MOSBRI
Molecular-Scale Biophysics
Research Infrastructure

ESC1:
Circular dichroism:
best practice and data analysis

Lecture 3: What can go wrong and common mistakes.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

Baseline scans

- A baseline scan for a sample should be performed on all other components than the e.g. protein in the sample (cell, buffer, other additives etc.). This is to ensure that if any of the other components have a CD signal that this can be subtracted out (as well as any contribution to absorbance).
- Good practice to:
  - Always take a baseline scan before a sample.
  - Measure the baseline and sample scans in the same cell.
  - Take at least 3 scans.
  - Measure out to long wavelengths where there is no CD signal from the sample, and check that the sample and baseline scans match at these long wavelengths.
- There's no guarantee that you will get the same baseline the next day, and without a matching baseline you can't trust your sample data!

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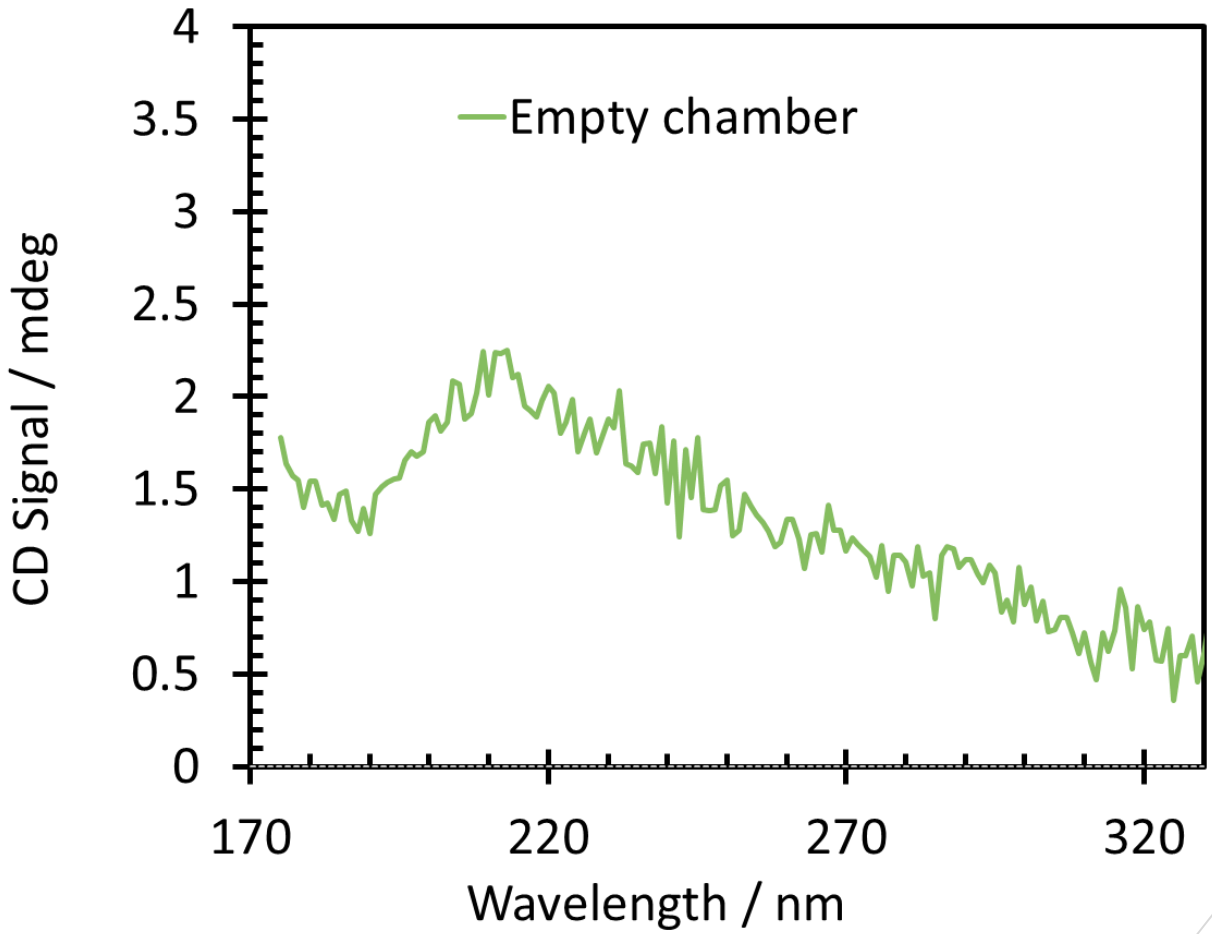
CD Baseline

