

Challenges in measuring comparability of aggregation and conformation for biosimilars (and often innovator products too!)

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ABSTRACT: There is much current interest in analytical methods for demonstrating comparability of biosimilars to the reference product. Heightened concerns about potential differences in immunogenicity make it particularly important to show comparability of product molecular conformation as well as aggregate content and distribution. However key biophysical tools such as analytical ultracentrifugation, light scattering, and circular dichroism (CD) may not be applicable to the final product due to interference from excipients. In such cases some biosimilar developers have tried to re-create the innovator's bulk drug substance by purifying the active ingredient out of the reference final product. While this approach may give material suitable for some analytical methods, we show that for erythropoietin (EPO) this creates a drug substance surrogate that is damaged and not equivalent with respect to aggregation. Further, in this case subjecting two different final products to the same re-purification protocol does not produce equivalent changes and thus would give a potentially invalid comparison.

Some specifics about excipient interference with AUC, light scattering, CD, and/or SEC will be discussed, with examples. Polysorbate 20 or 80 can be particularly troublesome when present at levels above ~0.3% and may cause false peaks in SEC, sedimentation velocity, or DLS. Further, because the detergent-associated impurities in product samples typically differ from those present in the formulation buffers it is often not possible to correct for the detergent effects by running control samples or buffer blanks. For CD the UV absorbance of simple salts such as citrate can cause problems. The strong UV absorbance of bacteriostatic agents present in multi-dose formulations can pose severe challenges for CD and AUC using absorbance detection.

Challenge 1: When bulk drug substance (DS) is not available for comparability, trying to re-create it from drug product may produce a compromised DS surrogate, as happened for EPO

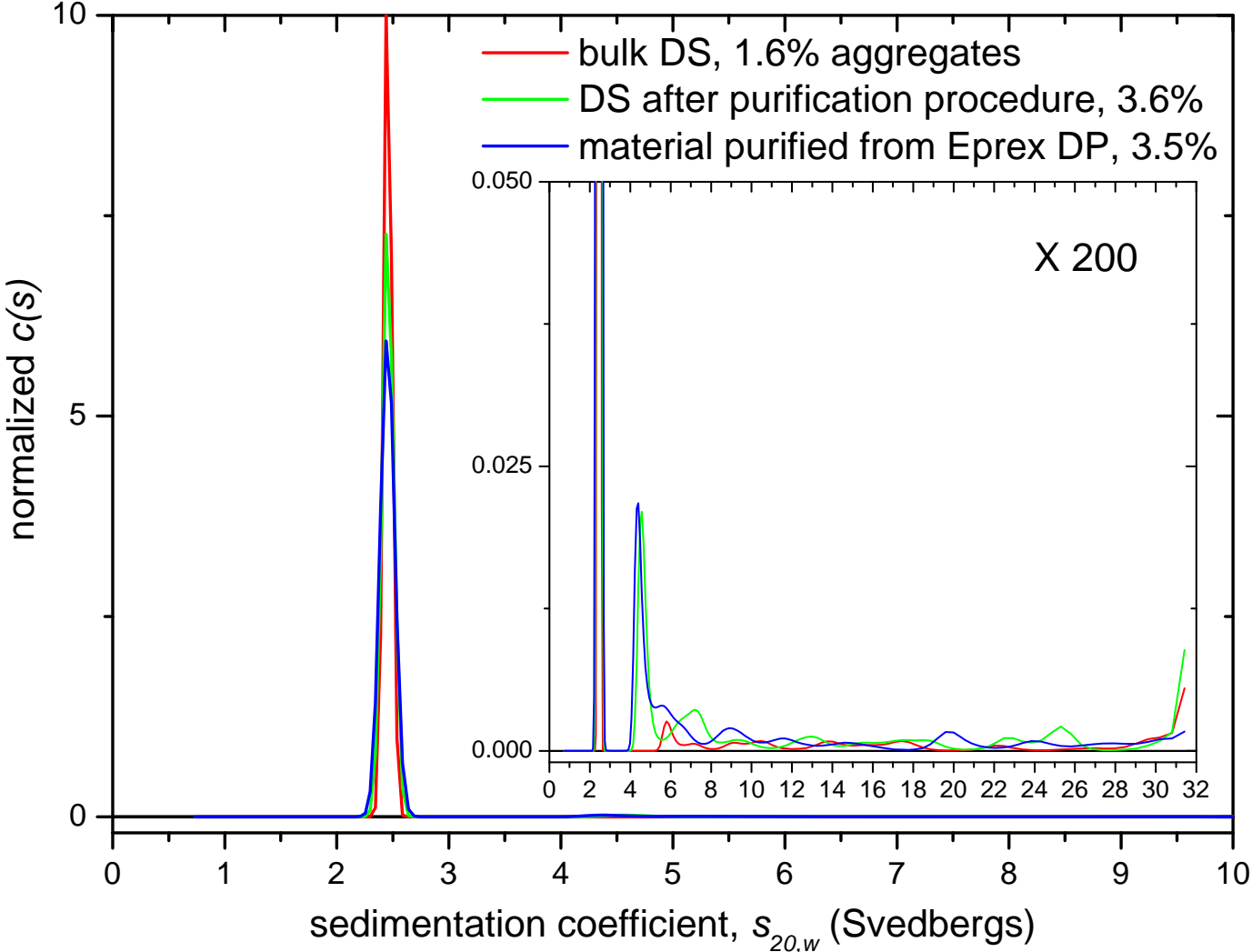
Amgen scientists purified EPO from Eporex[®] drug product (DP) for purposes of comparison to the DS for Amgen's EPO product, Epogen[®], and reported differences in conformation, aggregation, and spectroscopic properties between these products.¹ Subsequently in a collaboration with scientists at Centocor we showed that this purification procedure had damaged the protein and created a DS surrogate that was not equivalent to true Eporex DS. Subjecting either Eporex bulk DS or Eporex DP to this purification protocol caused increased aggregate levels.

Further, the pH 8.4 elution used by Amgen caused degradation of some component of the Eporex formulation, creating a new non-protein peak in the elution profile. The fact that this peak was not seen when Amgen tried to re-create an Eporex-like formulation shows that the components of that formulation were not equivalent to those in the commercial EPREX product samples.

