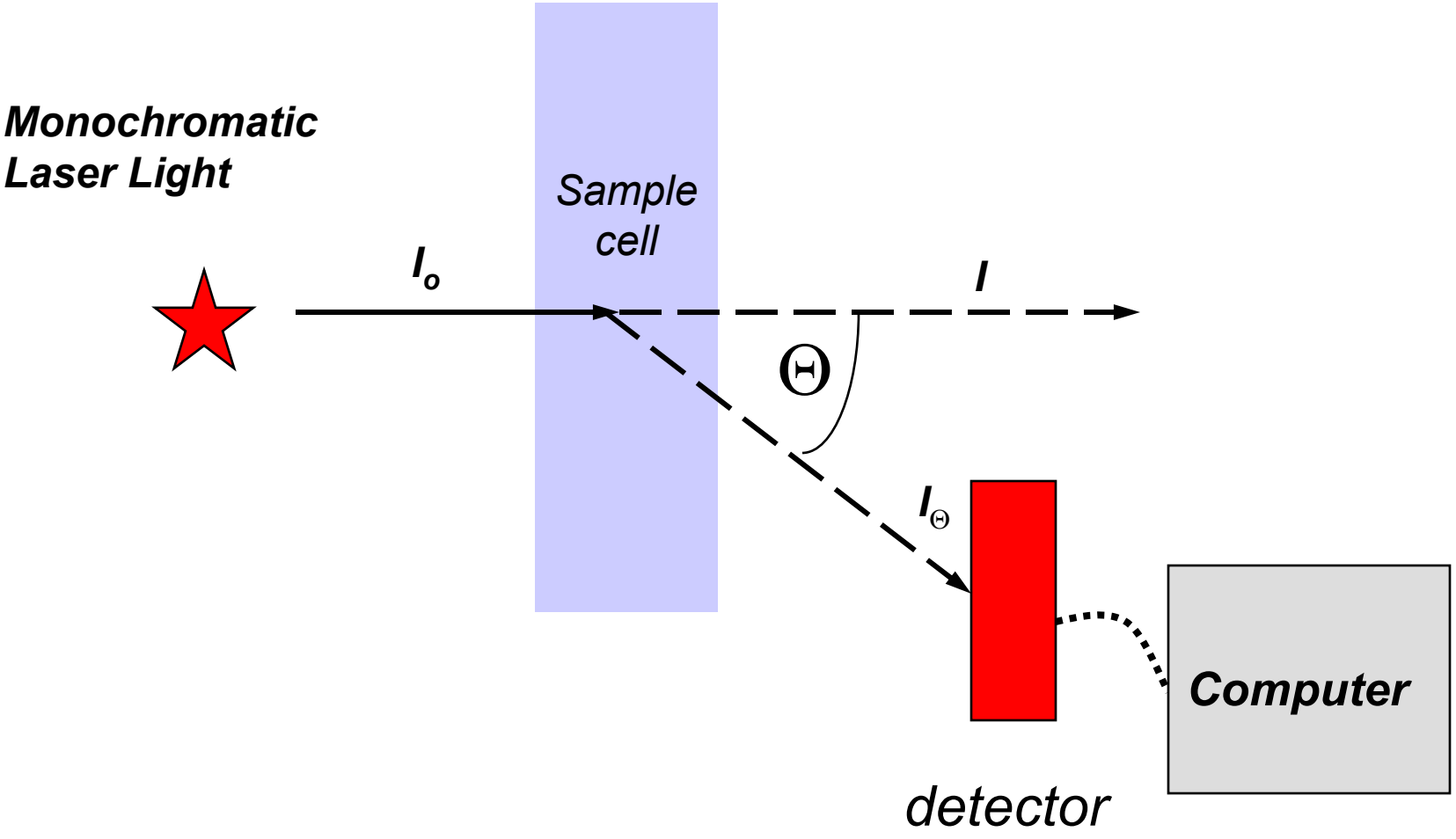
Light Scattering as a Tool for Assessing Protein Aggregates

Static and Dynamic LS

- Description of the technique
- Parameters derived from a LS measurement
- Strengths and Weaknesses illustrated by examples with emphasis on detection, quantitation and characterization of aggregates present
  - *What can it do and to what extent?*
  - *How it can be used to characterize a protein sample?*
  - *What is the analytical uncertainty?*
  - *Is the quantitation of the results straightforward and objective?*
  - *How sensitive is the technique to changes in the population?*
  - *To what extent can the technique indicate protein conformation?*
  - *What (typical) protein modifications can it detect?*
  - *Is side-by-side testing of comparator products with a reference standard beneficial or necessary?*

Light Scattering Experiments



Light Scattering Experiments

- ***Static (classical)***

  time-averaged intensity of scattered light

- ***Dynamic (quasielastic)***

  fluctuation of intensity of scattered light with time

Parameters derived:

- *Molar Mass (weight-average) accuracy ~5%*
- ($<r_g^2>^{1/2}$) root mean square radii for ($<r_g^2>^{1/2}$)> *($\lambda/20$) ~ 30 nm*

Parameters derived:

- D_T translation diffusion coefficient
- R_h *hydrodynamic* radius (Stokes radius)

  Uncertainty of ~10% for monodisperse sample

Light Scattering Experiments

- ***Static (classical)***

  time-averaged intensity of scattered light

- ***Dynamic (quasielastic)***

  fluctuation of intensity of scattered light with time

Measurements:

- ***batch mode***
- ***"in-line" mode combined with a fractionation step,***

  i.e. chromatography, mainly Size Exclusion Chromatography, Flow Field Fractionation

How it can be used to characterize a protein sample?

Static LS can easily detect aggregates

Light Scattering Signal $R(\Theta) \sim Mw*c$

because of their big Mw, aggregates scatter strongly

Angular variation of the scattered light is related to the size of the molecule

the light scattering signal from aggregates will show angular dependence, while LS signal produces by lower order oligomers like monomers, dimers et c. will not

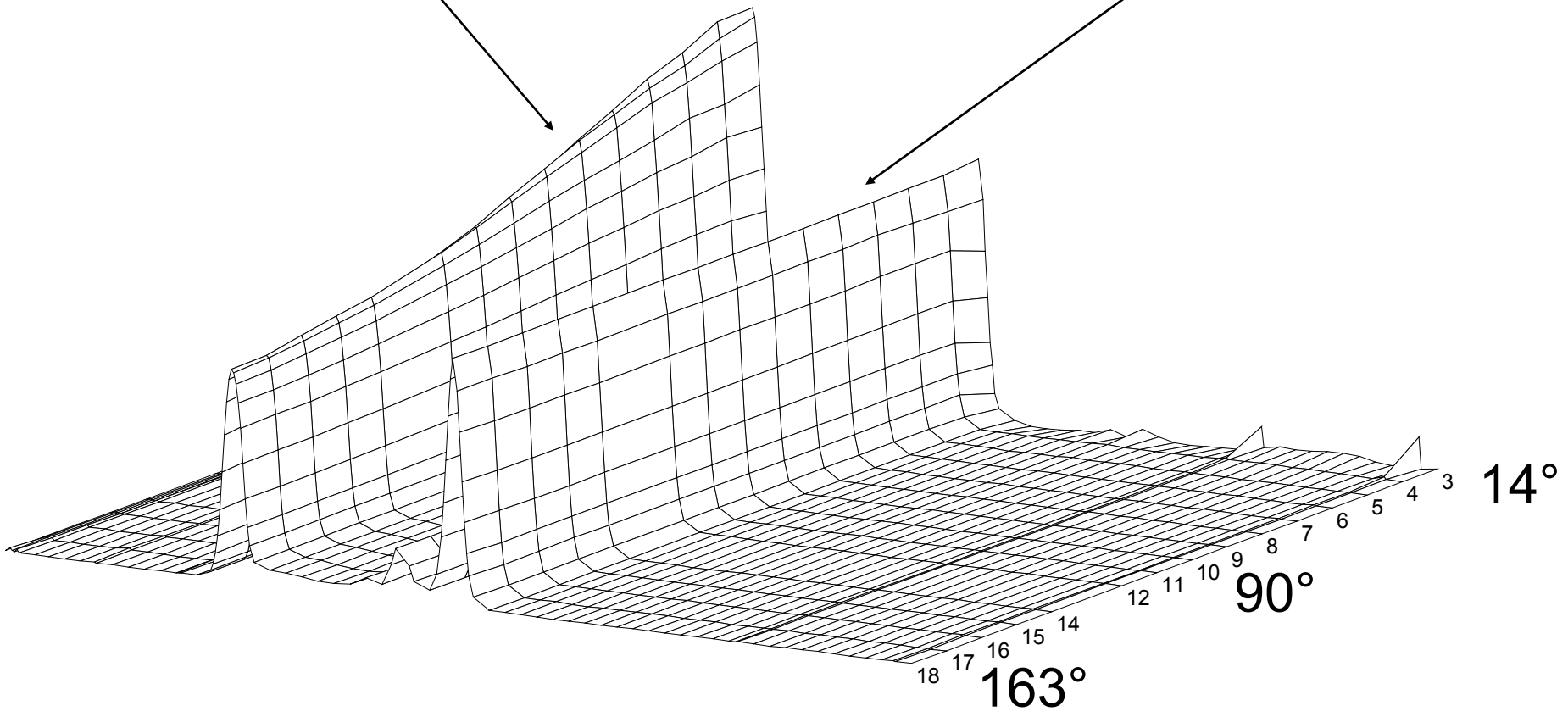
Ovalbumin 43 kDa

Aggregates

angular dependence of scattered light

Lower order oligomers

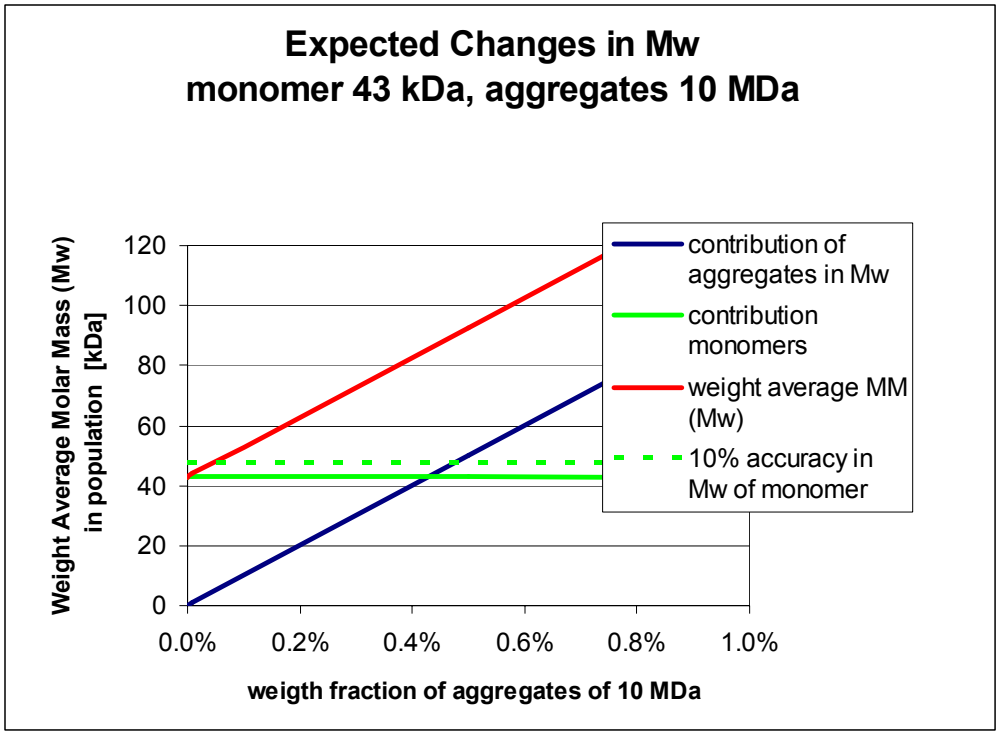
no angular dependence of scattered light



The Molar Mass measured in light scattering experiment is weight-average Molar Mass

For a simple, two component system with monomeric protein and aggregates:

$$Mw = f_{w(mono)} * MM_{(mono)} * + f_{w(agg.)} * MM_{(agg.)}$$



Batch Mode Static MALLS experiment

Monomer 14 kDa

Sample	Weight Average MM, Mw ± SD [kDa]	RMS [nm]
1	15 ± 1	0
2	126 ± 8	***40 ± 1***