The baseline of the spectrometer

- A scan of the system with no cell/sample



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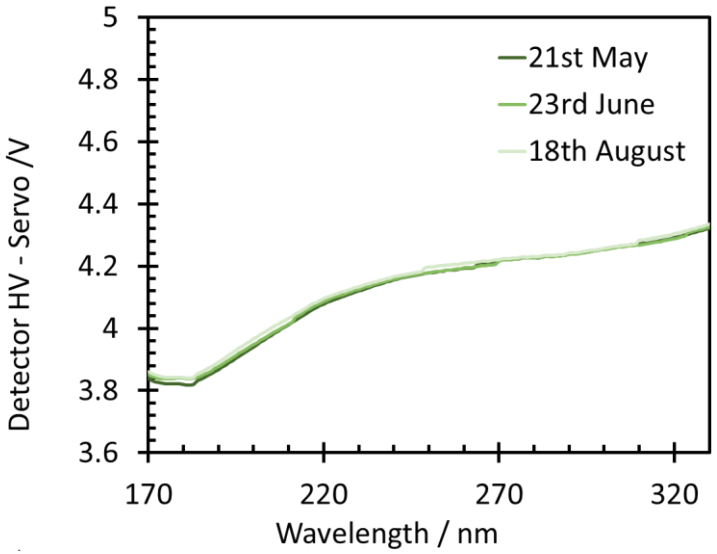
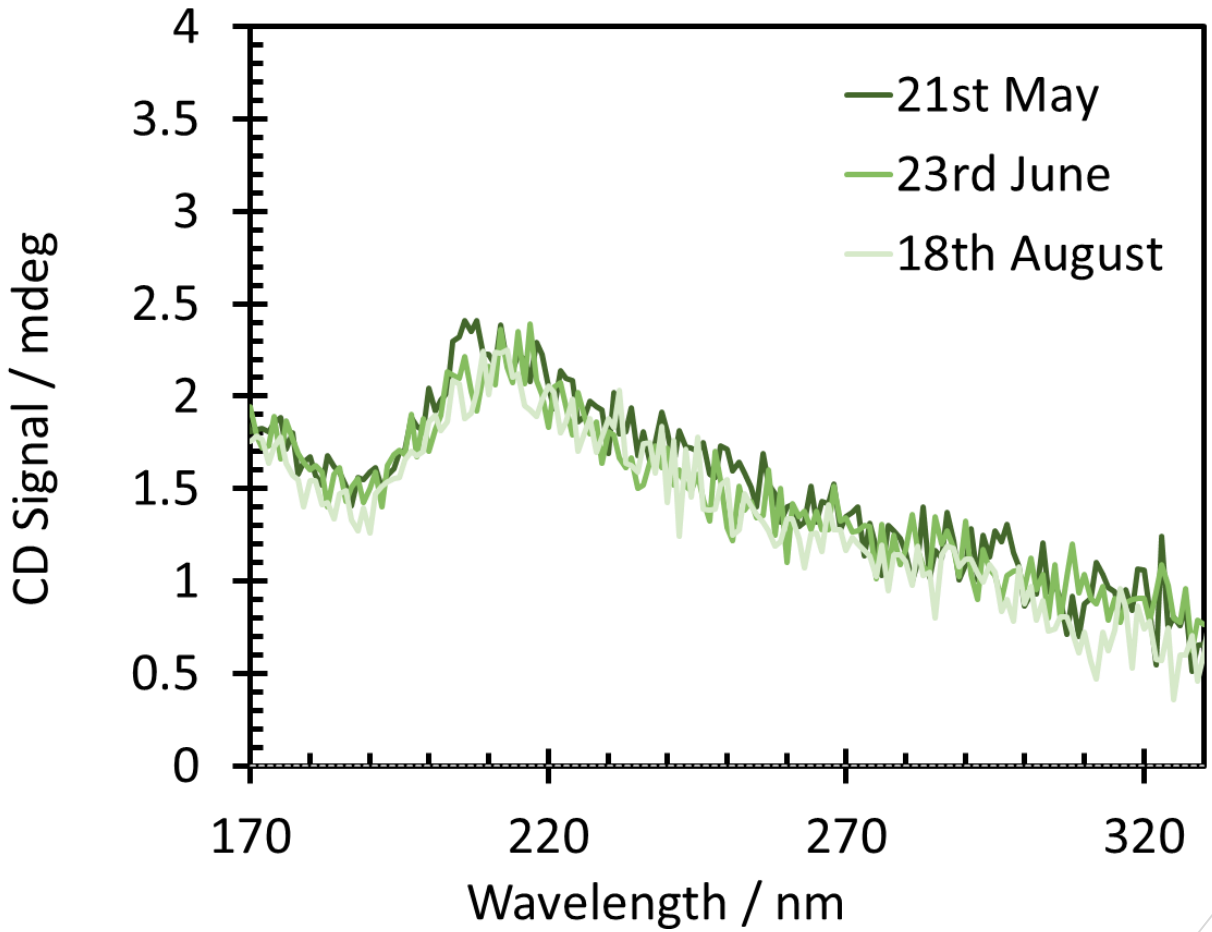
CD Baseline