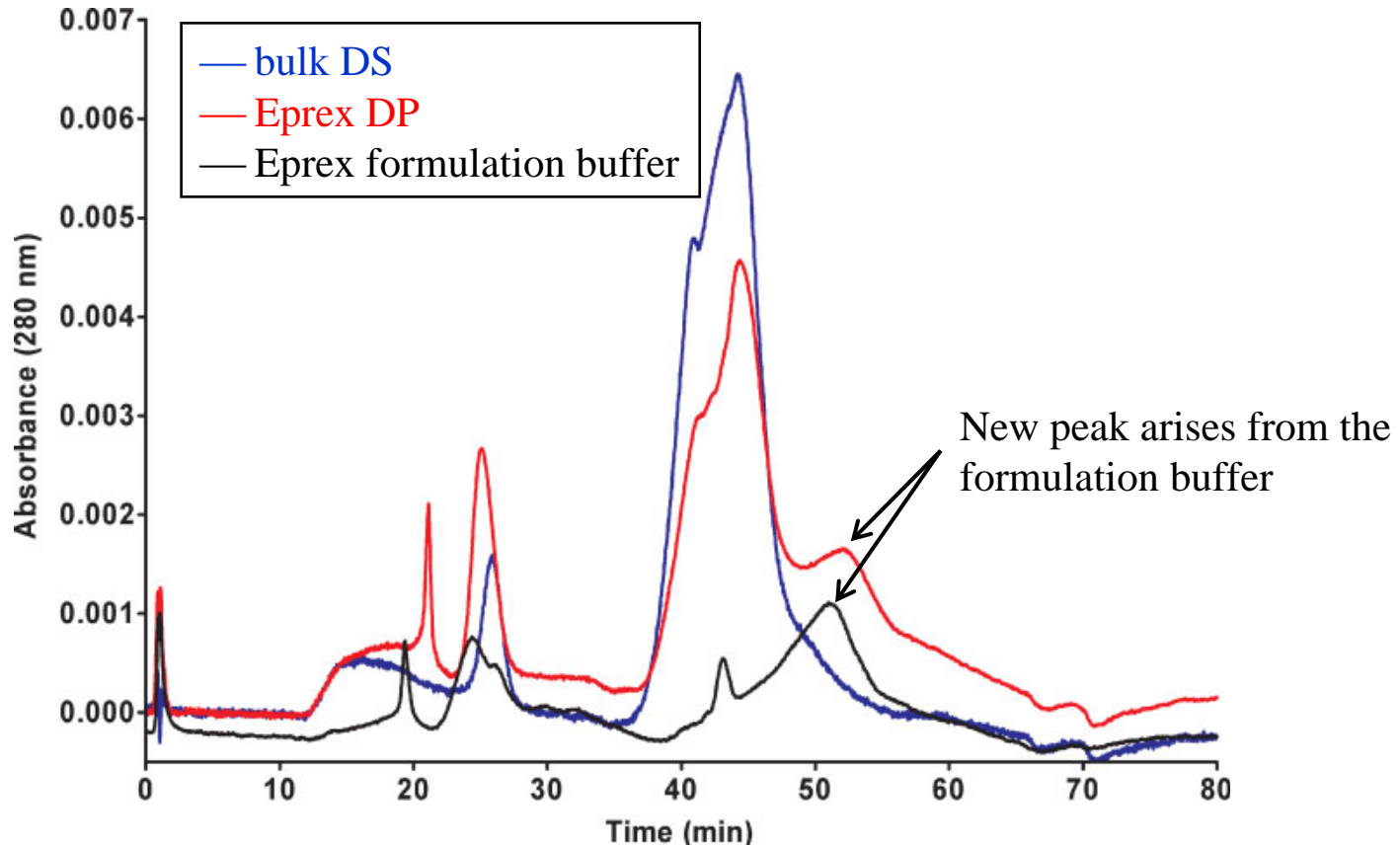
¹ Deechongkit, S., Aoki, K. H., Park, S. S., and Kerwin, B. A. (2006). Biophysical comparability of the same protein from different manufacturers: a case study using Epoetin alfa from Epogen and Eporex. *J. Pharm. Sci.* **95**, 1931-1943.

² Heavner, G. A., Arakawa, T., Philo, J. S., Calmann, M. A., and Labrenz, S. (2007). Protein isolated from biopharmaceutical formulations cannot be used for comparative studies: Follow-up to "a case study using Epoetin Alfa from Epogen and EPREX". *J. Pharm. Sci.* **96**, 3214-3225.

Sedimentation velocity shows that attempting to purify EPO from either DS or DP causes increased aggregation



The pH 8.4 elution buffer used to elute EPO in Amgen's purification protocol creates a new elution peak at 50 min that is due to chemical alteration of components in the formulation buffer.

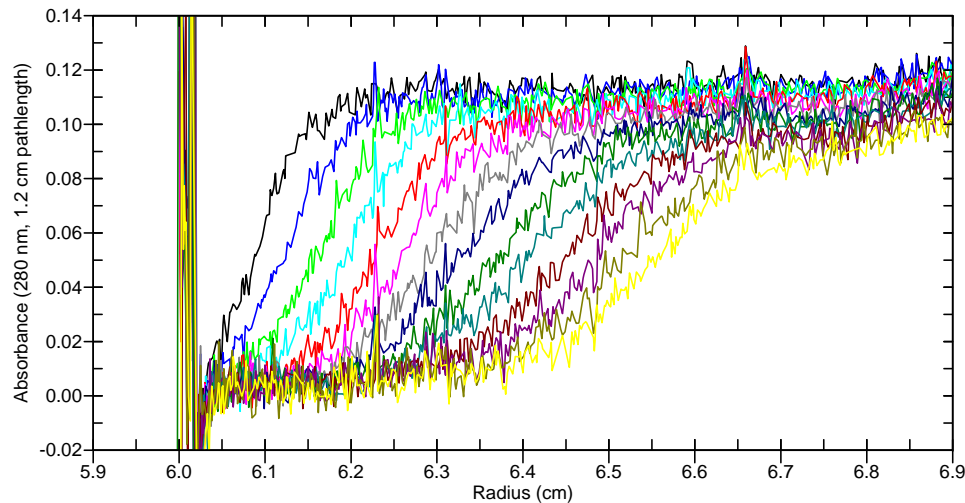


Hi-Trap elution profiles at 280 nm of EPREX bulk drug substance (blue), EPREX formulated product (red) and EPREX formulation buffer (black). The elution buffer is the Tris pH 8.4 buffer used by Deechongkit et al. (ref. 1) The non-protein, post-erythropoietin eluting peak observed by Deechongkit *et al.* at ca. 50 min is present in EPREX formulated product and EPREX formulation buffer but not in EPREX bulk drug substance, indicating that its source is the formulation buffer