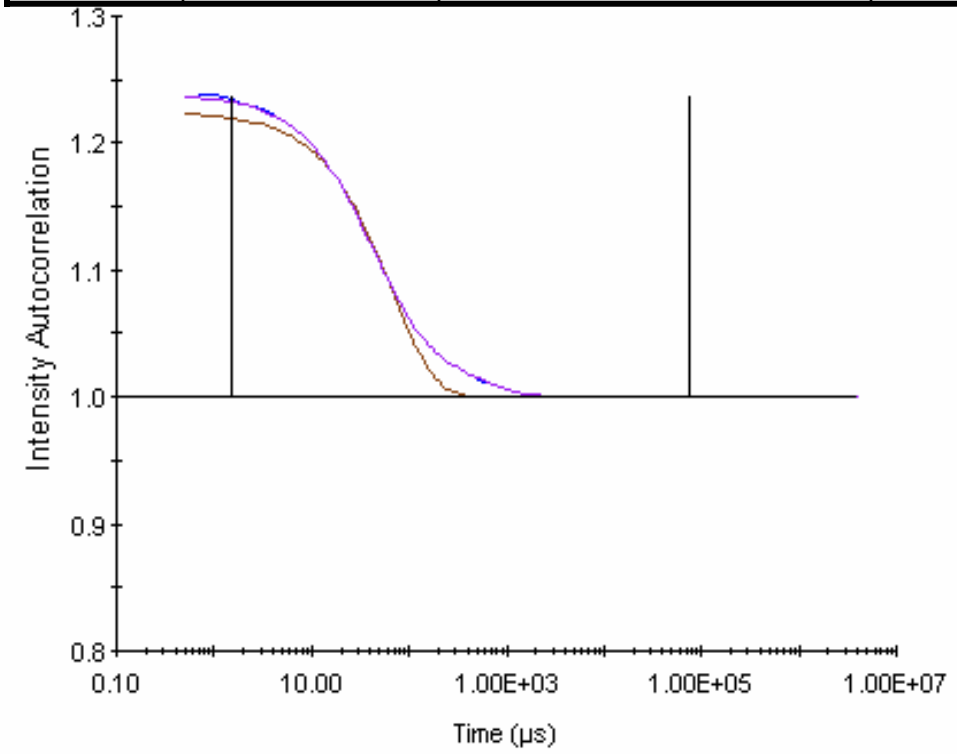
Angular dependence of scattered light clearly indicates presence of large molecules

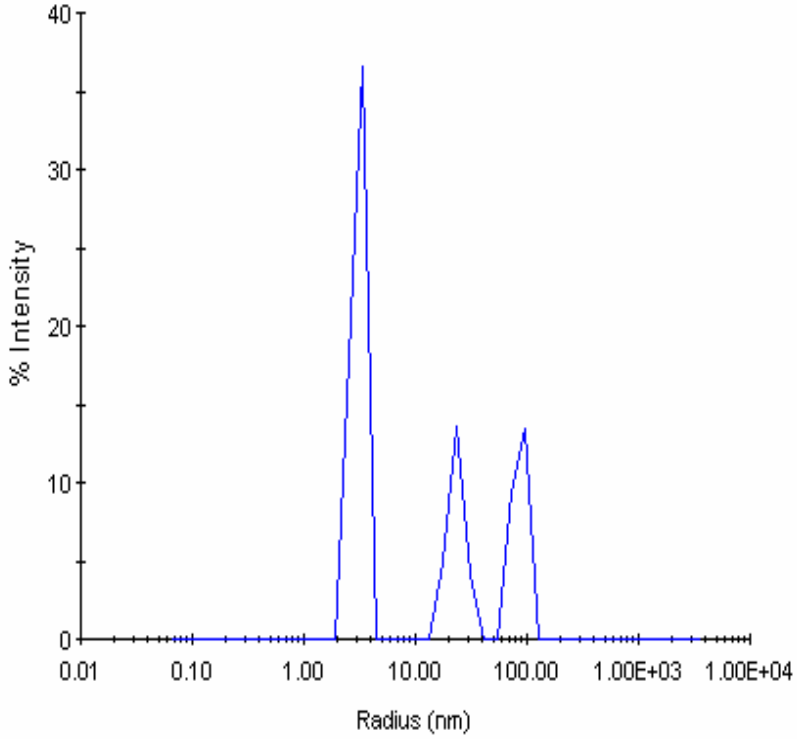
Average from three measurements at various concentrations

Results from Dynamic LS experiment: *Ovalbumin 43 kDa*

Regularization Fit *Rh = 8 nm from Cumulant Fit*

Peak	Rh (nm)	Polydispersity (%)	MW (R) kDa	% Intensity	% Mass
1	3.1	12.8	46	54	99.9
2	24	17.8	5599	23	0.1
3	86	13.4	113513	23	<0.1





Feature detected in a batch mode LS measurements for sample containing aggregates

- ***Static (classical)***

Aggregates present:

- elevated weight average Molar Mass (M_w weight average)
- angular dependence in scattered light

- ***Dynamic (quasielastic)***

Aggregates present:

- autocorrelation function cannot be described by single exponential (cumulant fit)

Feature detected in a batch mode LS measurements for sample containing aggregates

- ***Static (classical)***
- ***Dynamic (quasielastic)***

Aggregates present:

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- angular dependence in scattered light

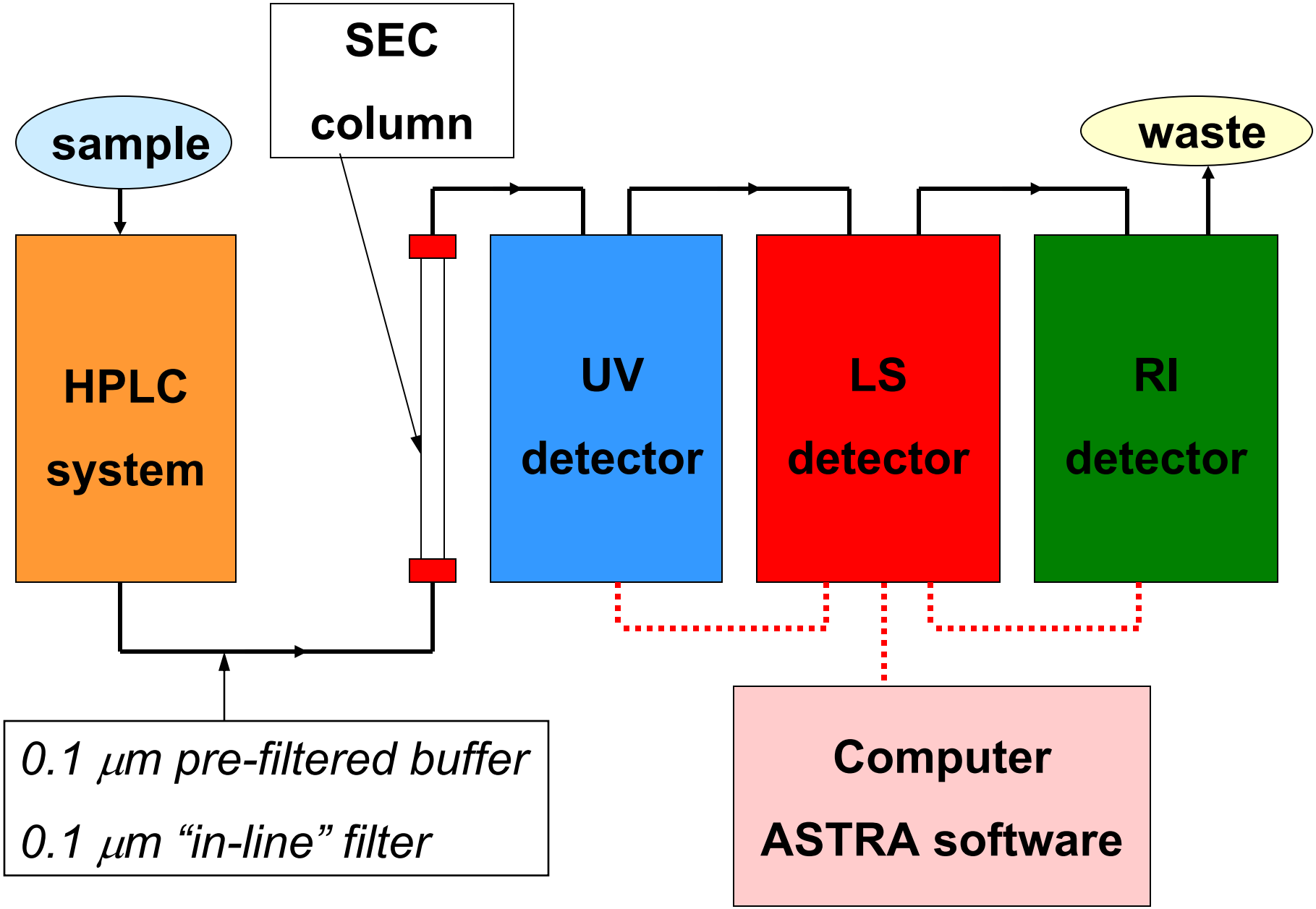
Aggregates present:

- autocorrelation function cannot be described by single exponential (cumulant fit)

Missing information: how much and what size?

- Fractionate Sample

- Combine LS measurement with a fractionation step; SEC/ MALS


SEC
column
sample
waste
HPLC
system
UV
detector
LS
detector
RI
detector
0.1 μm pre-filtered buffer
0.1 μm "in-line" filter
Computer
ASTRA software

