Sensitivity and Reproducibility of Protein Aggregate Analysis by Sedimentation Velocity

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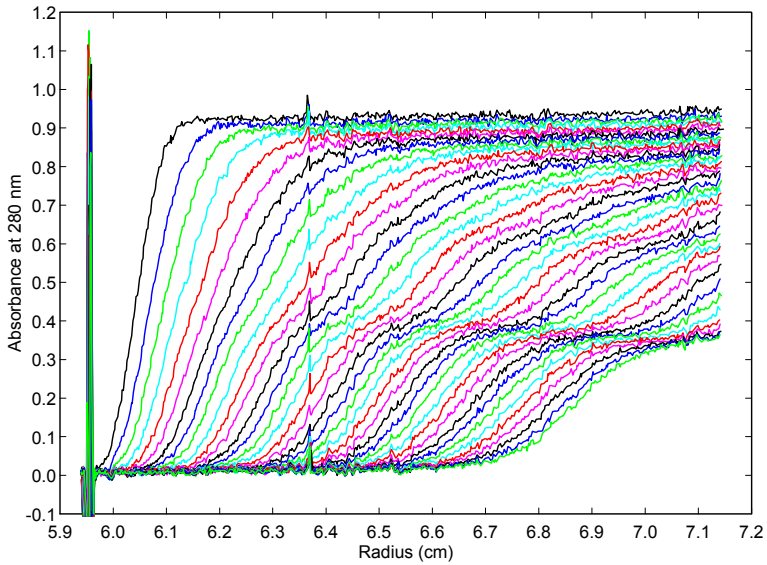
Although use of sedimentation velocity (SV) for aggregate analysis is becoming fairly common, there is little data available about the sensitivity and reproducibility of this method. Replicate samples demonstrate that the standard deviation for the fraction main peak can be 0.3% or less, with comparable or lower uncertainties for individual aggregate peaks. In some cases larger aggregate species that are well separated from the main peak can be detected at levels well below 0.1%, as demonstrated by the presence of the same aggregate species in multiple lots and/or increases in the fraction of that species with time or stress.

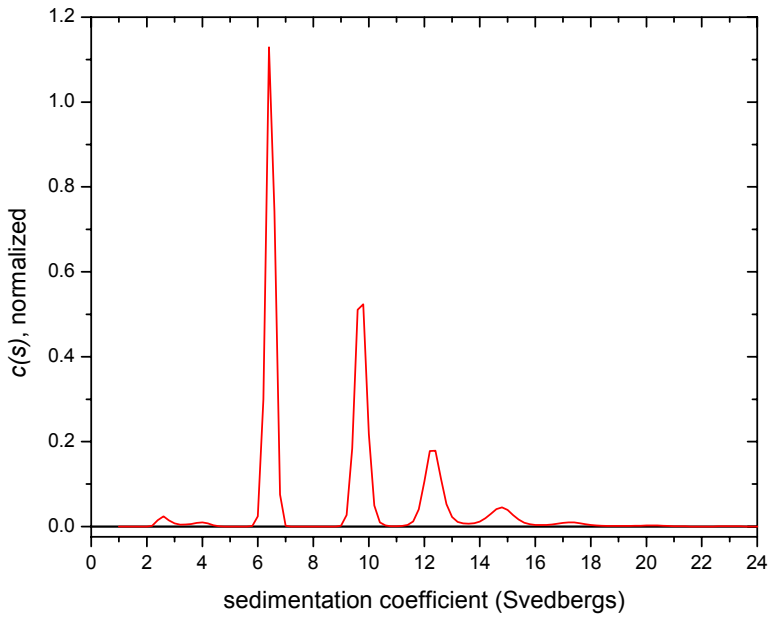
While modern SV data analysis methods can provide size distributions that resemble chromatograms, that similarity can be deceptive because the nature of the "noise" in the data is often quite different than for chromatography. Examples will illustrate that it is normal for the peak positions for very minor peaks to shift from sample to sample. It is also normal for peaks near the threshold of detection to appear or disappear in different replicates of the same material, or for two minor peaks to sometimes be merged into one average species by the software. Those features can make it difficult to reproducibly assign and quantitate individual aggregate components when there are many minor species, but those features do not compromise the assessment of fraction main peak. Further, it is nonetheless always clear whether large aggregate species are present which are lost or poorly resolved by SEC.

Modern methods of sedimentation velocity data analysis[1] allow us to separate and quantitate long-lived aggregates with high resolution and sensitivity, giving a distribution much like a chromatogram.