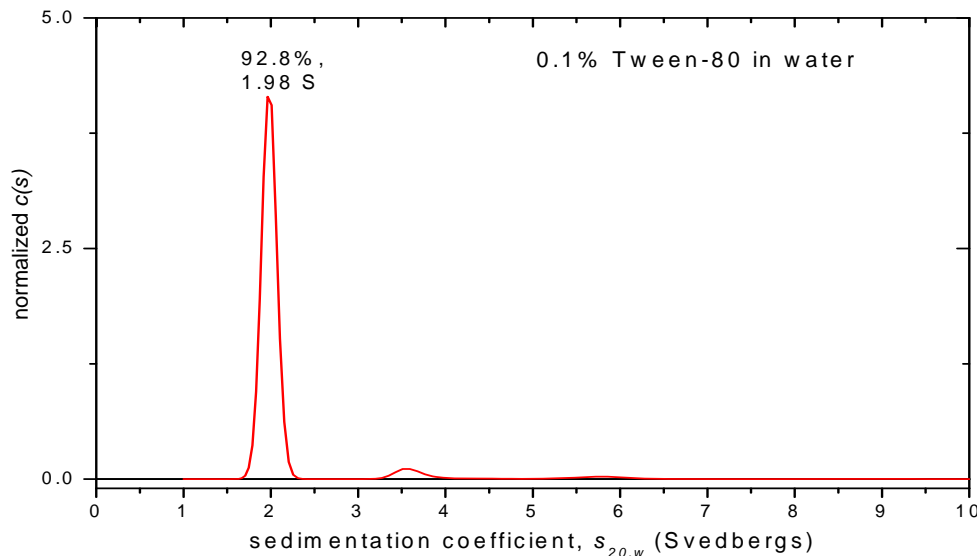
Challenge 2: Interference from Tween

Tween-20 and/or Tween-80 are common excipients that can interfere with comparability analysis or product characterization via AUC, light scattering, and SEC.

In sedimentation velocity with absorbance detection, only those Tween micelles that contain UV-absorbing impurities are detected

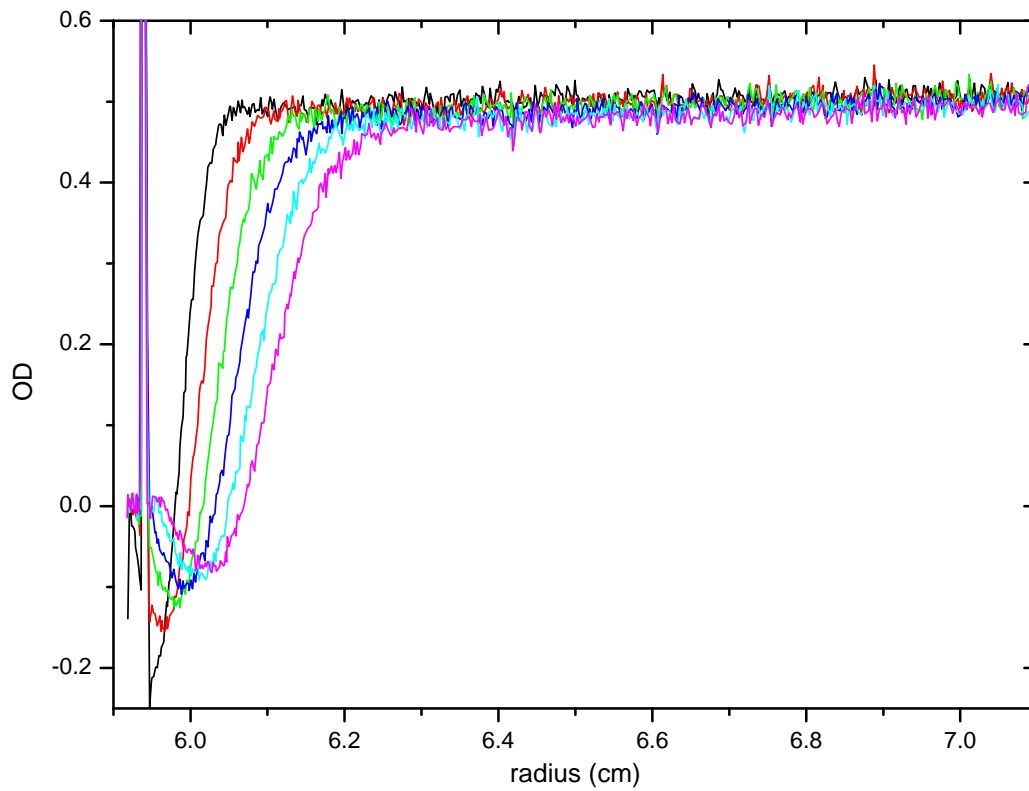


Some velocity scans at 280 nm and 60,000 rpm for a Tween-80 lot containing relatively high levels of impurities at 0.1% (wt/vol) in water.