Ovalbumin 43 kDa

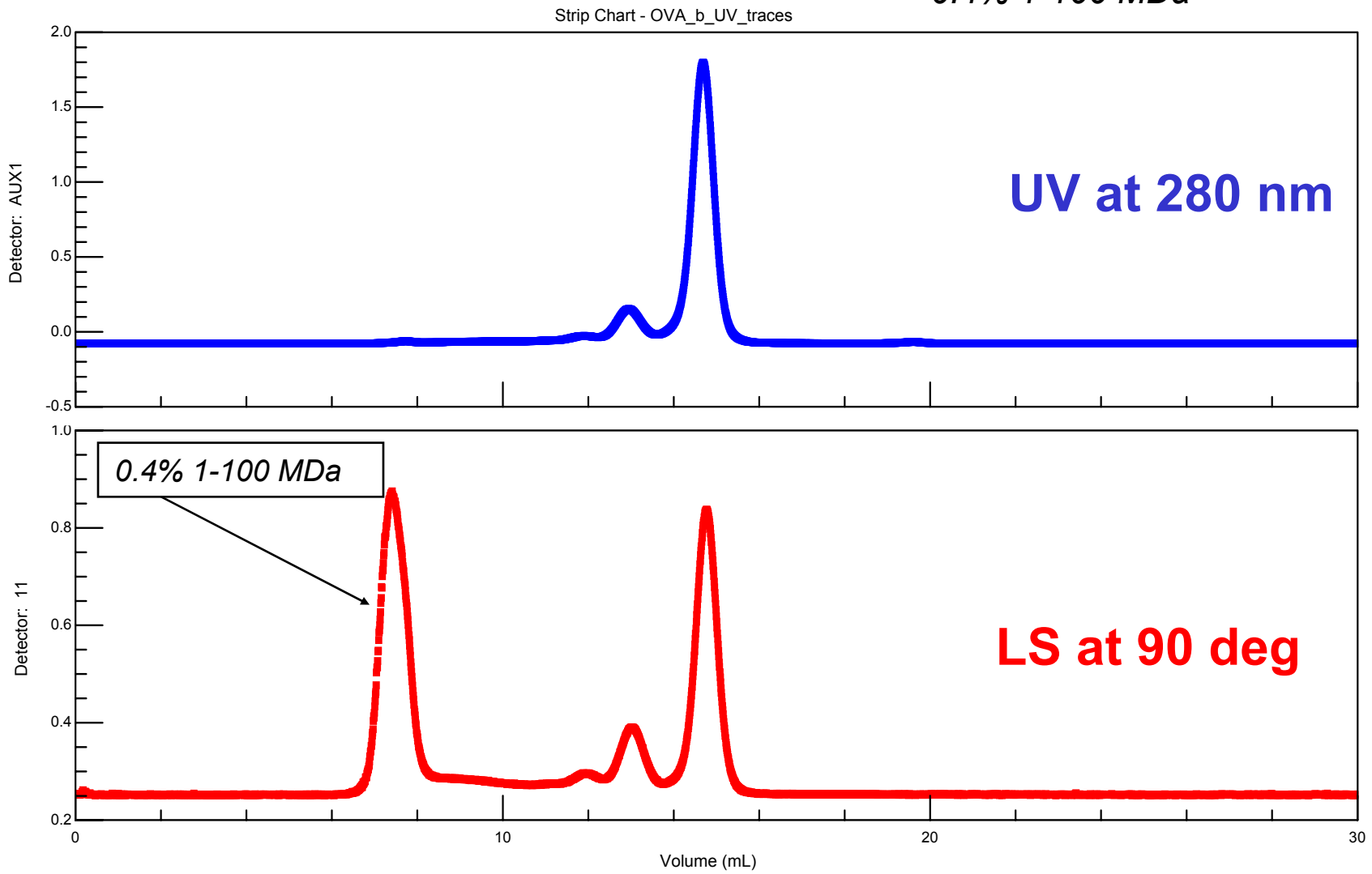
88% monomer

8% dimer

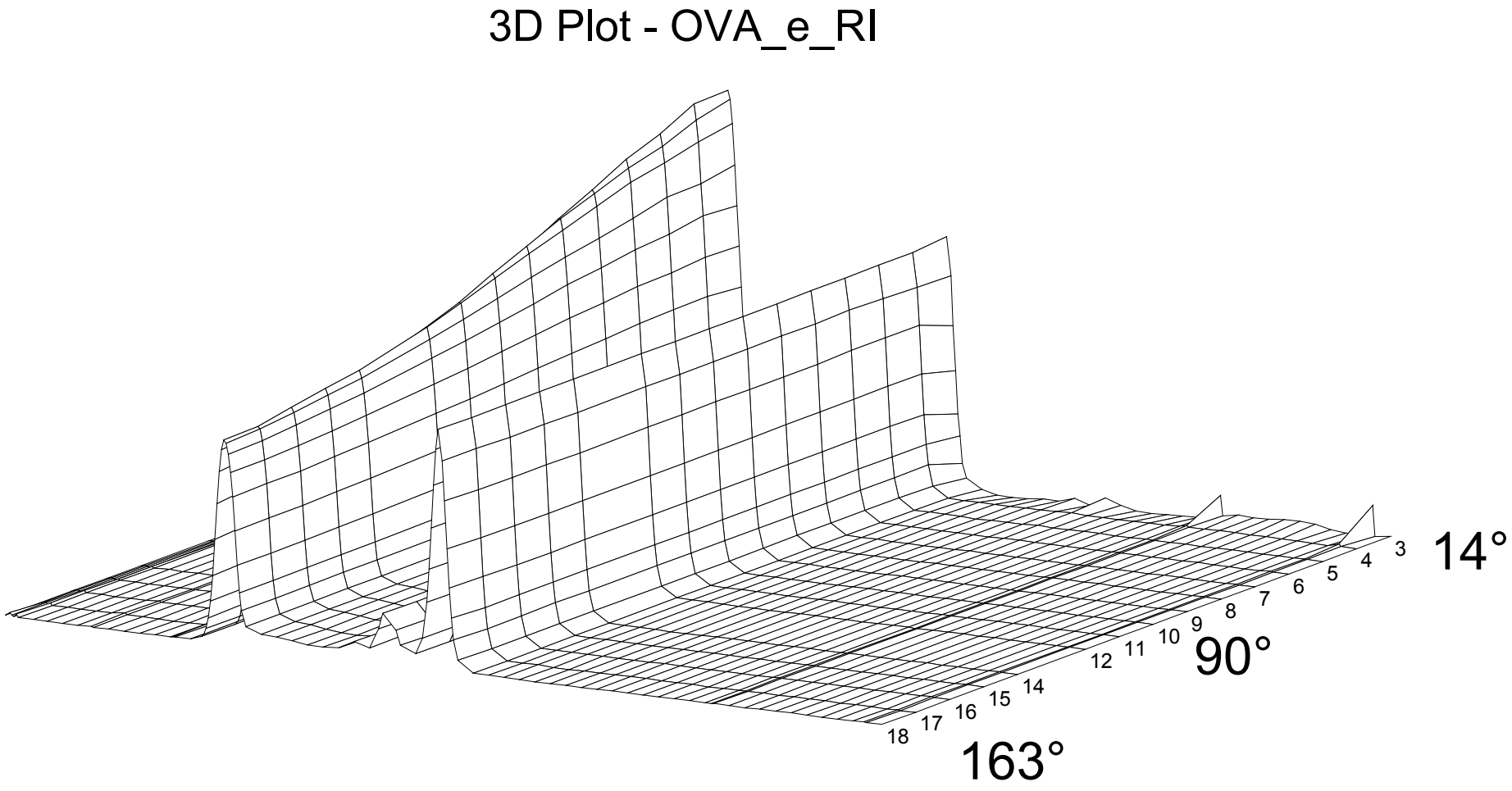
1.5% trimer

3% aggregates < 1MDa

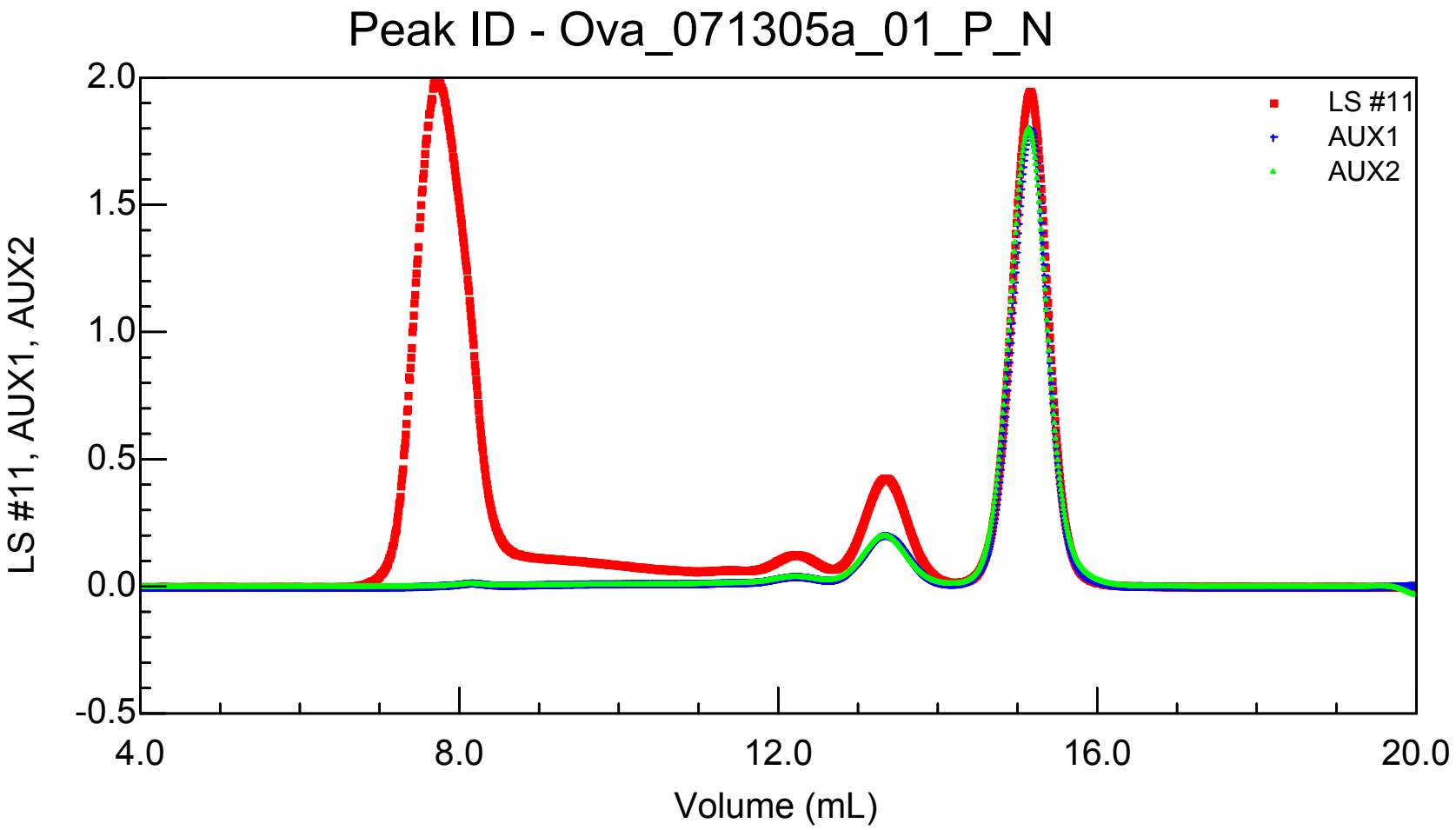
0.4% 1-100 MDa



Ovalbumin 43 kDa



Three Detector monitoring



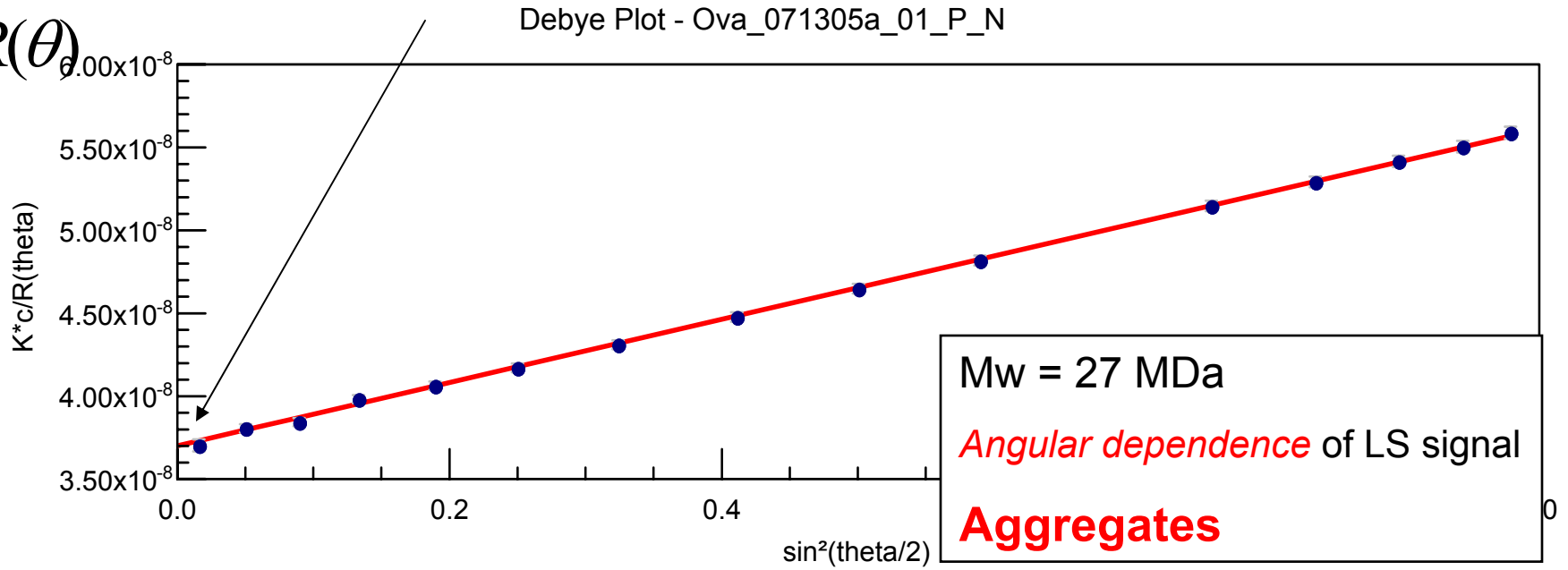
UV at 280 nm RI LS at 90°

Zimm Plot Ovalbumin (43 kDa)

$$\frac{K^*c}{R(\theta)} = \frac{1}{Mw}(1 + f(\sin^2(\frac{\theta}{2})))$$

$\frac{K^*c}{R(\theta)}$

1/(Mw)

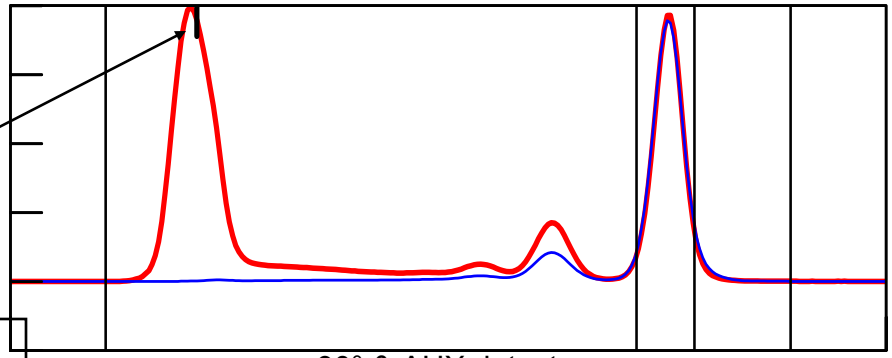


Mw = 27 MDa

Angular dependence of LS signal

Aggregates

Peak, Slice : 1, 938
Volume : 7.817 mL
Fit degree : 1
Conc. : (1.768 ± 0.021)e-6 g/mL
Mw : (2.702 ± 0.033)e+7 g/mol
Radius : 51.3 ± 0.2 nm