raw data



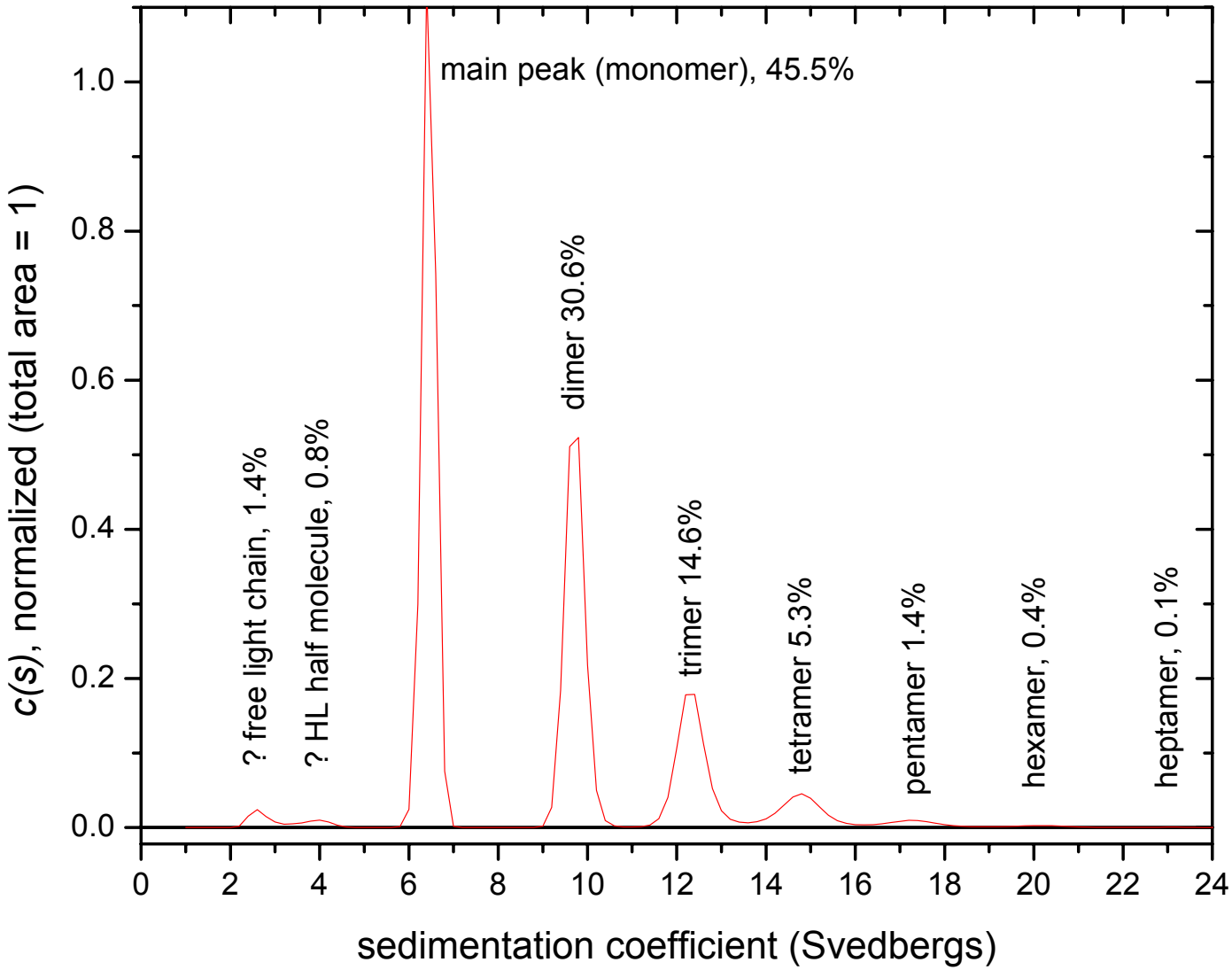
sedimentation coefficient distribution



[1]Schuck, P. (2000). Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. *Biophys. J.* 78, **1606-1619.**

THE PROMISE

These data for a highly stressed antibody sample illustrate excellent resolution, sensitivity, and range of size