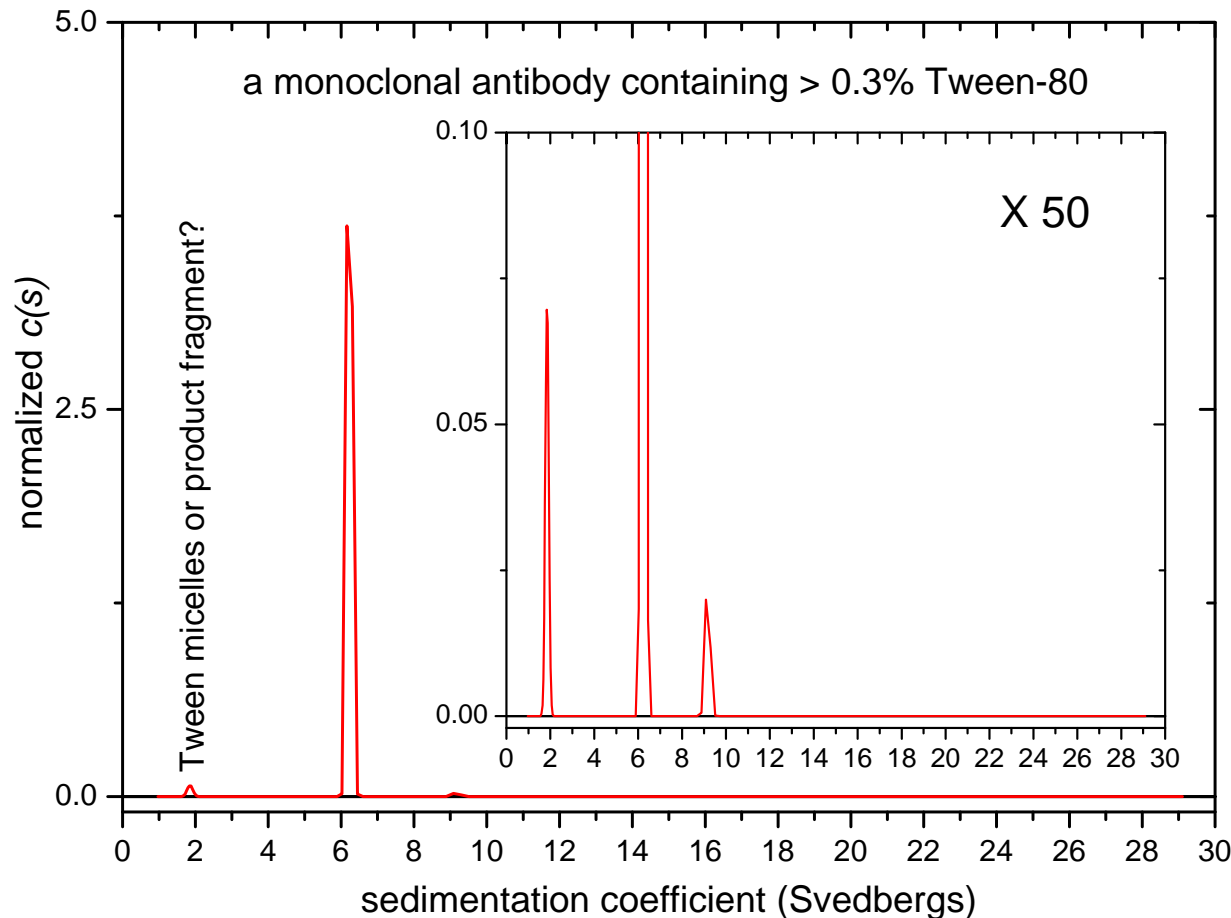
Tween-80 micelles typically sediment at about 2 S, a rate comparable to that for a protein of about 20 kDa. As we see here minor amounts of faster-sedimenting forms are also sometimes detected.

Because it is really the UV-absorbing impurities in Tween that are detected (possibly including species mobilized from container surfaces), and because those impurities affect the micelle sedimentation coefficient, it is quite difficult to provide a matching reference sample to cancel out the Tween signals

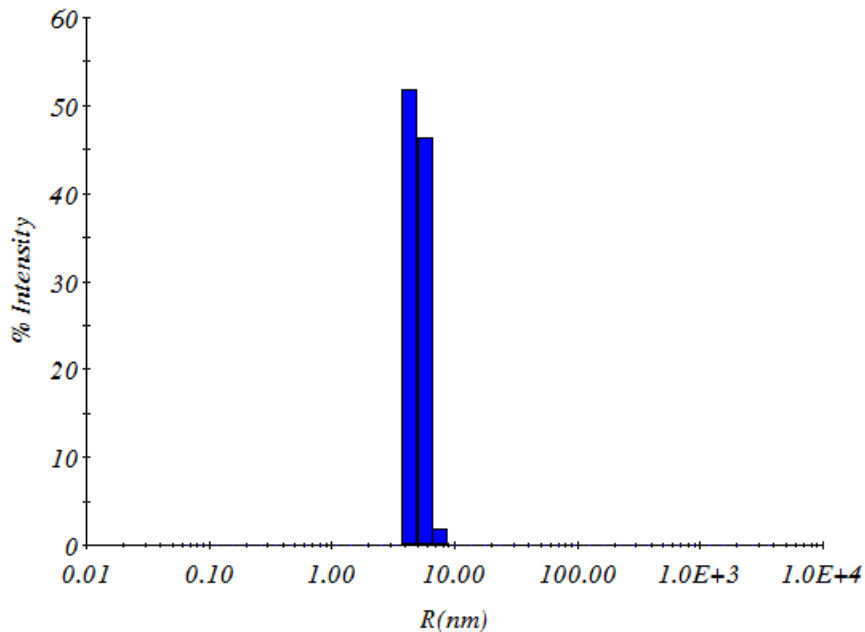


Some sedimentation velocity scans early in the run for a protein containing relatively high levels of Tween-20 (more than 0.2%). The buffer sample loaded into the reference channel, whose OD is subtracted point-by-point during data acquisition, was a poor match for the actual protein sample, leading to the negative OD readings seen here.

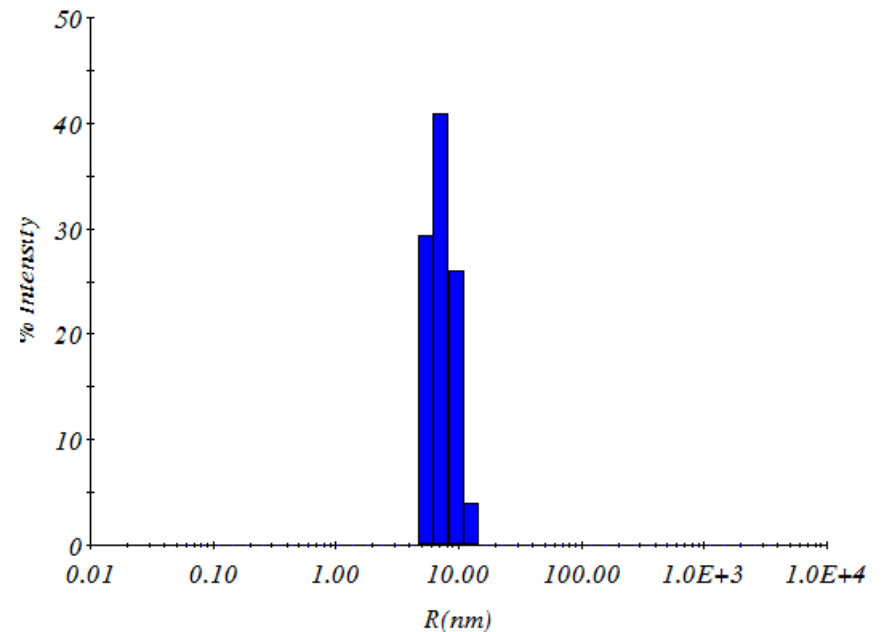
Therefore for SV analysis of samples containing 0.1% or more of Tween it is not unusual to see a peak at a sedimentation coefficient near that expected for the micelles (~2 S). Thus for a peptide product the micelles could be mistaken for an aggregate, while for antibodies they could be mistaken for a product fragment, as shown here.