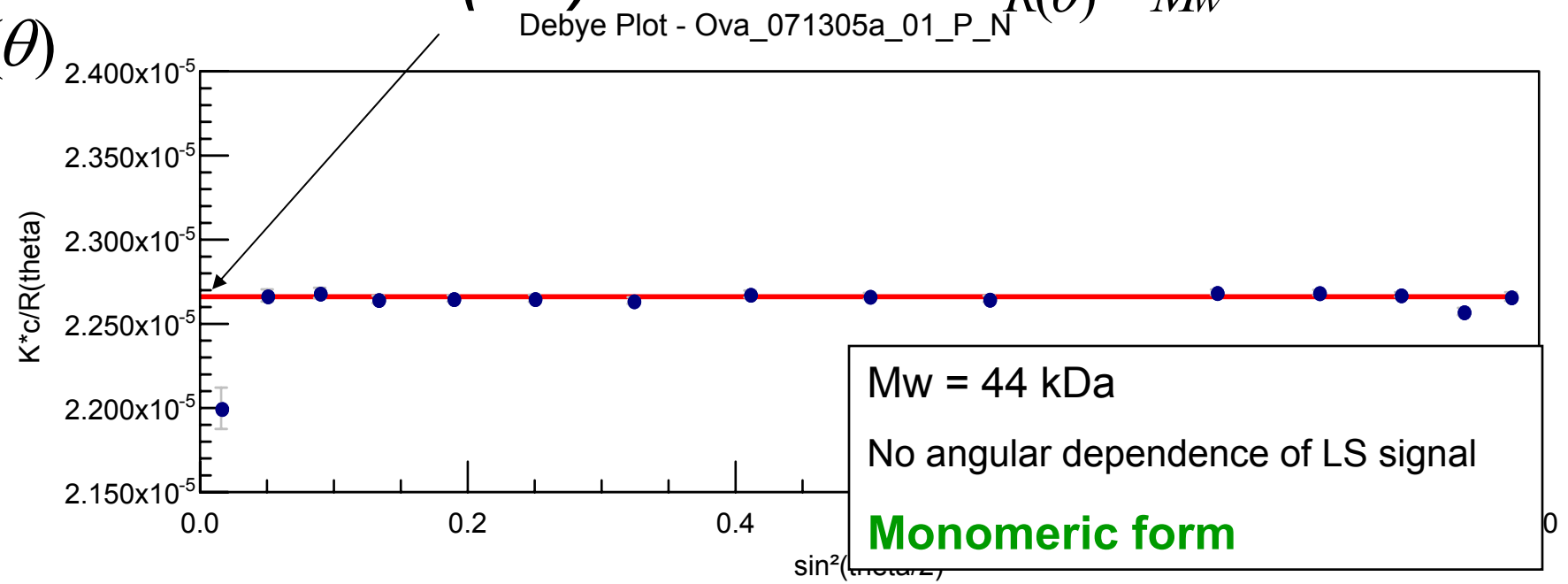
Results for initial peak in elution profile

Zimm Plot Ovalbumin (43 kDa)

$$\frac{K^*c}{R(\theta)}$$

$$\frac{K^*c}{R(\theta)} = \frac{1}{Mw}(1 + f(\sin^2(\frac{\theta}{2})))$$

1/(Mw)

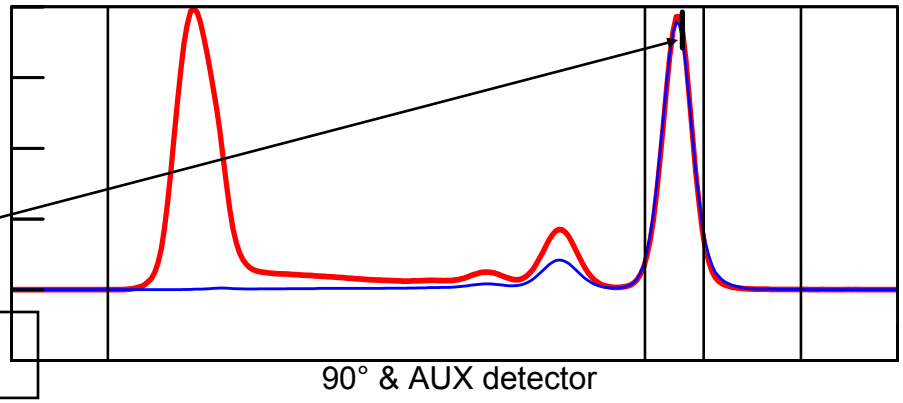


Mw = 44 kDa

No angular dependence of LS signal

Monomeric form

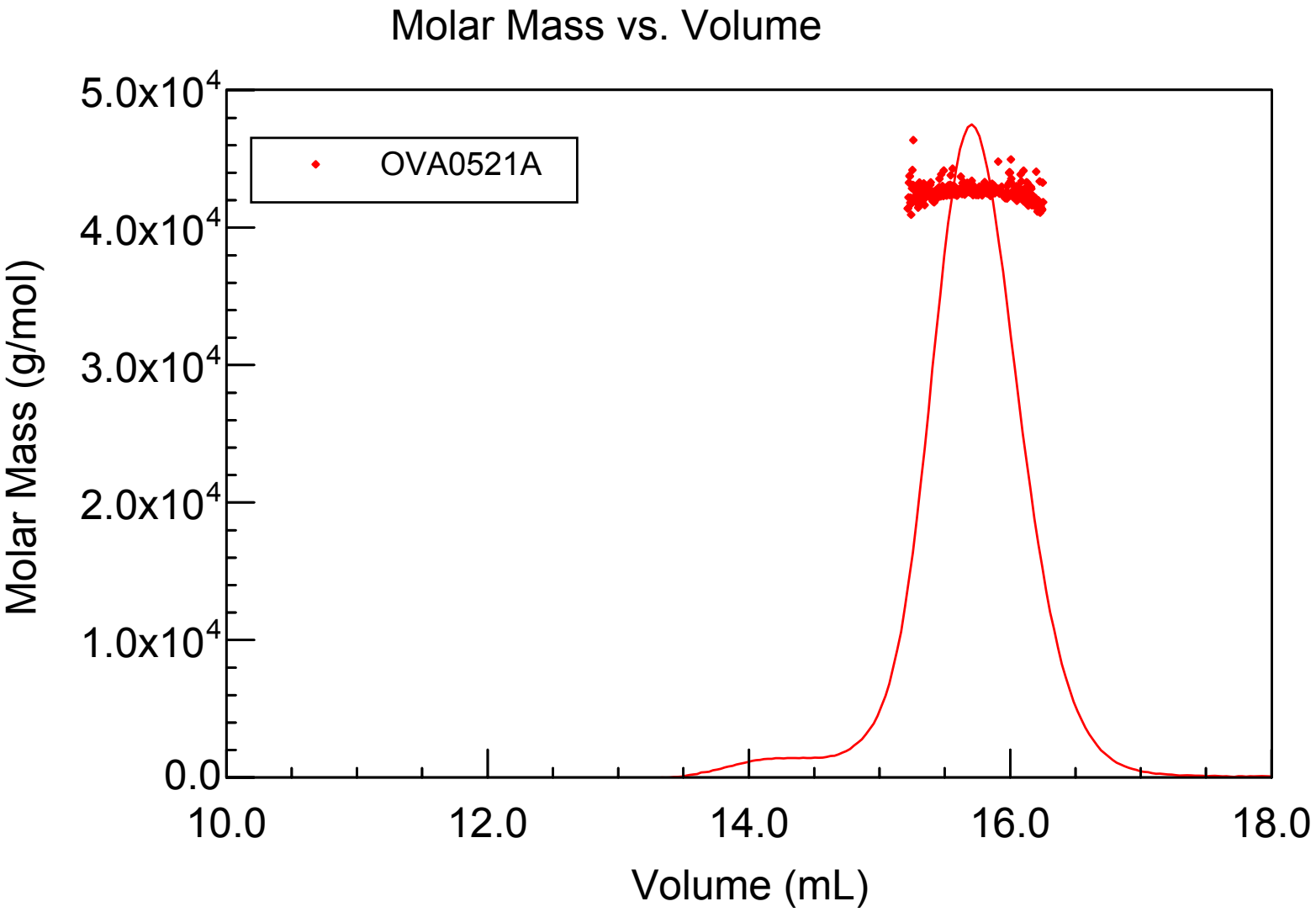
Peak, Slice : 2, 1826
Volume : 15.217 mL
Fit degree : 0
Conc. : (8.320 ± 0.000)e-4 g/mL
Mw : (4.413 ± 0.002)e+4 g/mol
Radius : 0.0 ± 0.0 nm



Results for last peak in elution profile

Molar Mass Distribution Plot

Ovalbumin 43 kDa



What is the analytical uncertainty?

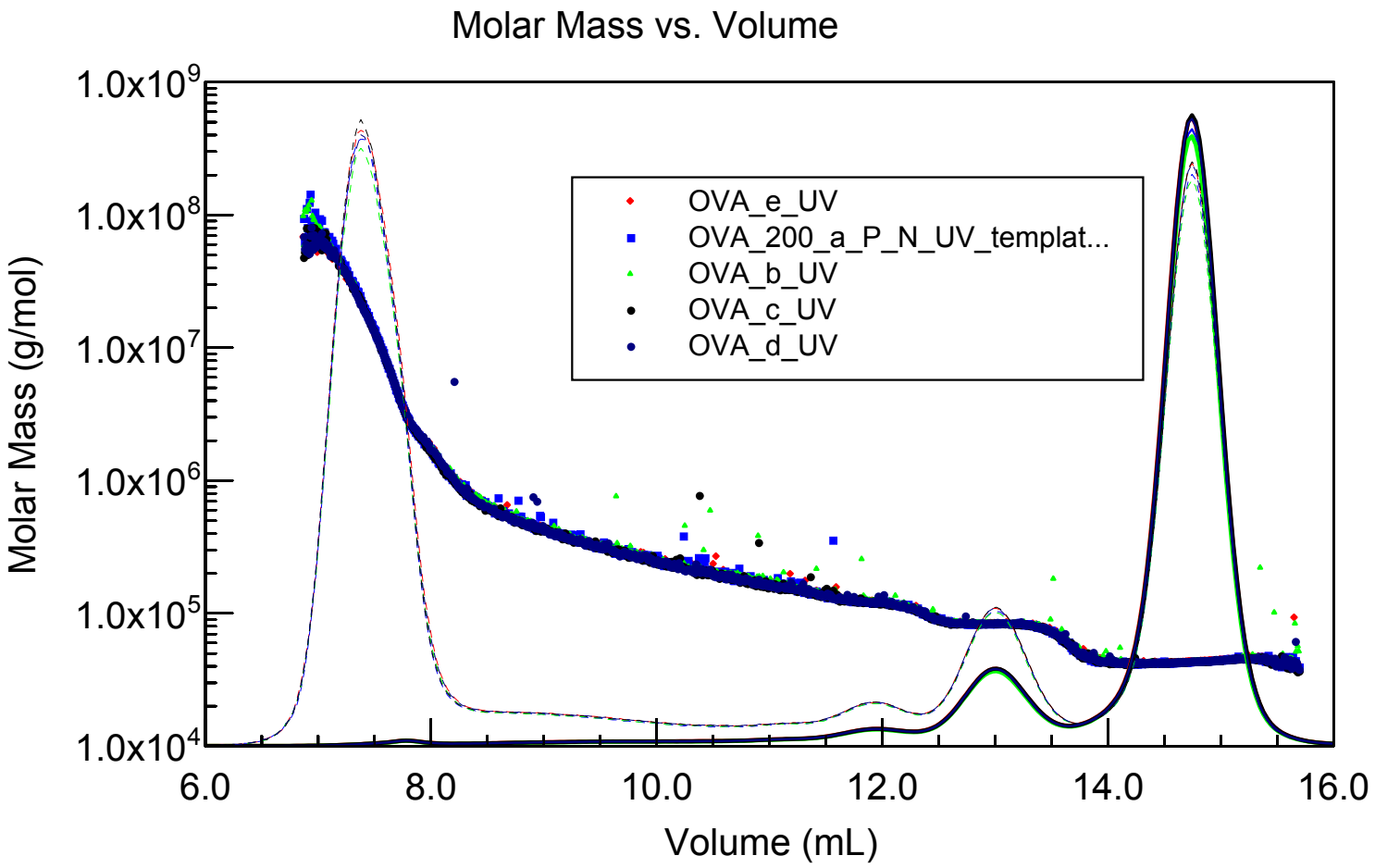
Molecular Weights Determined from "in line“ analyses static LS in line with SEC fractionation