The baseline of the spectrometer – stability over time

- A scan of the system with no cell/sample.
- Can also check the light intensity





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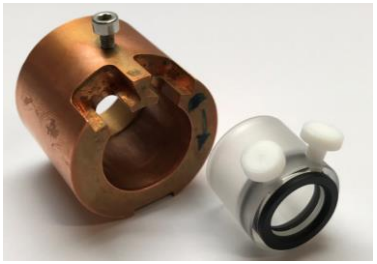
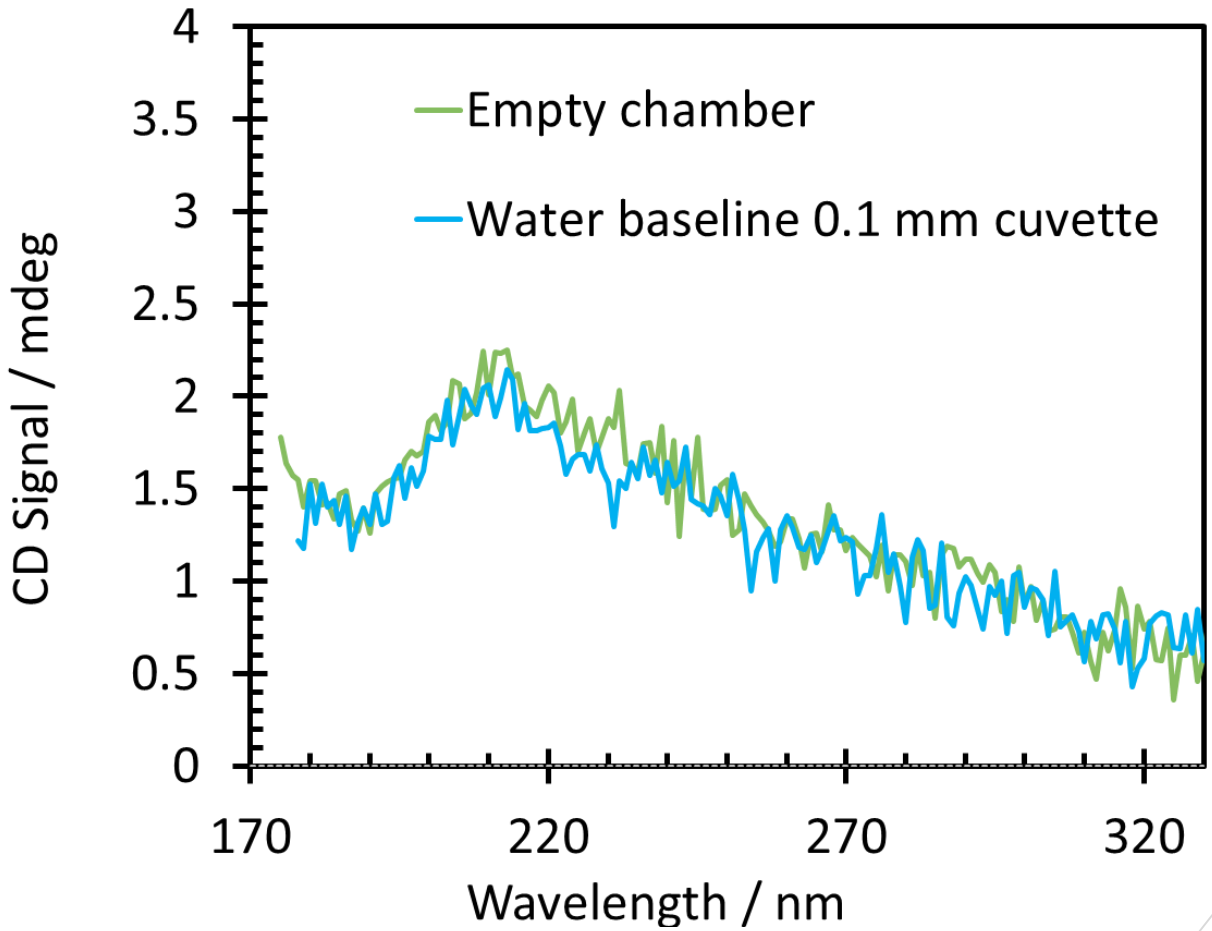
CD Baseline

The baseline of the spectrometer – with the cell



- A scan of the system with no cell/sample
- Ideal situation - the same (or at least similar) baseline is reproduced when measuring with a cell with water in