In SV the Tween micelles sediment like a small protein, but in dynamic light scattering (DLS) they have a hydrodynamic size near that for an antibody monomer. The extent of Tween interference for DLS depends strongly on the product molecular weight and concentration.

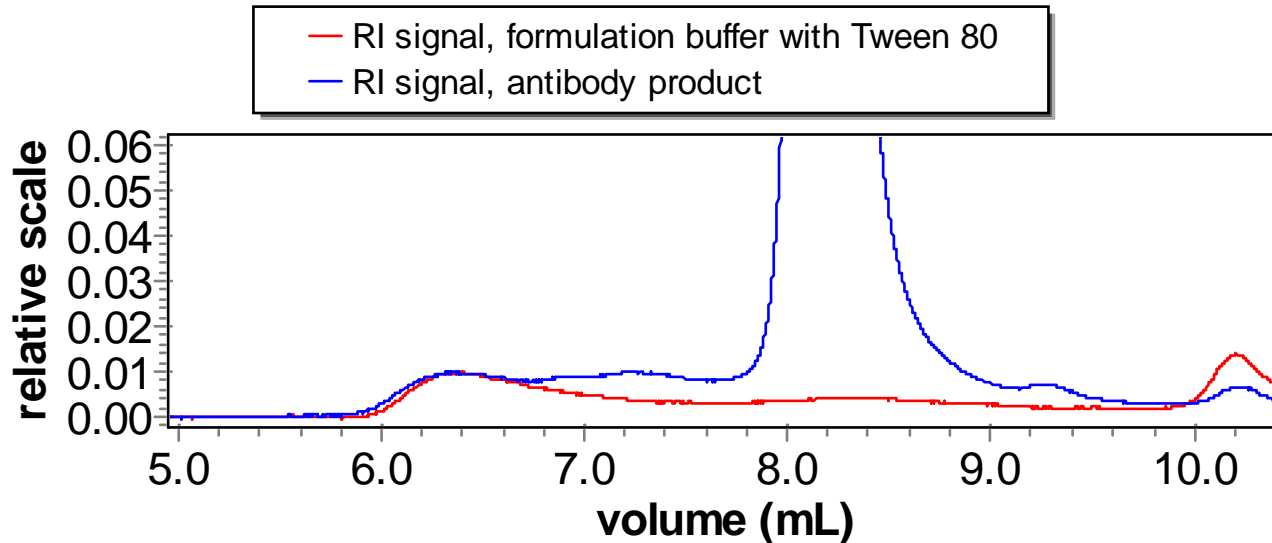


The micelles in this formulation buffer containing 0.1% Tween-80 show up clearly at a mean radius of 5.06 nm, corresponding to a globular protein of about 150 kDa.

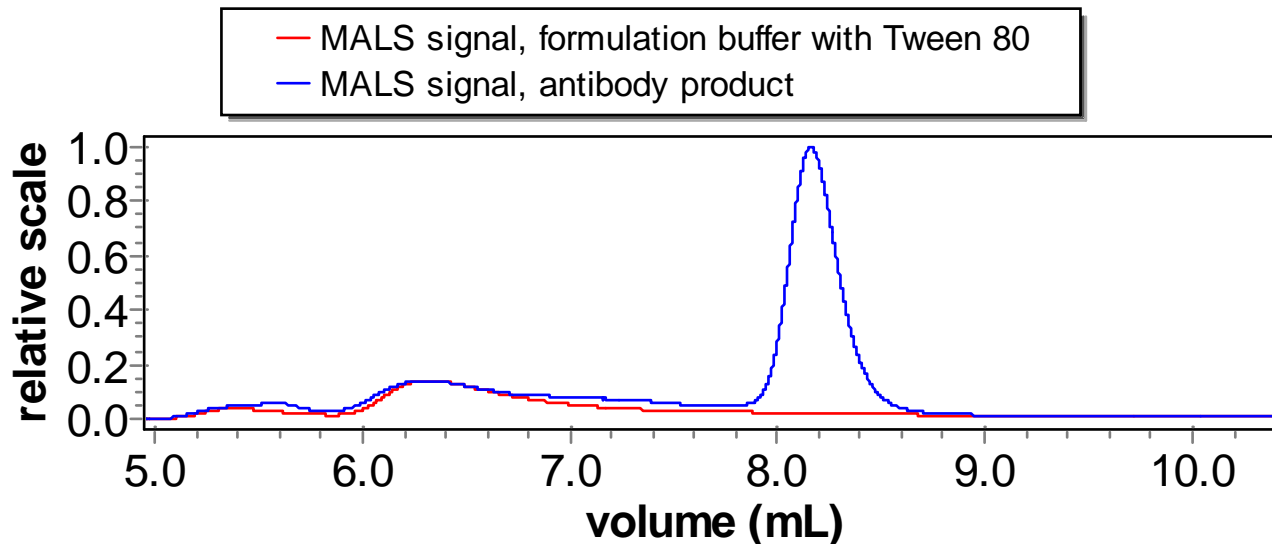


Clearly the micelle peak overlaps somewhat with this product peak (mean radius 7.59 nm). However in this case the distortion of the product peak should be quite small because the scattering intensity from the product is ~25X higher.

Tween can also cause significant interference for SEC and SEC-MALS, often eluting in many regions of the chromatogram.