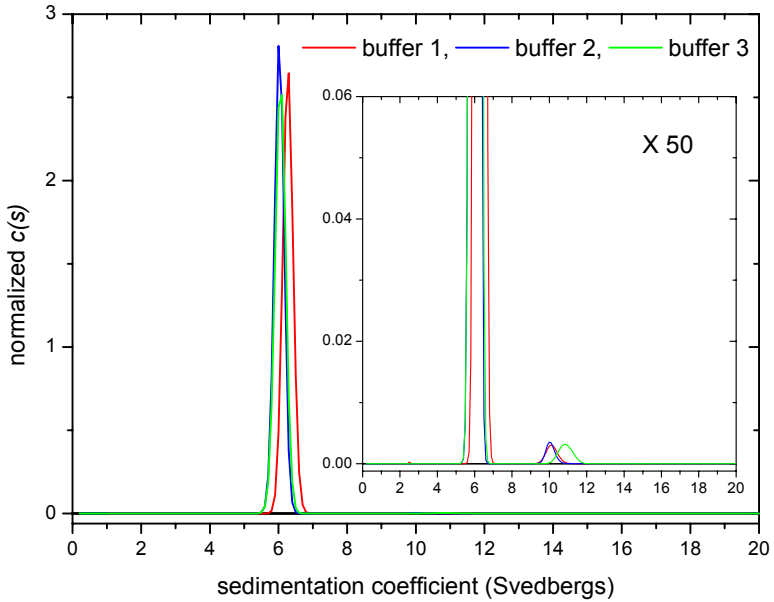


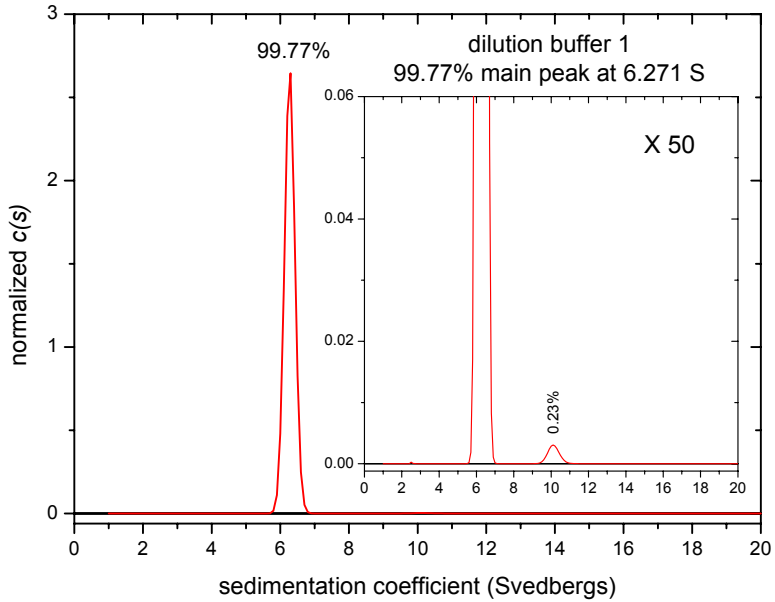
THE PERIL

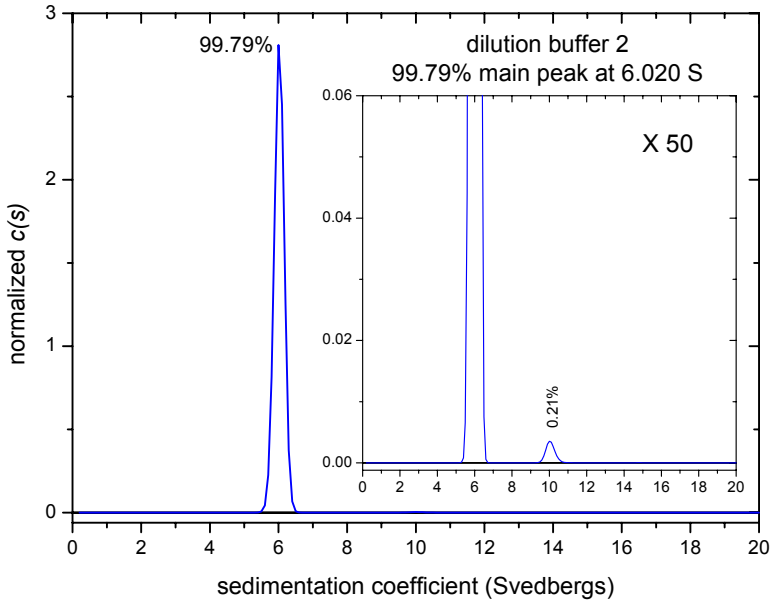
The similarity of these distributions to chromatograms can be deceptive, and the software is easily miss-applied

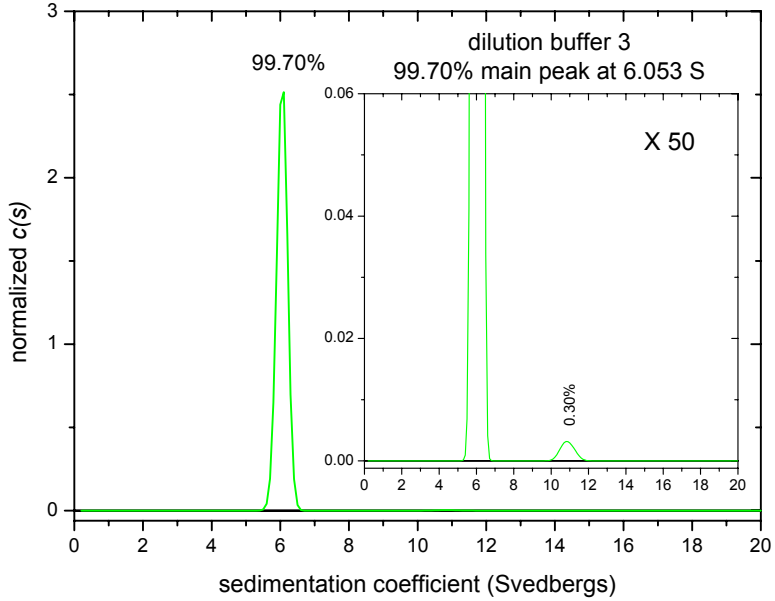
1. the effective resolution is a function of signal/noise ratio and goes down as the fraction of minor peaks goes down
   - the resolution you can achieve for a 150 kDa antibody is also much greater than for a 20 kDa cytokine
2. the nature of the noise (variability) is very different than in chromatography
3. false peaks are possible, especially when the software is applied inappropriately

Three measurements of the same monoclonal antibody stock diluted into 3 different buffers give 0.25% ± 0.05% aggregate