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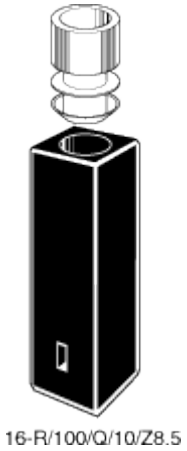
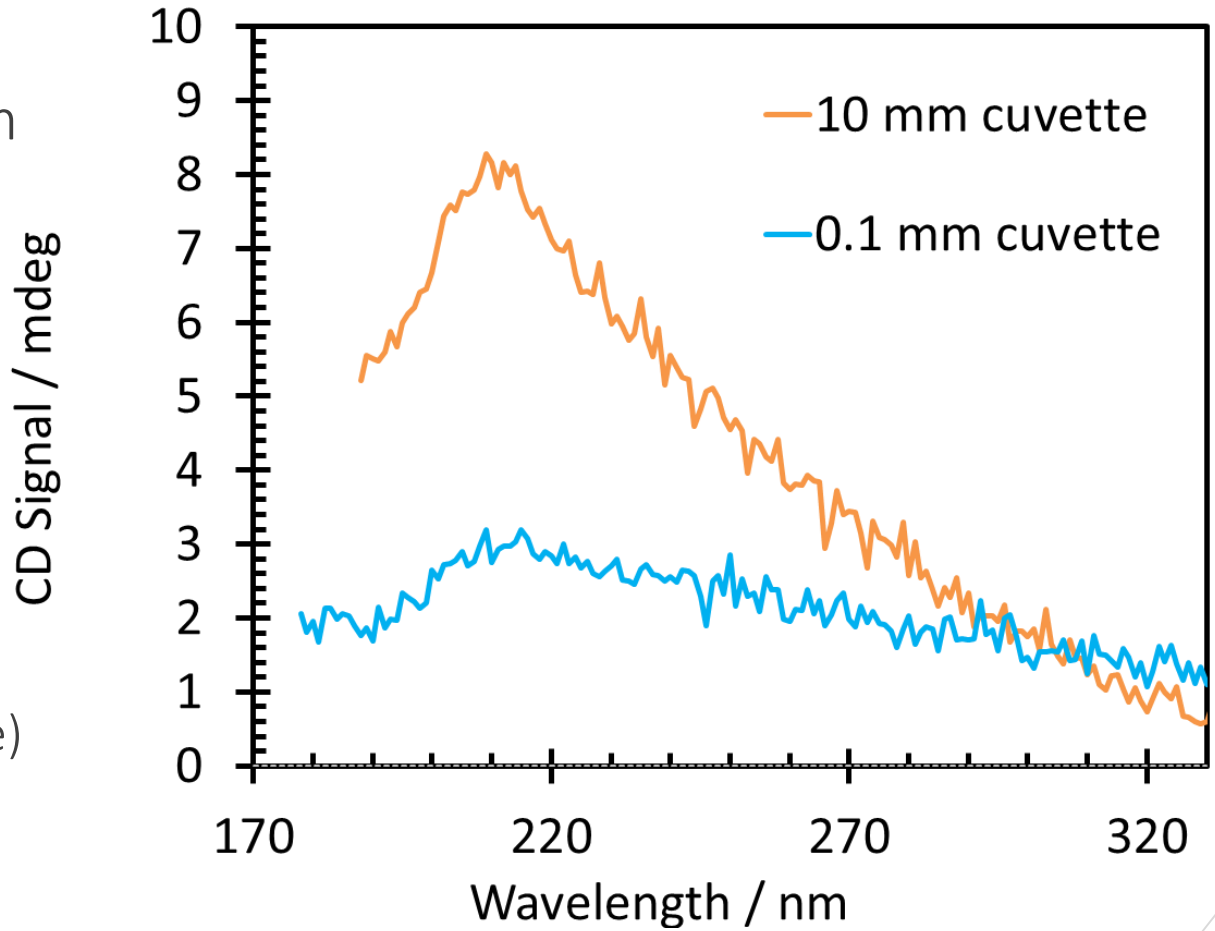
CD Baseline

The baseline of the spectrometer – with the cell

- Not all cells will have the same baseline as the system



- (10 mm sub-micro quartz cuvette)