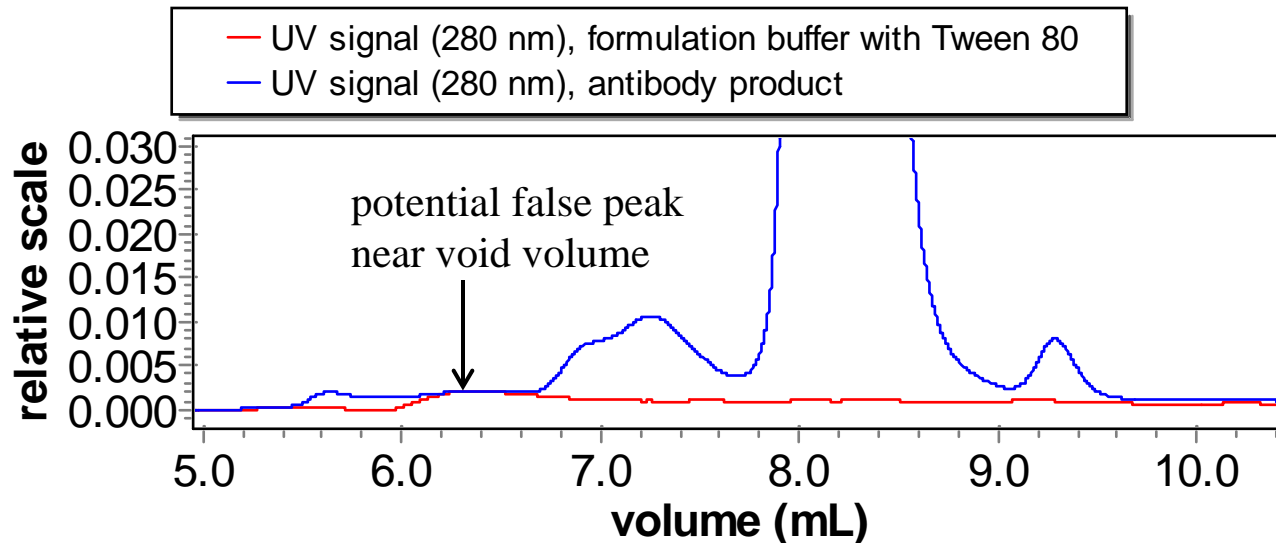


The RI detector shows that Tween 80 is eluting across the whole chromatogram, producing signals comparable to those from the minor components in the product.



The Tween contributions to the light scattering signals are even larger. The high level of Tween in this product makes it impossible to identify the stoichiometry of the aggregates via MALS, and even distorts the data for the main peak.

Tween can even cause problems for standard SEC with absorbance detection at 280 nm



See also:

Hermeling, S., Schellekens, H., Crommelin, D. J. A., and Jiskoot, W. (2003). Micelle-associated protein in epoetin formulations: A risk factor for immunogenicity? *Pharm. Res.* **20**, 1903-1907.

Heavner, G. A. (2006). Reaction to the paper: Interaction of polysorbate 80 with erythropoietin: A case study in protein-surfactant interactions - Rebuttal letter. *Pharm. Res.* **23**, 643-644.

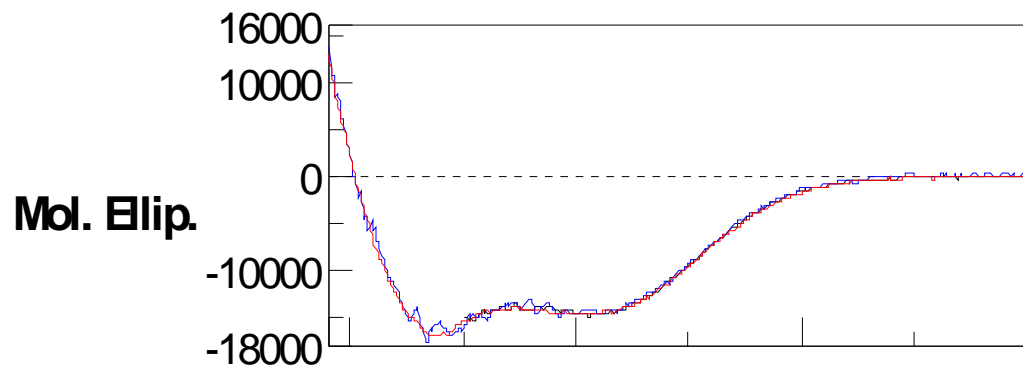
Challenge 3: Excipient interference with circular dichroism (CD) due to high background absorbance

Circular dichroism spectra, as well as thermal unfolding measured by CD, are often used to establish comparability of protein conformation. However even NaCl produces significant absorbance that may preclude collecting far-UV data below 200 nm, and common buffers such as acetate and citrate absorb strongly at much higher wavelengths.

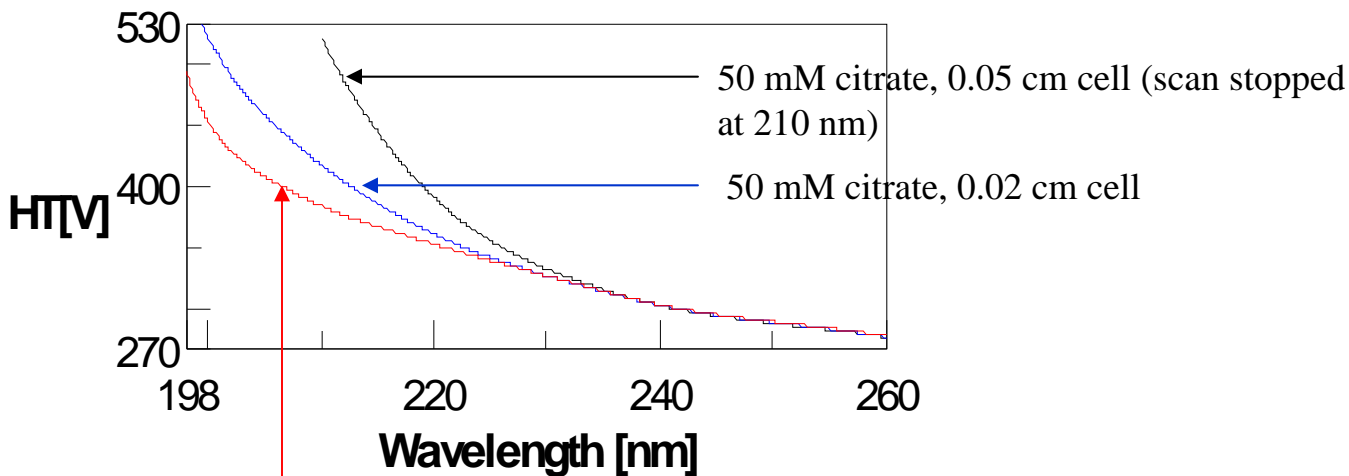
The absorbance of impurities present in Tween are also potentially problematic, particularly if Tween is present at levels above ~0.2%. Common anti-microbials such as benzyl alcohol or phenol not only introduce significant absorbance, but if they actually bind to the protein it is possible that an asymmetric binding environment will induce CD signals arising from the bound preservative.

To some extent this interference can be reduced by using CD cells with very short pathlengths (0.01-0.02 cm), but that then requires correspondingly higher protein concentrations (which may not be available for high-potency drugs like cytokines).

For 50 mM citrate buffer the far-UV CD spectra are noisy even when using a cell pathlength of only 0.02 cm; for PBS 0.05 cm gives good data.