Protein	Oligomeric state	# Runs	Pred. MW (kDa)[a]	Average MW ± St. Dev. (kDa)	Average error (%)
Aprotinin	monomer	2	6.5	6.8 ± 0.5	4.6
Cytochrome C	monomer	5	12.3	12.01 ± 0.57	2.4
α Lactalbumin	monomer	2	14.2	14.32 ± 0.01	0.9
Myoglobin	monomer	3	17.0	14.19 ± 0.91	16
β-Lactglobulin	monomer	2	18.3	20.06 ± 0.33	9.7
Tripsin inhibitor	monomer	1	20.0	20.50	2.3
Carbonic anhydrase	monomer	4	29.0	29.22 ± 0.20	0.8
Ovalbumin	monomer	10	42.8	42.52 ± 0.68	1.4
BSA (monomer)	monomer	5	66.4	66.41 ± 1.00	1.2
Transferrin	monomer	2	75.2	76.92 ± 0.98	2.3
Enolase (yeast)	dimer	3	93.3	80.74 ± 1.18	13
Enolase (rabbit)	dimer	4	93.7	86.44 ± 1.90	7.8
BSA (dimer)	dimer	5	132.9	137.10 ± 3.93	3.2
Alc. dehydrogenase	tetramer	4	147.4	144.02 ± 0.86	2.4
Aldolase (rabbit)	tetramer	2	156.8	153.7 ± 1.91	1.1
Apo-ferritin	24 x monomer	2	475.9	470.3 ± 2.62	1.2
				Median error:	**2.3**

Is the quantitation of the results straightforward and objective?

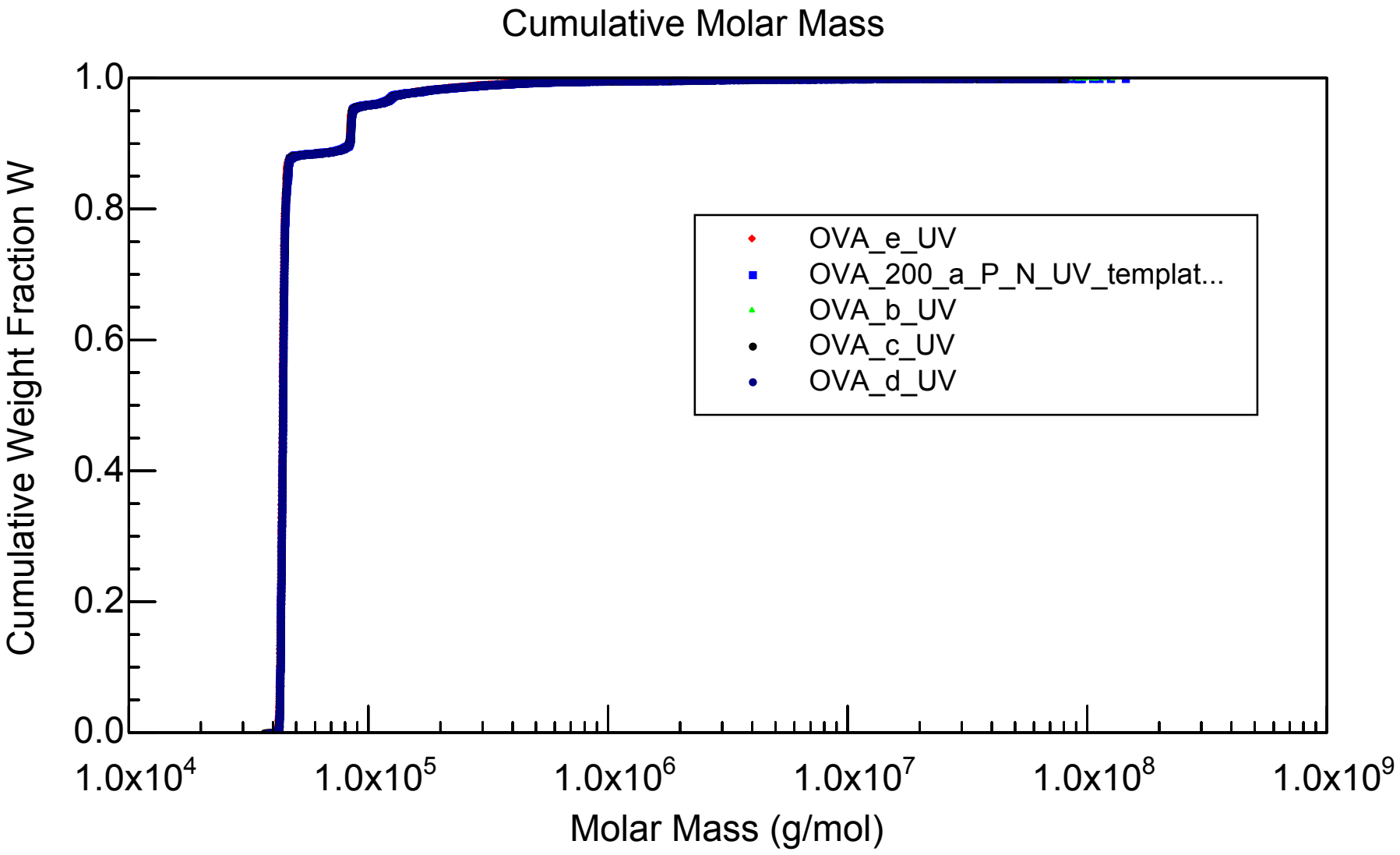
Molar mass distribution as provided by ASTRA software

Ovalbumin 43 kDa *template processing of five data sets*



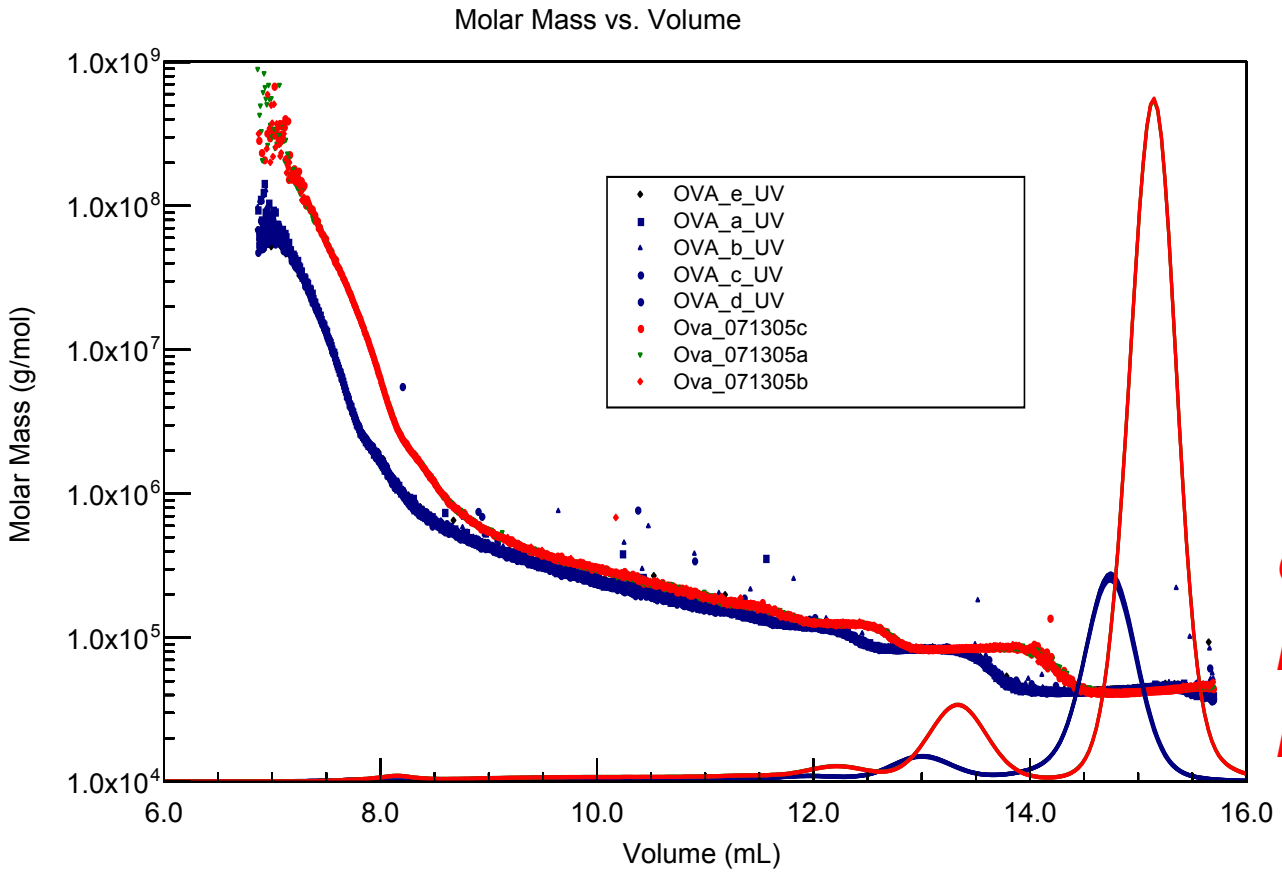
Is the quantitation of the results straightforward and objective?

Determination of Weight Fractions (ASTRA software)



how sensitive is the technique to changes in the population?

Differences in population based on molar mass distribution



Ovalbumin (5 runs)

Mw = 108 ± 17 kDa

Polydispersity Mw/Mn

2.3 ± 0.4

Ovalbumin (3 runs)

Mw = 141 ± 3 kDa

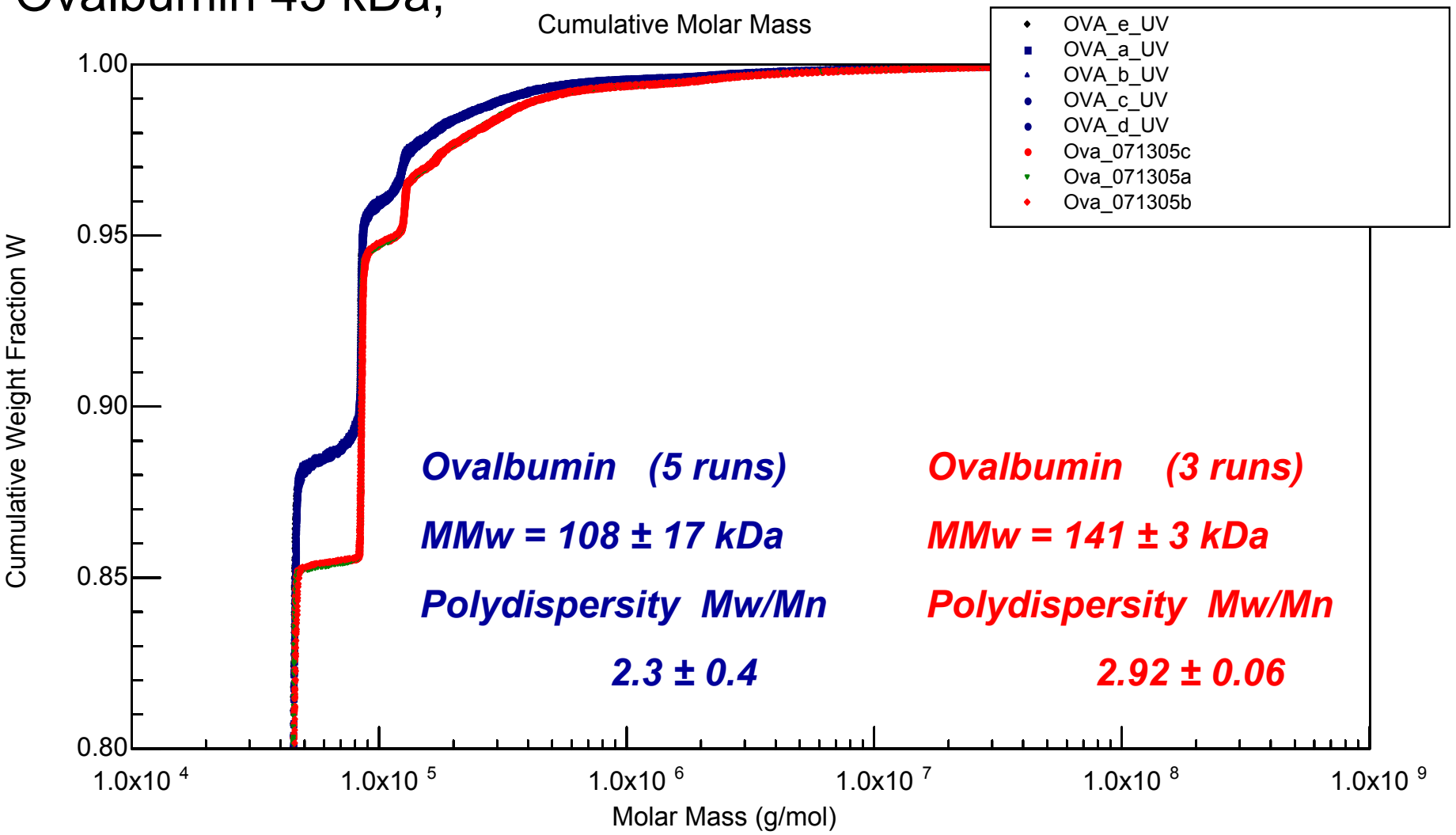
Polydispersity Mw/Mn

2.92 ± 0.06

how sensitive is the technique to changes in the population?