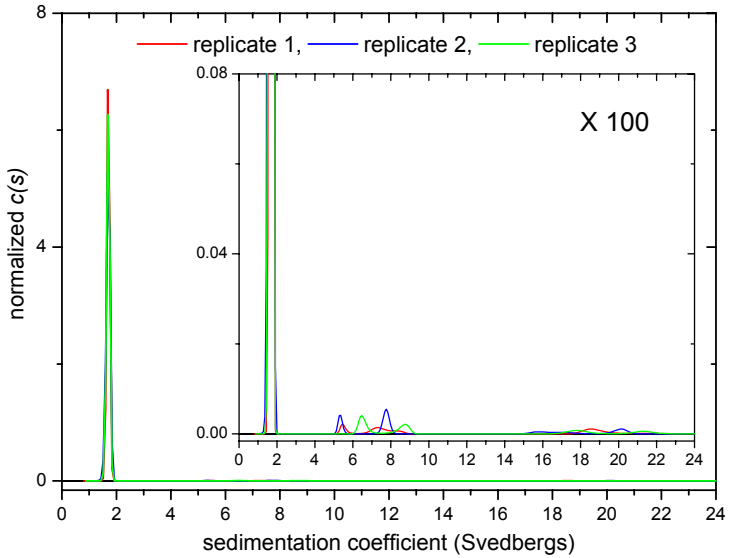


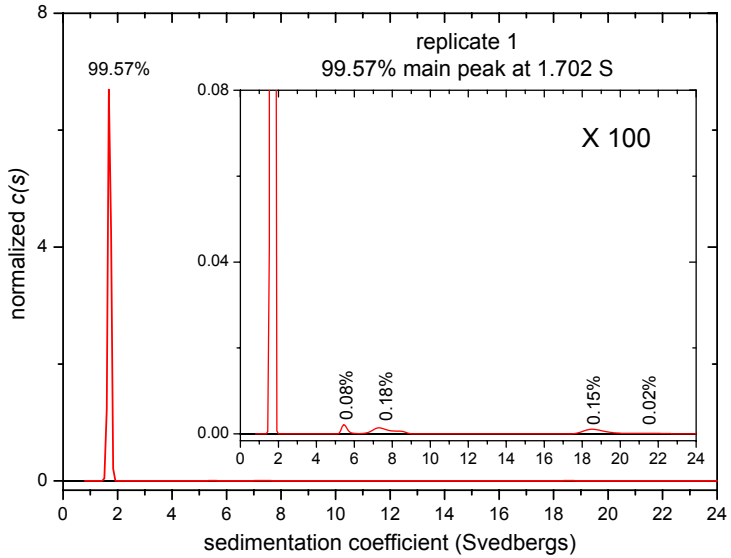


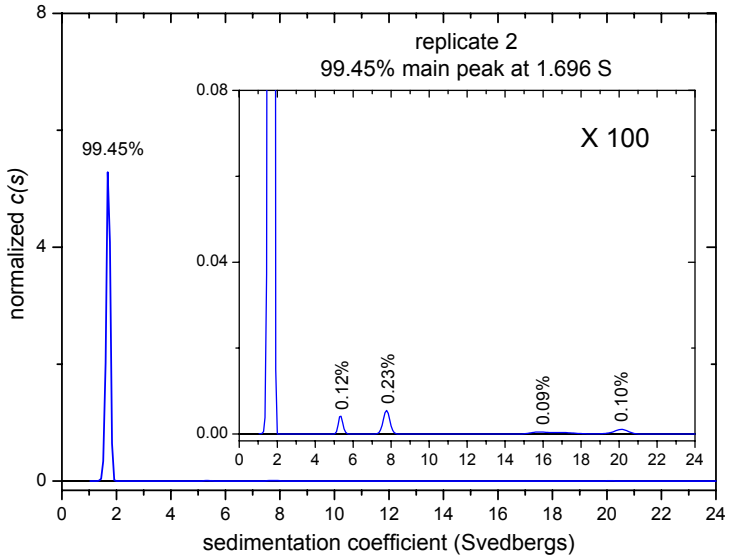


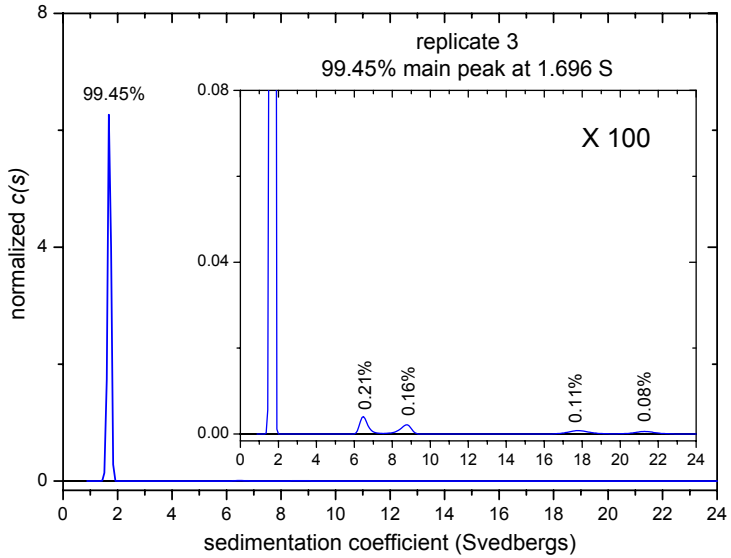


Three replicates of Protein X (monomer < 20 kDa) give 0.48% ± 0.06% total aggregate, 0.14% ± 0.07% species 1, 0.19% ± 0.04% species 2, 0.12% ± 0.07% species 3, 0.07% ± 0.04% species 4