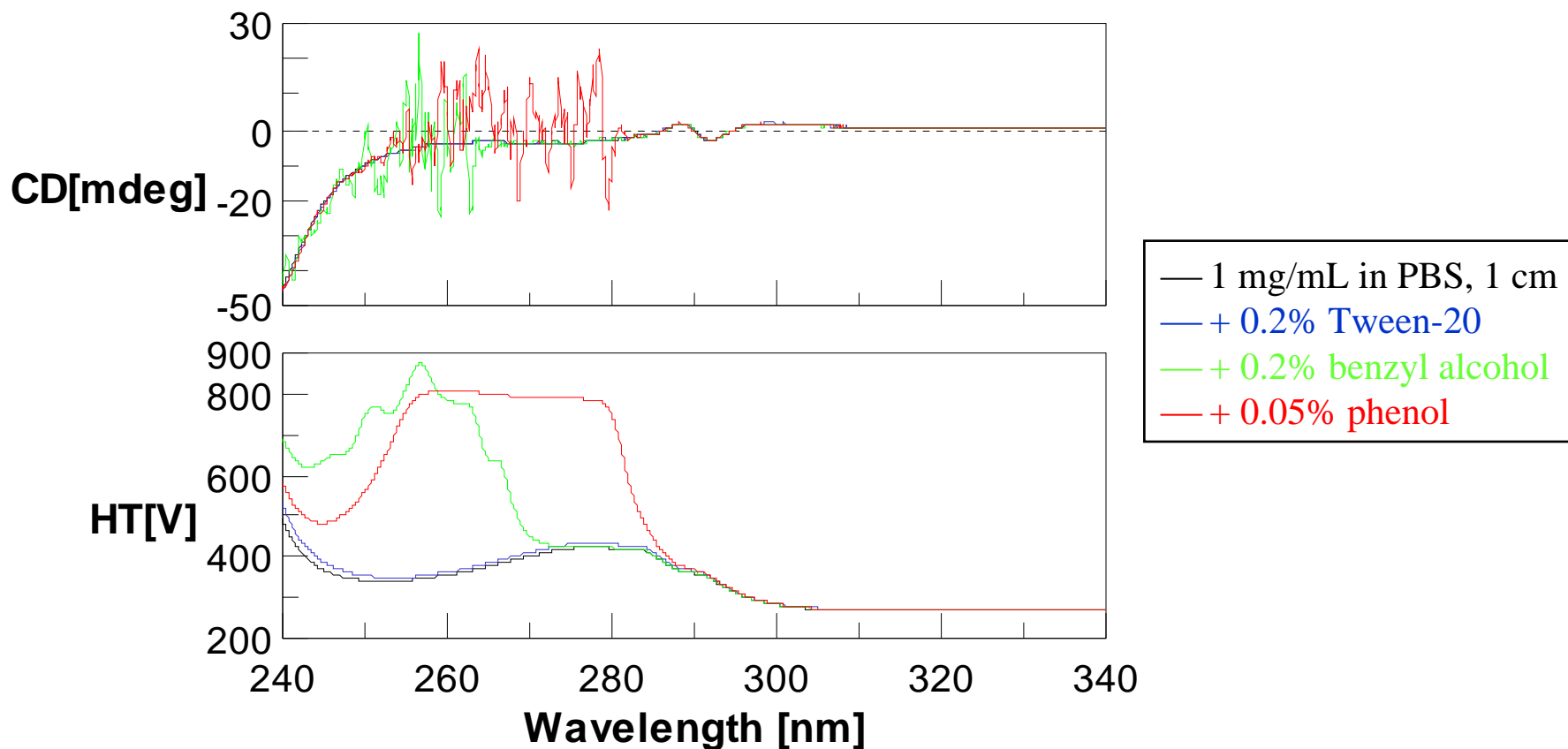


Note: this graph monitors the photomultiplier voltage, which rises as the OD rises.



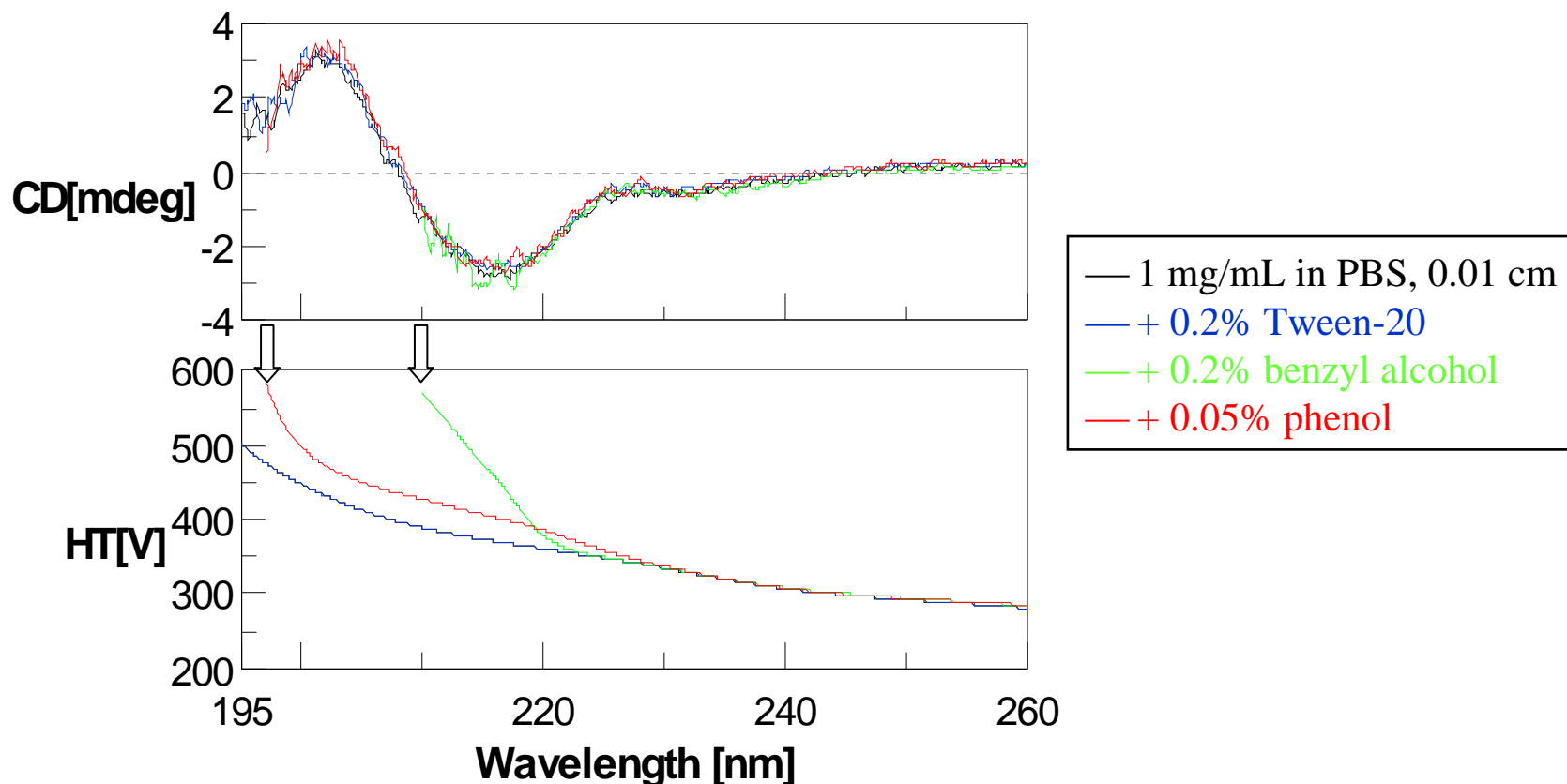
8 mM phosphate, 137 mM NaCl, 0.05 cm cell