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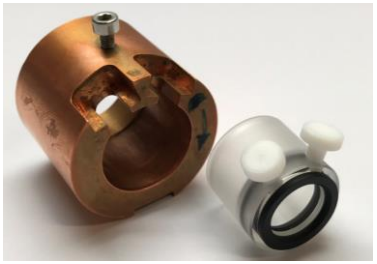
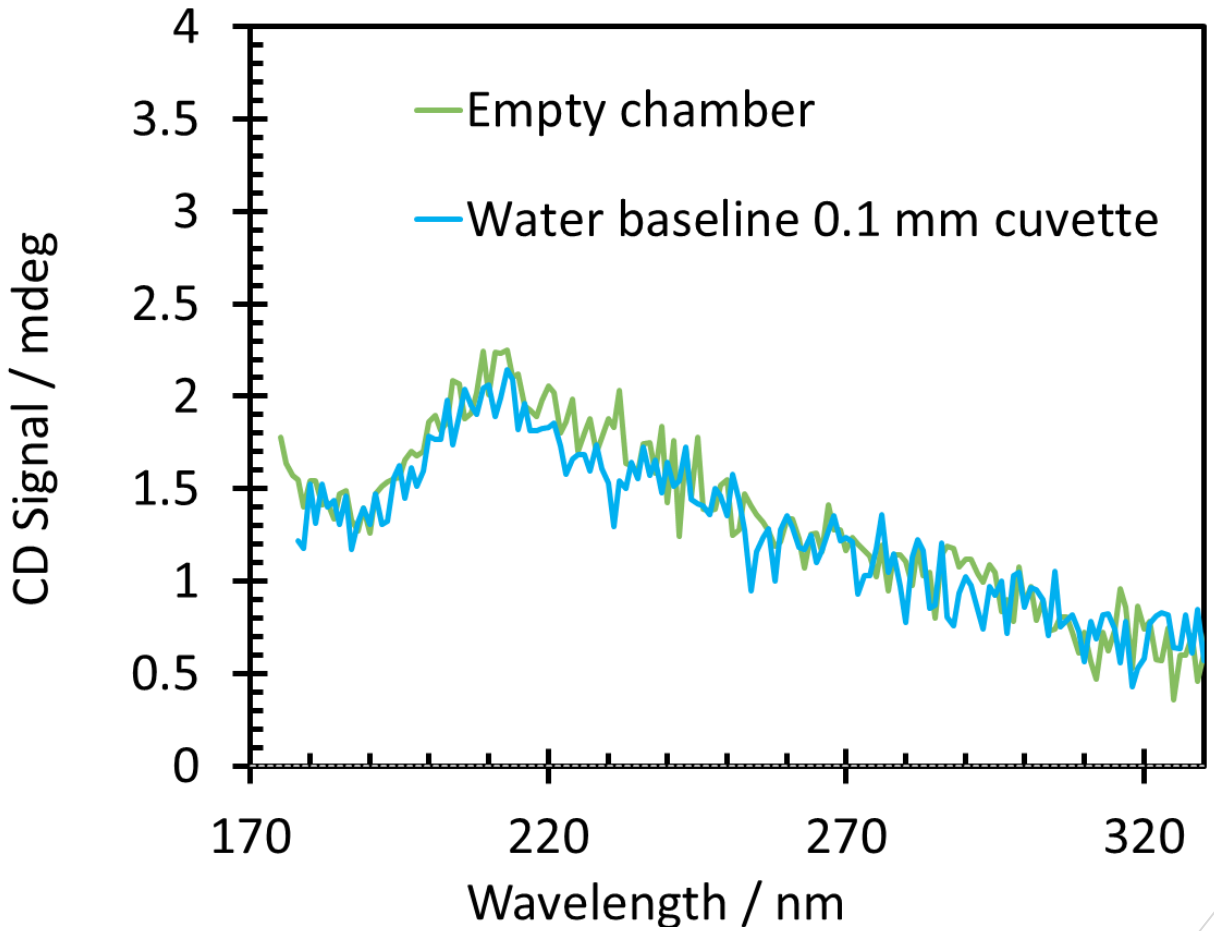
CD Baseline

The baseline of the spectrometer – with the cell



- A scan of the system with no cell/sample
- Ideal situation - the same (or at least similar) baseline is reproduced when measuring with a cell with water in
- Want to ensure that this underlying baseline of the cell is consistent for all sample measurements