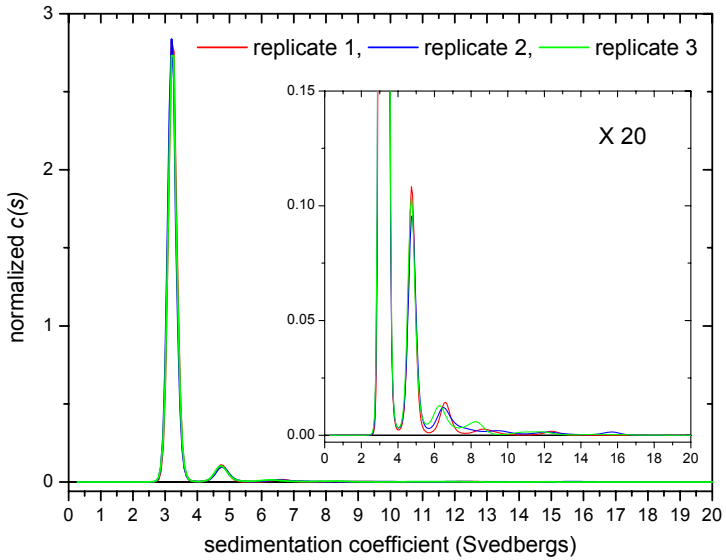


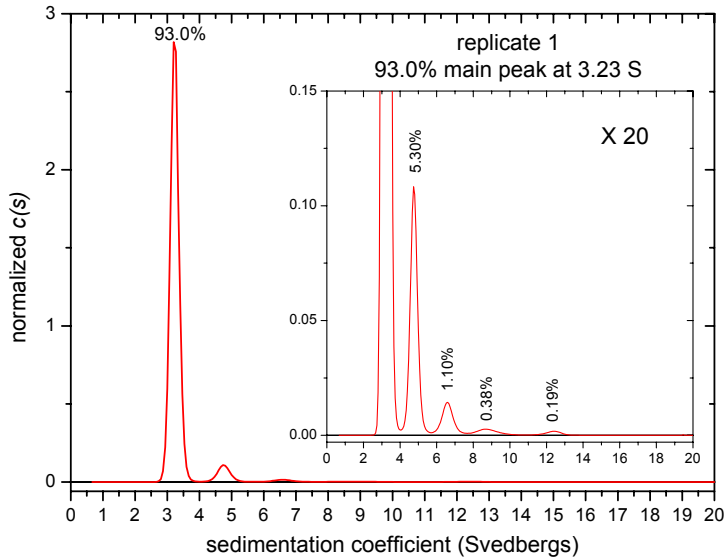


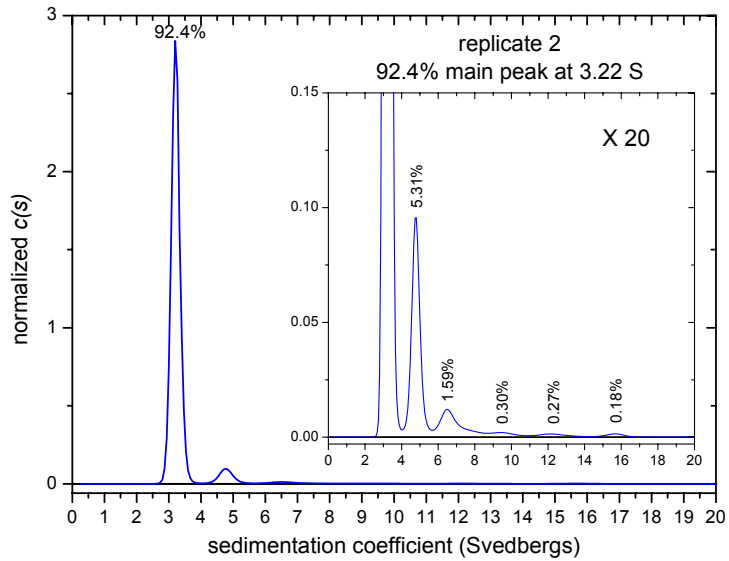


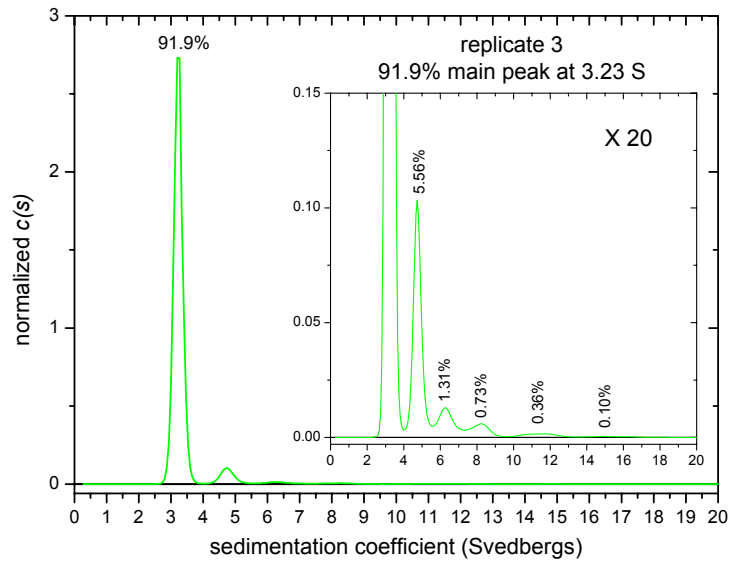


Three replicates of Protein Y (monomer < 50 kDa) give 7.6% ± 0.55% total aggregate, 5.4% ± 0.15% dimer, 1.33% ± 0.25% tetramer, 0.47% ± 0.23% hexamer, 0.27% ± 0.09% octamer, 0.09% ± 0.09% larger aggregates