Differences in population based on molar mass distribution

Ovalbumin 43 kDa,



how sensitive is the technique to changes in the population?

Differences in population based on molar mass distribution

Ovalbumin 43 kDa

Oligomeric state	Average Mw ± SD [kDa] (5 analyses)	Average Mw ± SD [kDa] (3 analyses)	Fraction of Mass [% of total] (5 analyses)	Fraction of Mass [% of total] (3 analyses)
	Mw = 108 ± 17	Mw = 141 ± 3	Mw = 108 ± 17	Mw = 141 ± 3
Mono (20-50 kDa)	43.0 ± 0.1	42.80 ± 0.02	88.1 ± 0.1	85.23 ± 0.06
Di (50-96 kDa)	82.7 ± 0.4	84.1± 0.2	7.68 ± 0.04	9.4 ± 0.0
Tri (96-130 kDa)	114 ± 4	121.8 ± 0.7	1.54 ± 0.05	1.9 ± 0.0
Agg. (0.13 –1 MDa)	270 ±10	284 ± 2	2.18 ± 0.08	2.87± 0.06
Agg. (1 –100 MDa)	10±1 x10^3	10.9±0.4 x10^3	0.4 ± 0.0	0.6 ± 0.0

Population in Ovalbumin sample

(averages from five analyses of 200 ug of protein total)

UV used as mass detector

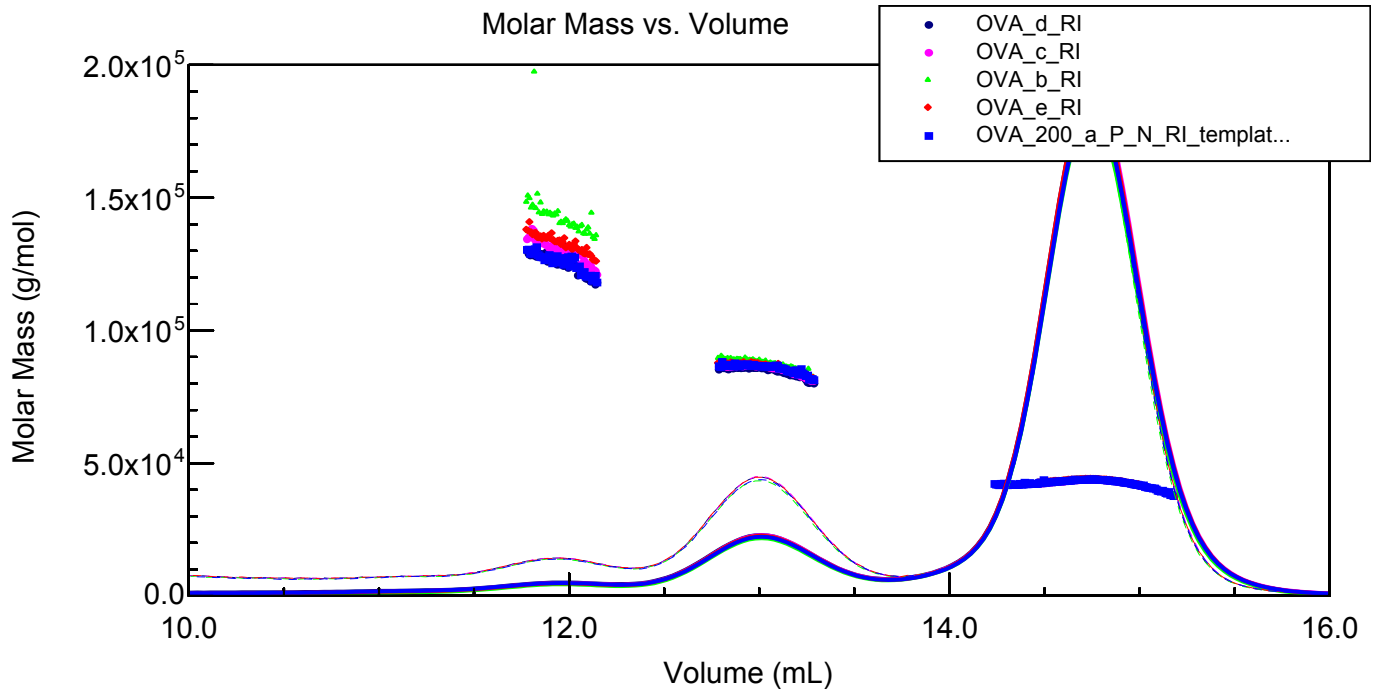
Molar Mass	Monomer	Dimer	Trimer	Aggregates	
	20-50 kDa	50-96 kDa	96-130 kDa	130 kDa-1MDa	1-100MDa
Average ± SD	88.1 ± 0.1 %	7.68 ± 0.04 %	1.54 ± 0.05 %	2.18 ± 0.08 %	0.40 ± 0.00 %

RI used as mass detector

Molar Mass	Monomer	Dimer	Trimer	Aggregates	
	20-50 kDa	50-96 kDa	96-130 kDa	130 kDa-1MDa	1-100MDa
Average ± SD	89.2 ± 0.4 %	7.48 ± 0.08 %	0.9 ± 0.2 %	2.1 ± 0.2 %	0.32 ± 0.08 %

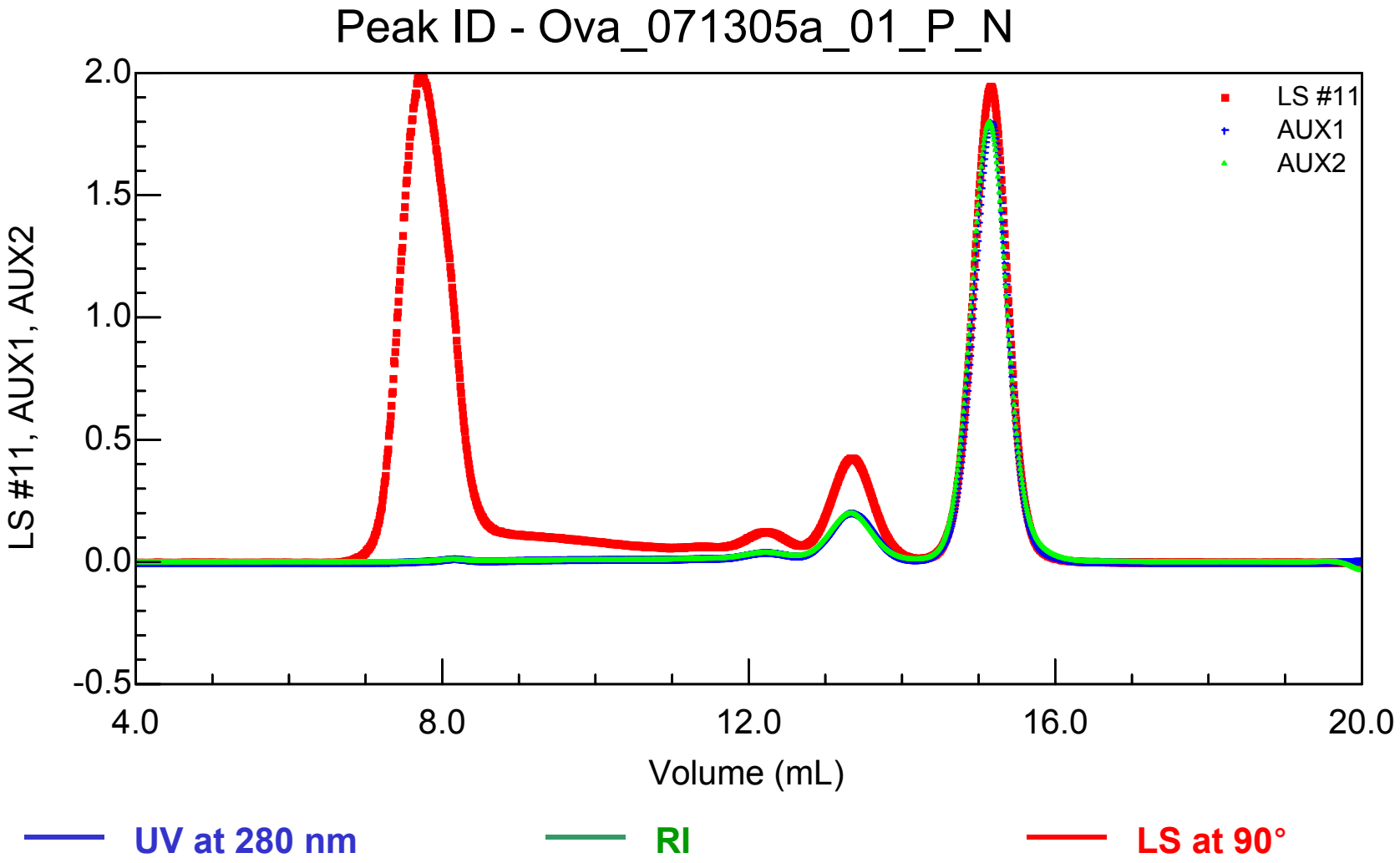
What is the analytical uncertainty? Precision and accuracy

Ovalbumin (expected MM) total mass in eluting peak	MM ± SD (5 analyses)	Precision SD (%)	Accuracy
Monomer (42.8 kDa) 178ug	43.0 ± 0.7	0.2%	0.4%
Dimer (85.6 kDa) 25ug	82.7 ± 0.4	0.5%	3%
Trimer (128.4 kDa) ? 5ug	114 ± 4	3%	11%



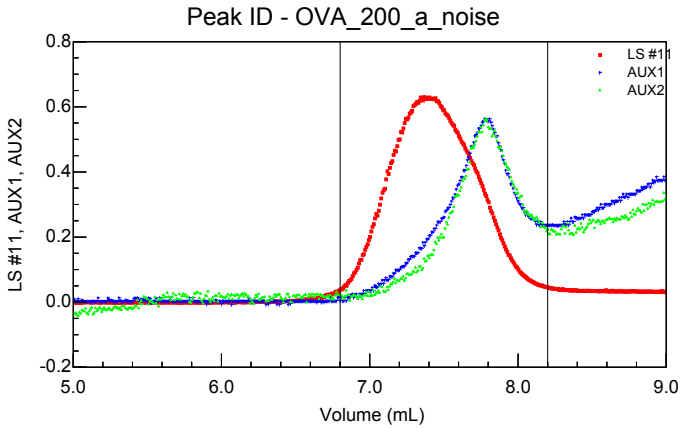
What is the analytical uncertainty?