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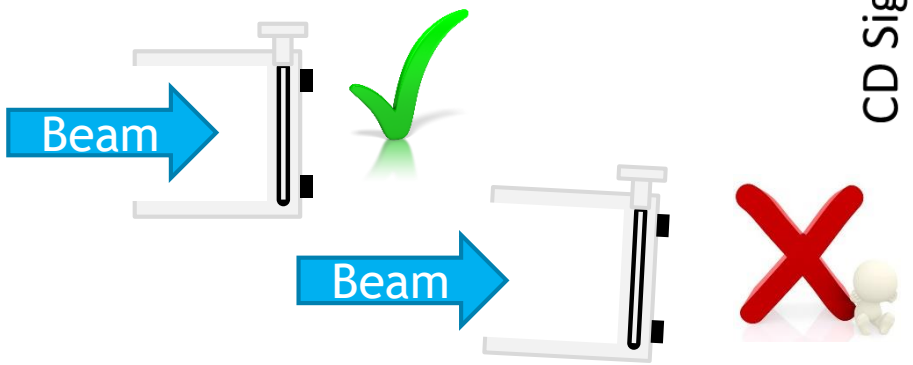
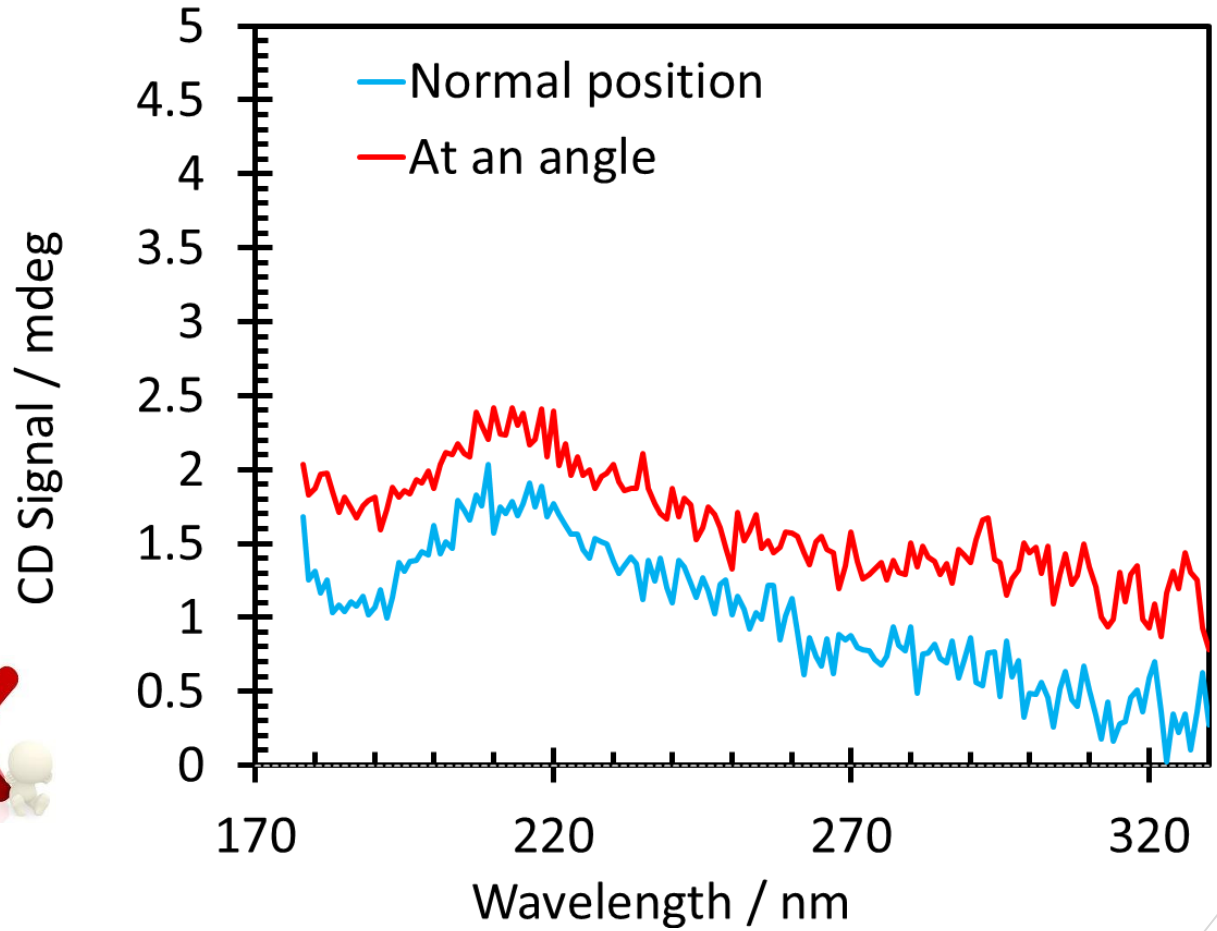
Baselines – what can go wrong?