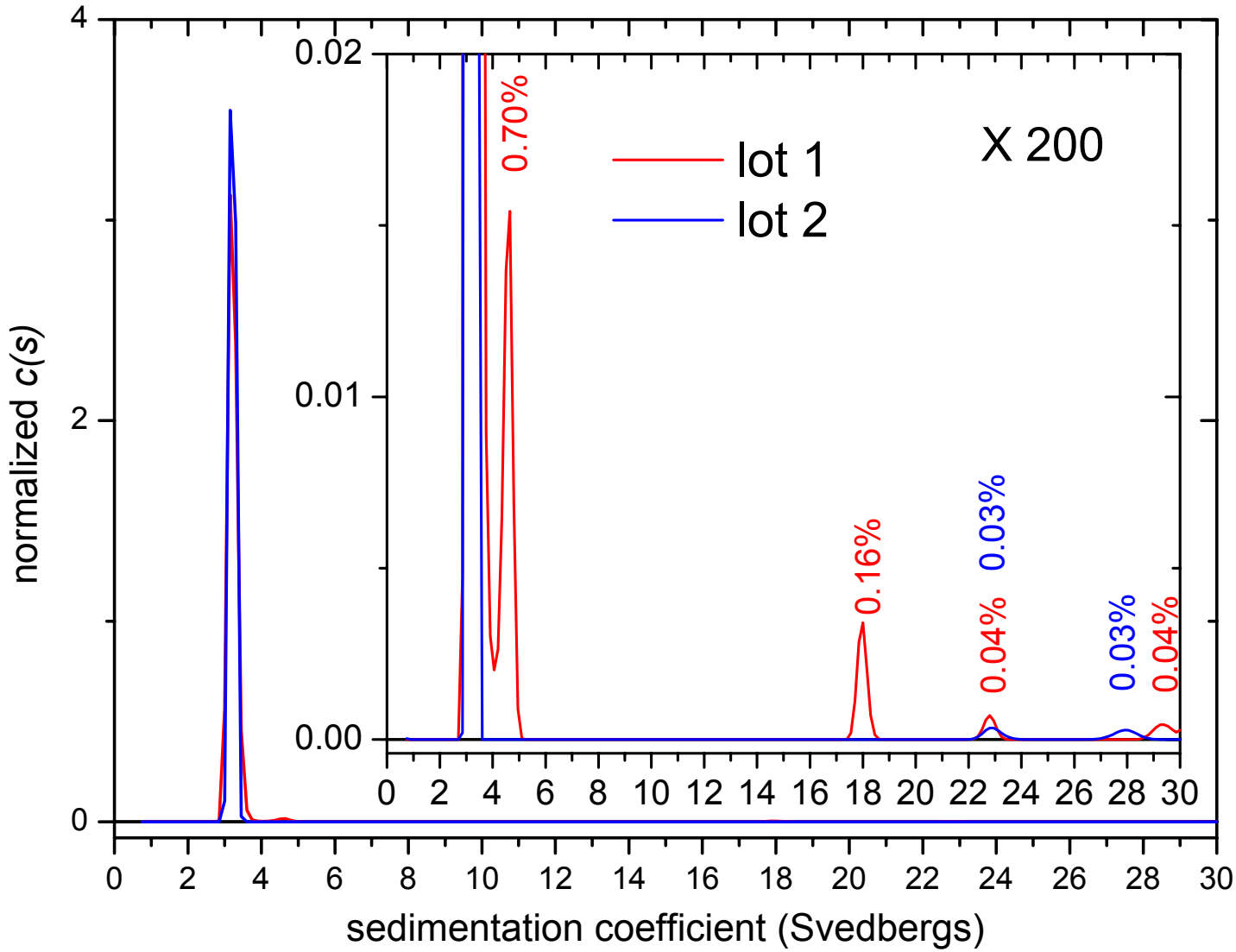




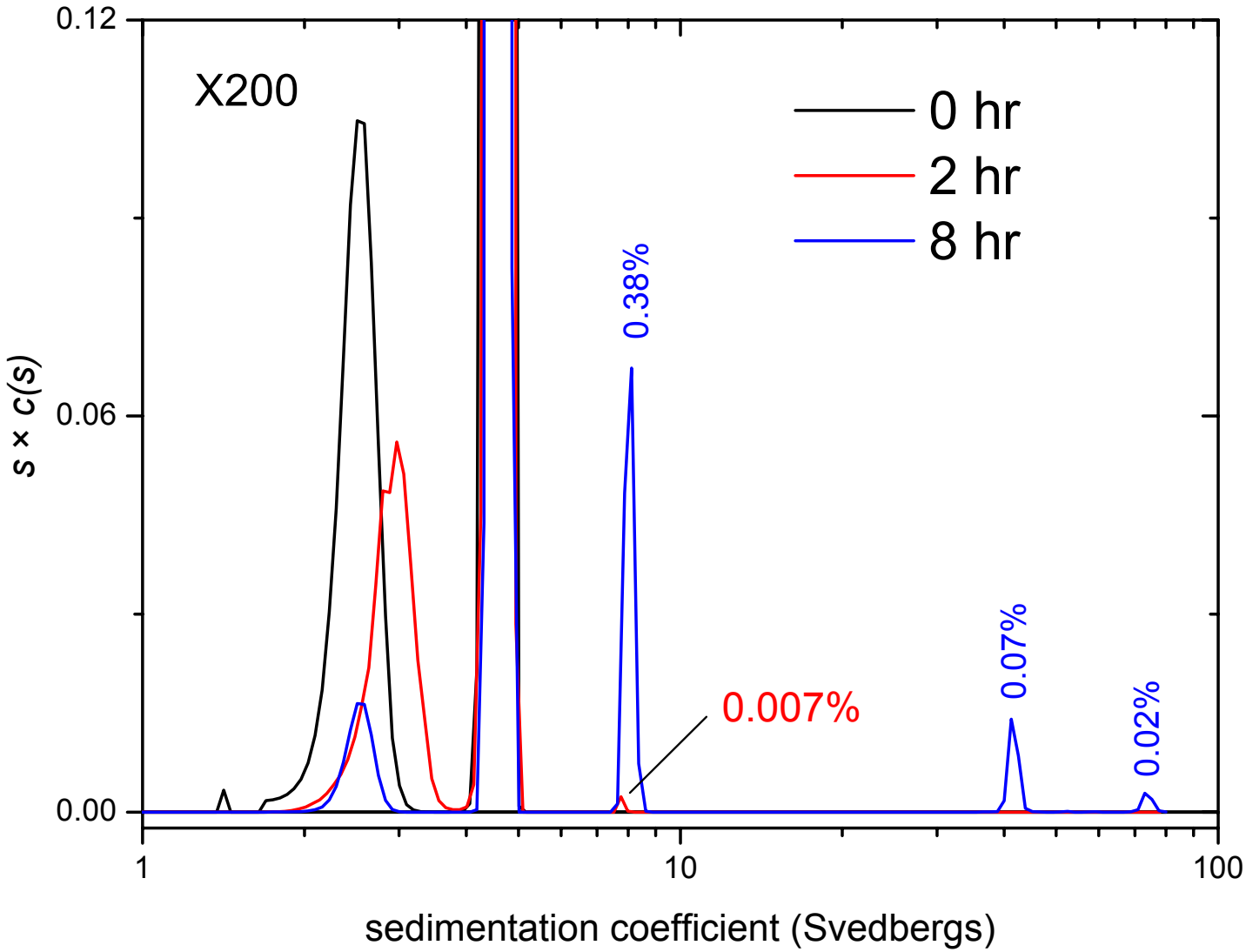




Protein Z: trace peaks (<0.05%) in multiple lots fall at similar positions, implying these are real species