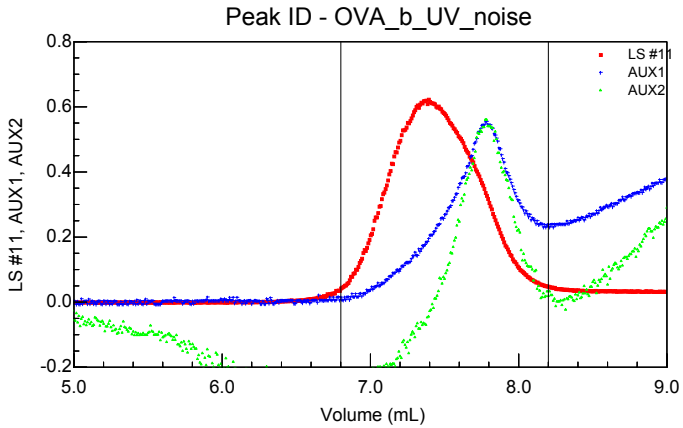
Looking at individual signals for peak containing aggregates in three detector monitoring

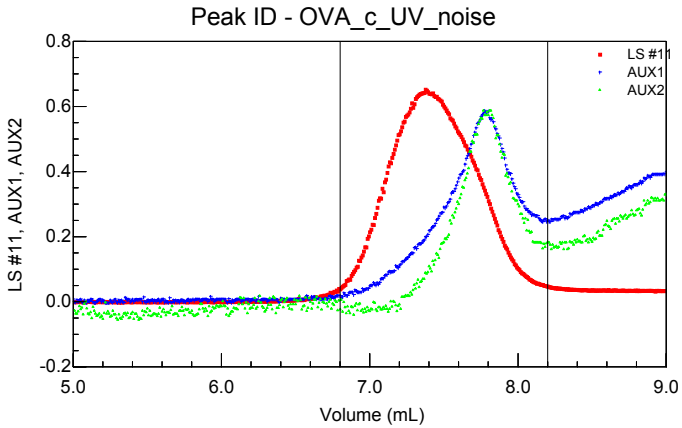


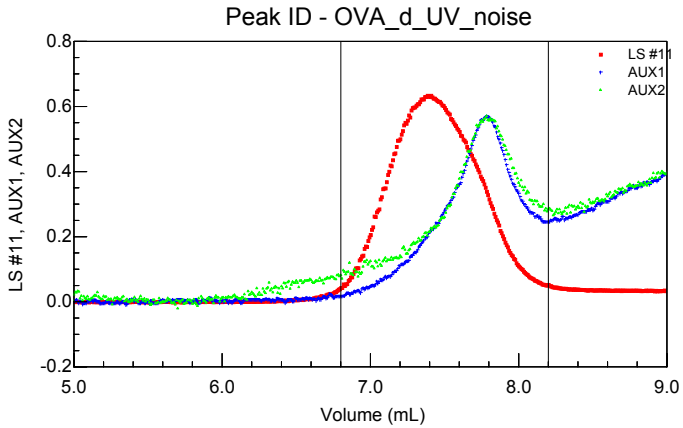
Aggregates ~1.5 micrograms	Volume ± SD	SD (%)	S/N at apex
UV	0.0100 ± 0.0003	3.2	33
LS	0.465 ± 0.006	1.4	316
RI	0.03 ± 0.05	166	1*