Factors affecting the baseline shape/stability of the system

- Cell position/**angle** with respect to the beam





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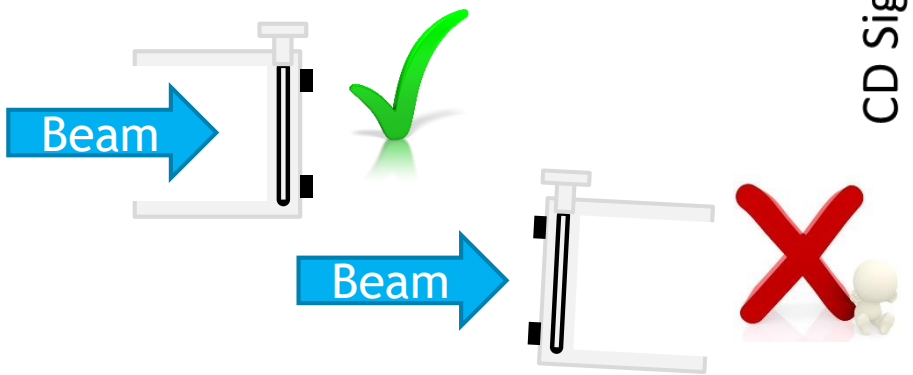
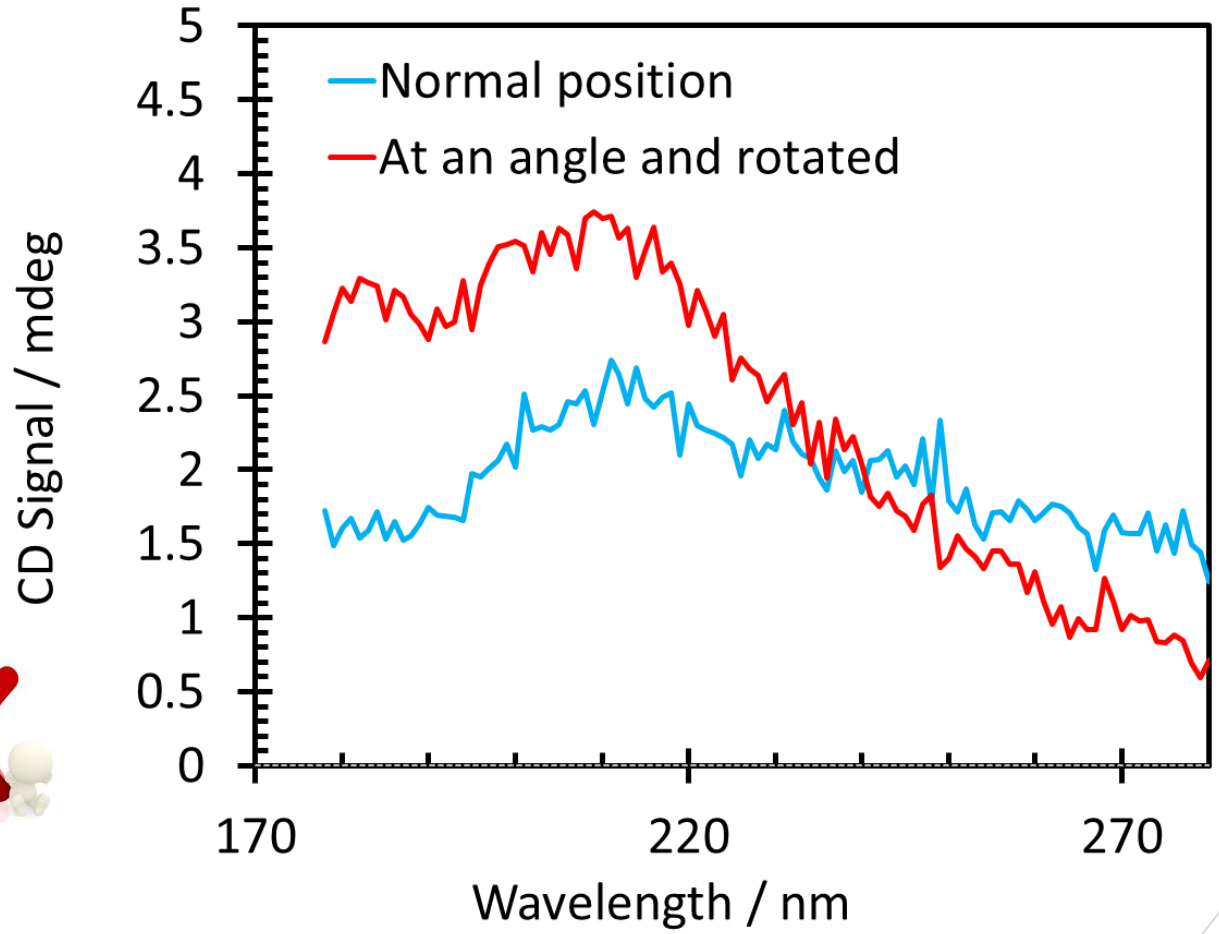