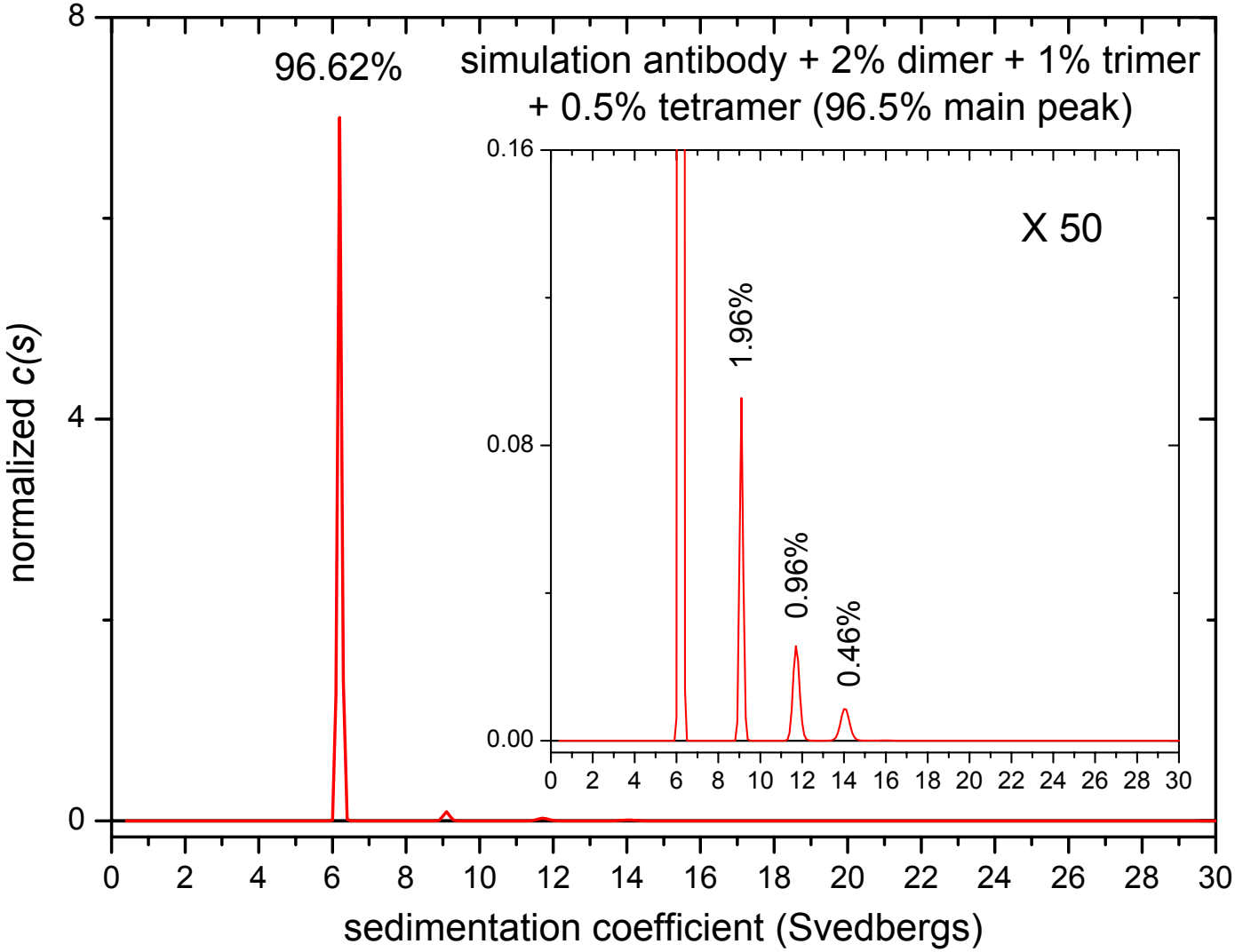
Changes in a lyophilized protein over time after re-hydration imply a peak initially detected at a level of only 0.007% is a real species



How can we predict the expected detection limits and reproducibility? We can exploit the fact that sedimentation velocity has a robust theory!

1. use the theory to simulate data for a known mixture, for example monomer + known percentages of irreversible aggregates
   - ★ mimic the data acquisition schedule, loading concentration, and noise level of real experiments
2. analyze the simulated data applying the same procedure as in real experiments, and see how well the results match the mixture that was simulated
3. repeat 1 & 2 several times to evaluate reproducibility

Analysis of simulated data for antibody + 2% dimer + 1% trimer + 0.5% tetramer



What sources of systematic error can prevent achieving high reproducibility and sensitivity?

1. Detergent micelles may produce peaks that overlap with species of interest
   - ★ Tween-80 micelles sediment around 2 S, and can often be detected by absorbance, particularly at wavelengths below 280 nm
   - ★ poor cancellation of detergent signals when detergent is present in both sample and reference channels is common
2. High levels of excipients (sucrose, sorbitol, amino acids) may produce significant density gradients at high r.p.m., slowing the sedimentation as the run proceeds
   - ★ in principal new models in the software can handle this, but we often lack the required data on the density and viscosity effects of the excipients

Other problems and sources of error, continued

3. Some proteins are easily damaged by UV exposure
   - ★ SV protocols now typically involve many more scans than they did in the past and hence much more UV exposure
   - ★ remember, the entire sample is exposed to light with every flash, not just the radius being measured at that time
4. Aggregates are generally quite sticky, and may get lost during transfer (on surfaces of vials, pipette tips, or the centrifuge cell)
   - ★ in theory multiple rinses should minimize this problem, if sufficient sample is available

Conclusions

1. Both experiment and theory indicate that individual aggregate species can be measured with a reproducibility of ±0.1% or better in some cases
   - resolution and reproducibility is generally higher for larger proteins like antibodies than for small cytokines
   - precision is poorer when many poorly-resolved aggregate species are present
   - it is normal for the positions of minor peaks to shift from sample to sample, and the software sometimes merges or omits minor peaks that are near the detection threshold
2. Large aggregates that sediment > ~3 times faster than monomer can generally be detected at levels of 0.1% or below
3. The levels of precision and sensitivity illustrated here cannot necessarily be achieved for all proteins, or by relatively inexperienced analysts