Effects of Tween-20 and preservatives on near-UV CD for an antibody:
high excipient absorbance gives high noise and non-linear CD signals.



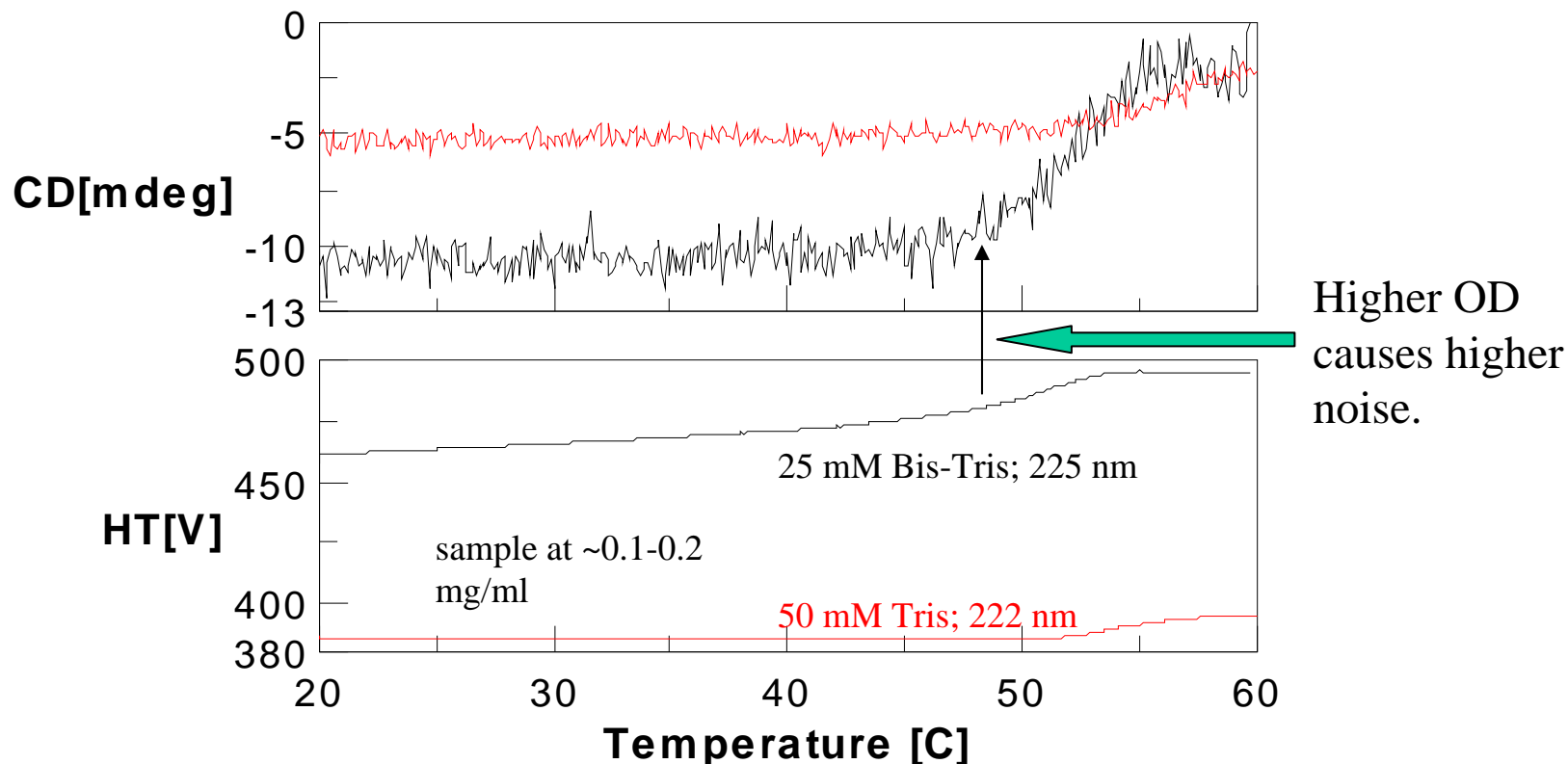
While the impurities in the Tween-20 do slightly increase the background absorbance, the CD spectrum is unaffected. Benzyl alcohol blocks nearly all the light from ~240-265 nm, while phenol does so from 250-282 nm, but the protein CD spectrum is identical outside those blocked regions.

By using a 0.01 cm cell for far-UV spectra 0.2% Tween-20 has no effect, and it is possible to acquire essentially full spectra with 0.05% phenol. However with 0.2% benzyl alcohol it is only possible to go down to 210 nm. The excipients do not alter the spectra over the regions that can be acquired.



Due to the high absorbance for the samples containing phenol or benzyl alcohol the scans were terminated at the wavelengths indicated by the arrows.

Unfortunately the shortest pathlength cells cannot be temperature controlled* so high excipient absorbance is more of a problem for thermal unfolding studies. Here is an example where 25 mM Bis-Tris causes problems for a thermal unfolding study in a 0.1 cm cell.



*For room temperature studies we have cells with 0.01, 0.02, 0.05, 0.2, and 1 cm pathlength; temperature-controlled cells are 0.1 or 1 cm pathlength.

Acknowledgements

- The EPO studies were done in collaboration with George Heavner (Centocor R&D), Melissa Calmann (Global Biologics Supply) and Steven Labrenz (Global Biologics Supply)