* Includes baseline instability in RI signal









Protein C 50 kDa + 8 x 8 kDa = 114 kDa

MMw = 126 ± 2 kDa

Polydispersity Mw/Mn

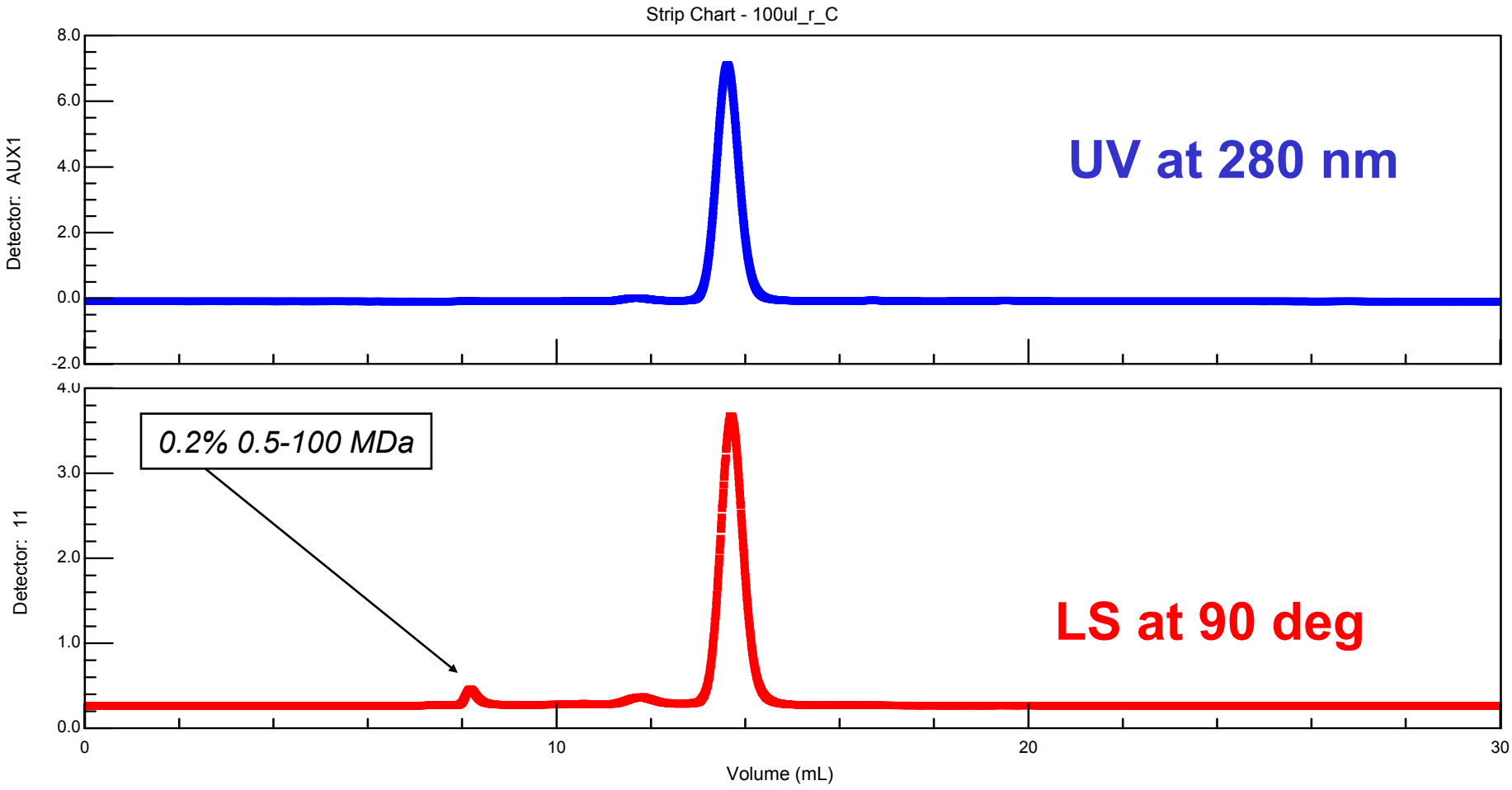
1.10 ± 0.01

97% at 113 kDa

1.6% at 280 kDa

0.6 % aggregates < 0.5MDa

0.2% 0.5-100 MDa



Protein K: octamer 8 x 16.3 kDa = 130 kDa

Mw = 137 kDa

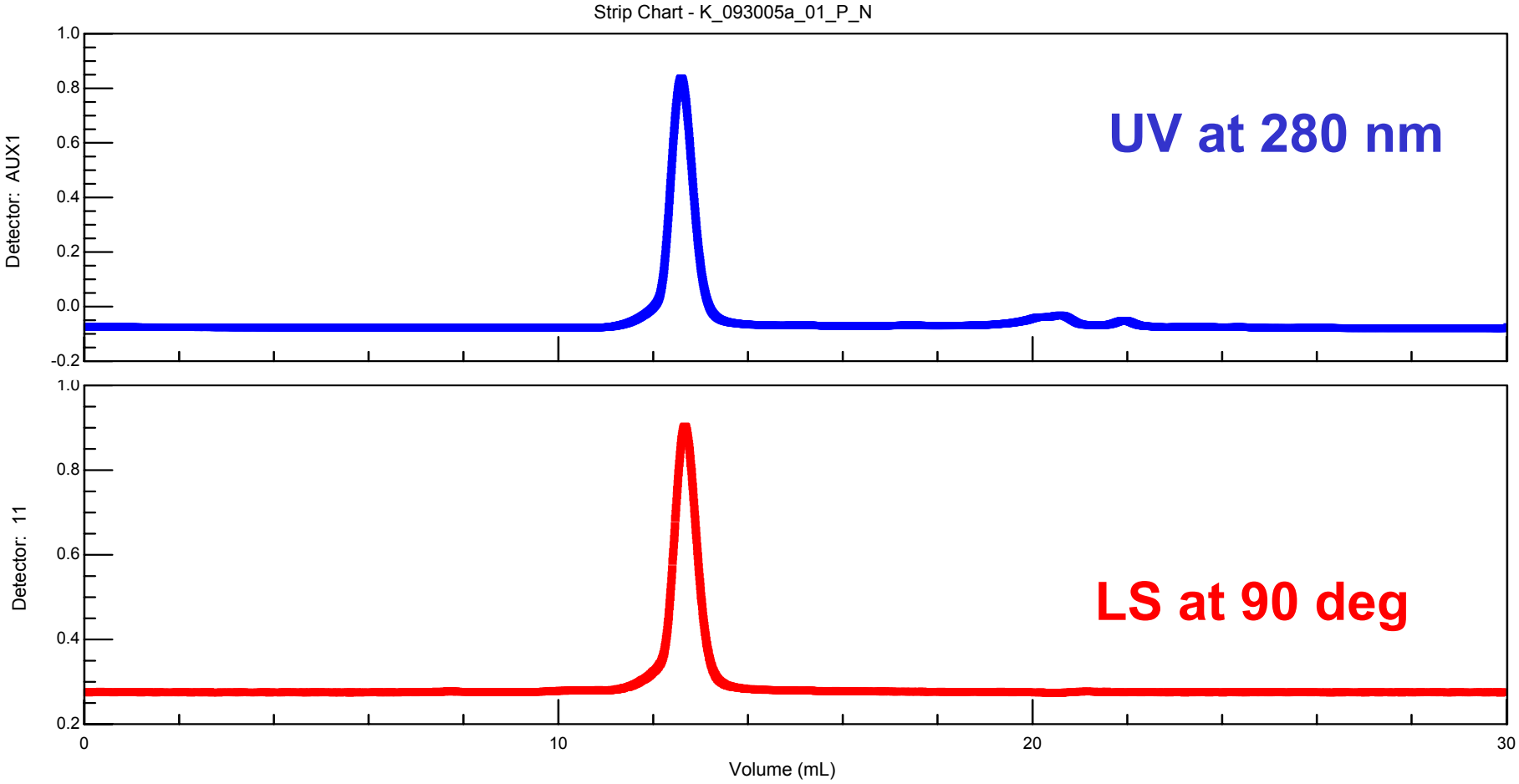
Polydispersity Mw/Mn 1.01

Concentration at apex = 0.09 mg/mL

98.9 % at 133 kDa

1.1 % at 192 kDa

0.0 % 0.5-100 MDa



Protein K: octamer 8 x 16.3 kDa=130 kDa

Mw = 141 kDa

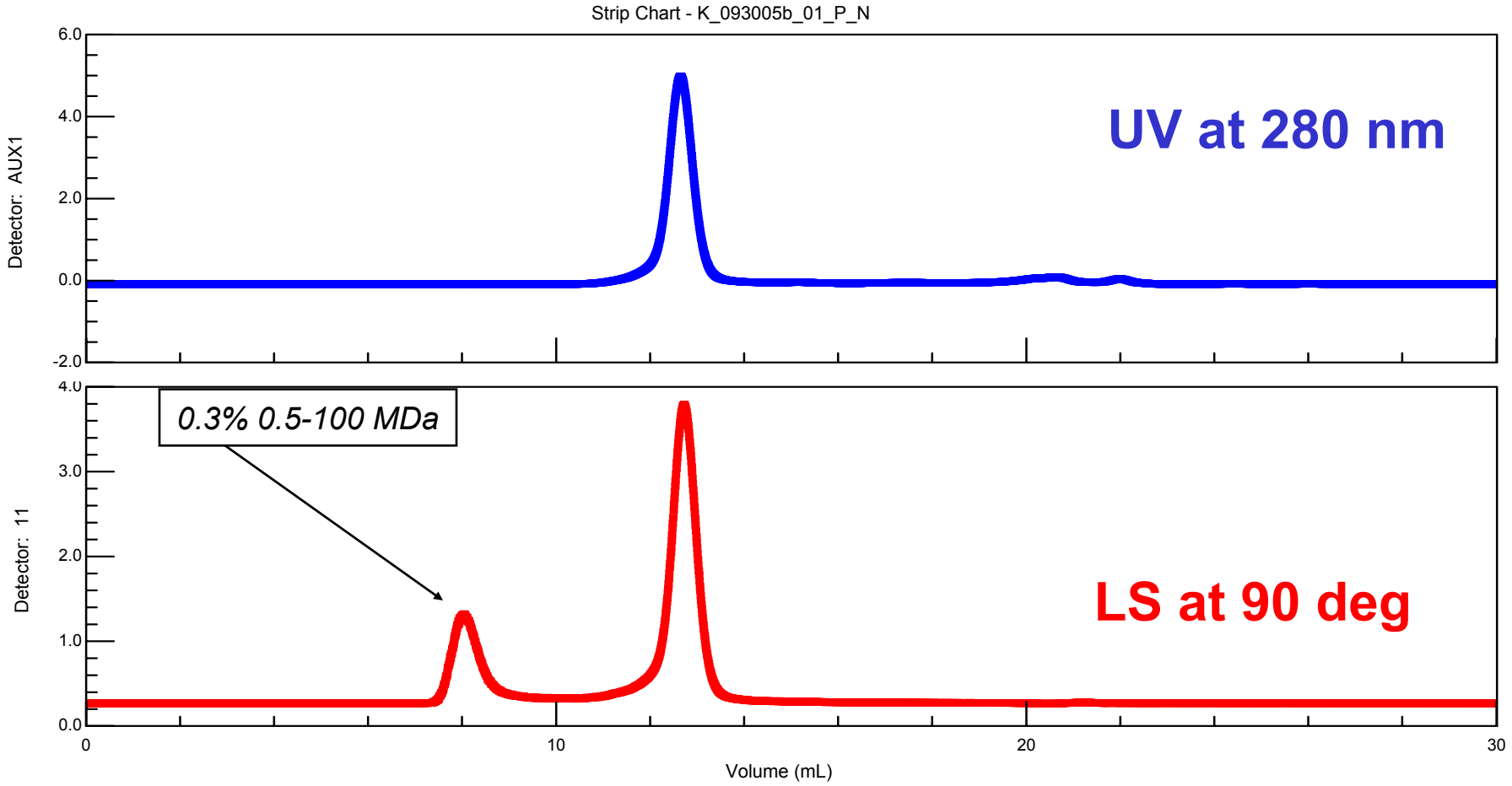
Polydispersity Mw/Mn 1.05

Concentration at apex = 0.5 mg/mL

95.8 % at 133 kDa

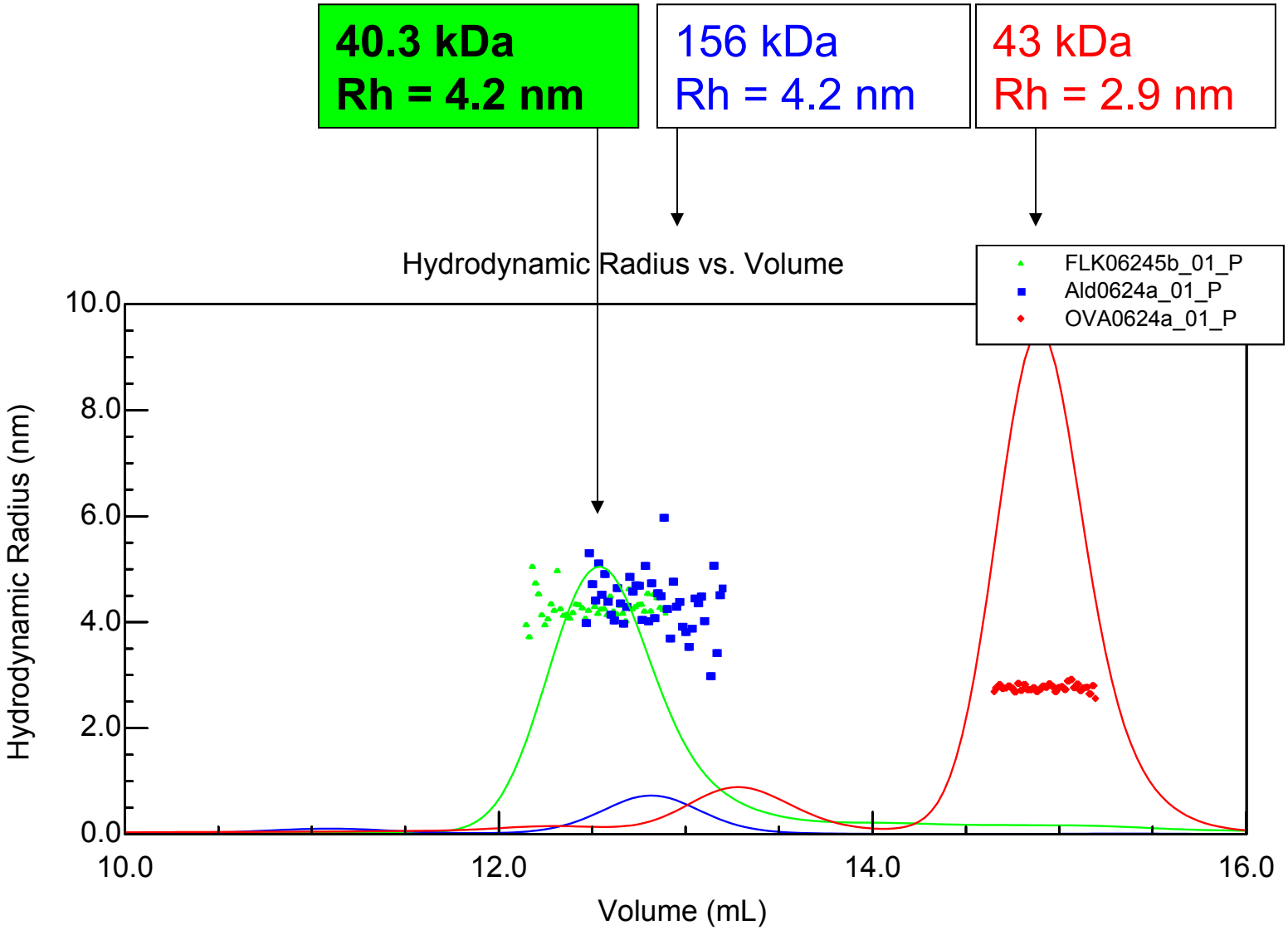
3.9 % at 217 kDa

0.3% 0.5-100 MDa



–To what extent can the technique indicate protein conformation?

Protein “F” frictional ratio $R_h/R_s = 1.85$ non-spherical shape



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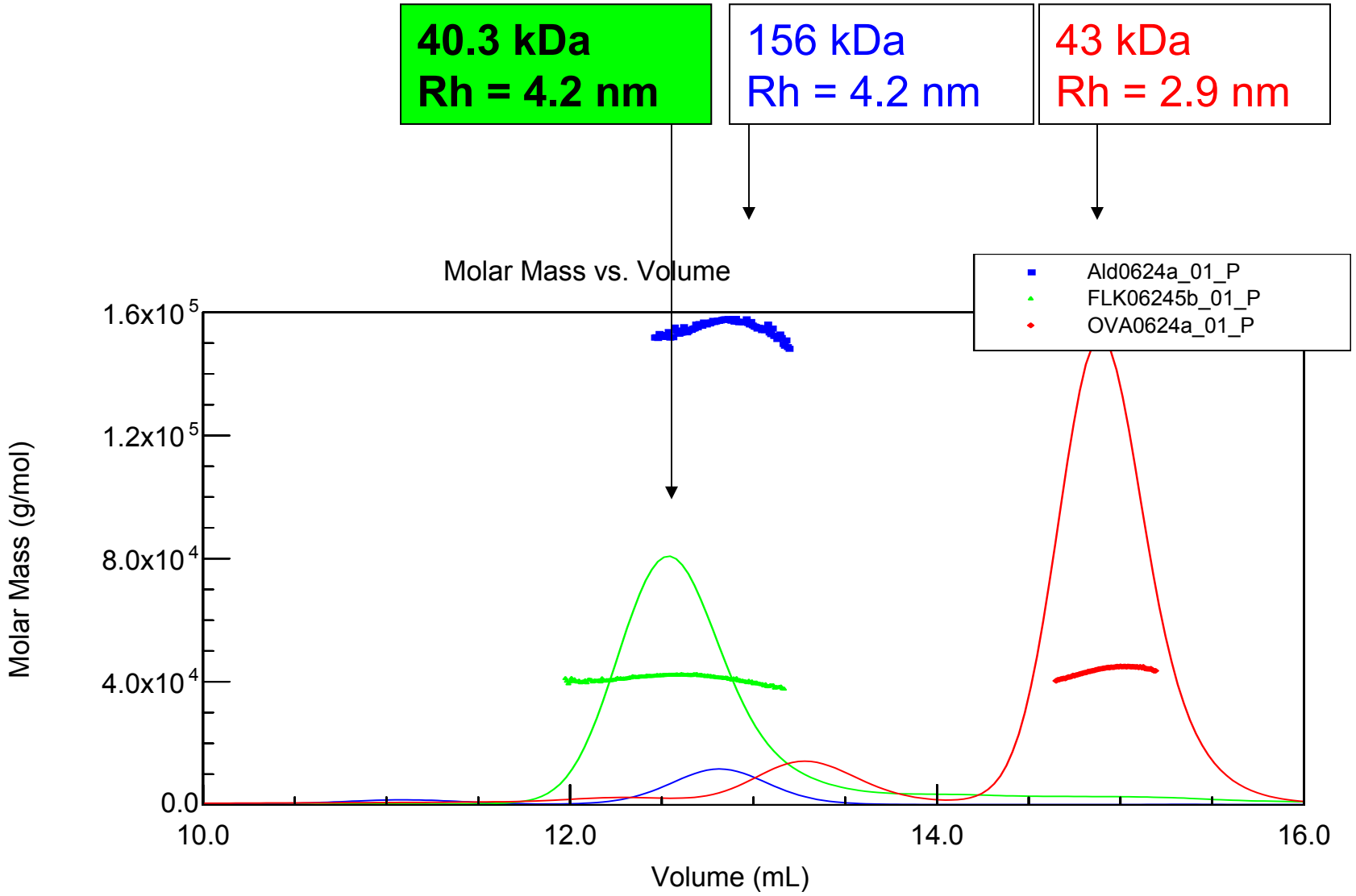

Various uses of Light Scattering for assessing protein aggregates

Experiment	Detects Aggregates	Information about population (distribution)	Challenge in use	Sample dilution	Speed
Batch or micro-batch MALS	Yes	No	High (for small sample volumes) Low (for large sample volumes)	No	Medium
SEC/MALS/DLS	Yes	Yes	Medium	Yes	Medium
DLS	Yes	No	Low	No	Fast

Capabilities

Static LS

- fast and accurate determination of molar masses (weight average) of macromolecules in solution
- single SEC/MALS measurement should be sufficient to determine Molar Mass with a precession of ± 5%
- angular dependence of LS signal easily detects presence of aggregates
- SEC/MALS excellent in detecting and quantifying population in protein samples based on differences in polydispersity and molecular weights
- can determine oligomeric state of modified polypeptide (glycosylated protein, conjugated with PEG, protein-lipids-detergent complexes, protein-nucleic acid complexes)

Dynamic LS

- in batch mode, very fast detection of aggregates and evaluation of polydispersity of sample with great dynamic range
- well suited to study kinetics of aggregation
- DLS detector available in a plate reader format for high volume analyses

Combined information about MM and Rh provides insight about shape via frictional ratio Rh/Rs

Limitations

Static LS

- Measures weight average molar mass – needs fractionation to resolve different oligomeric states or **fitting data to an association model**
- Possible losses of sample during filtration and fractionation
- Limitation on solvent choices (related to a fractionation step)
- When combined with SEC- dilution during experiment
- Needs extra hardware modification for samples that absorb laser light (633 nm)

Dynamic LS

- Measures hydrodynamic radius, which is affected by shape
- Cannot discriminate between shape effects and changes in oligomeric states, *i.e.* non-spherical shape mimics effects oligomerization
- Needs fractionation to resolve oligomers when present in mixture

Ken Williams
Director of W.M. Keck Biotechnology Resource Laboratory at Yale University School of Medicine

NIH

Users of SEC/LS Service

http://info.med.yale.edu/wmkeck/biophysics