Cell at an angle and rotated



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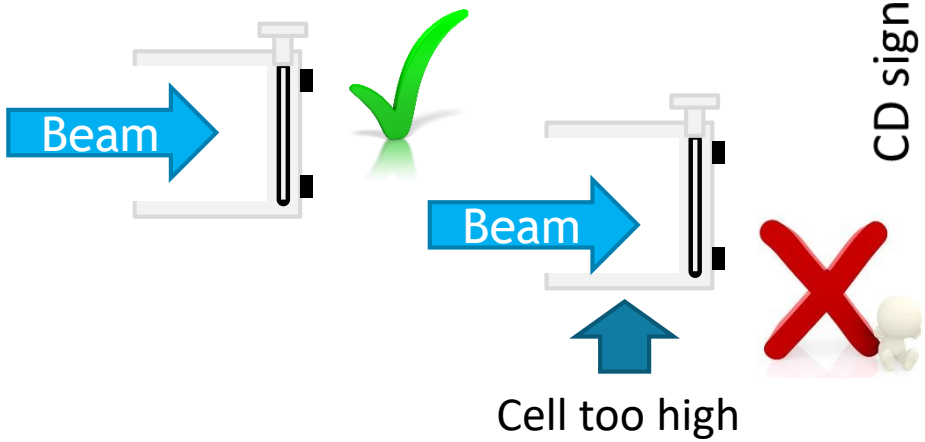

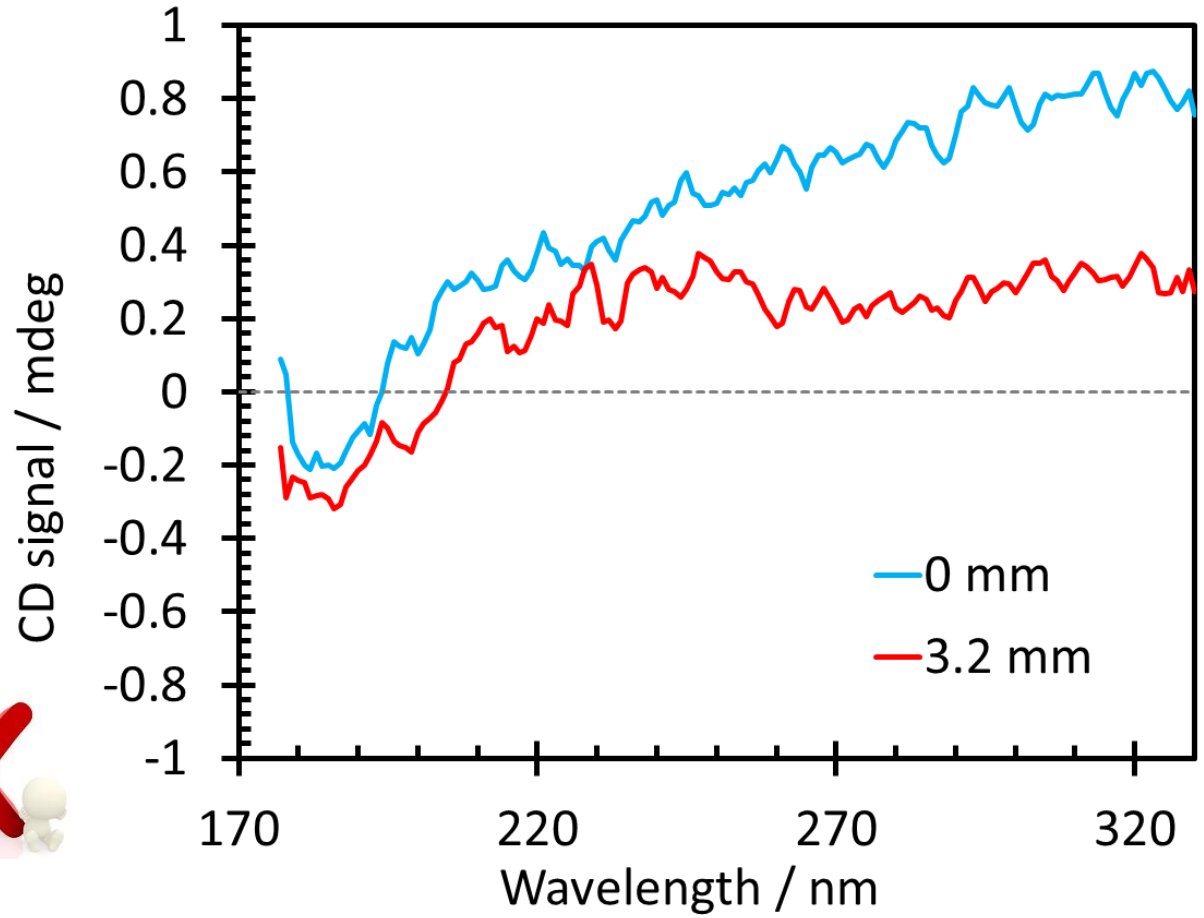

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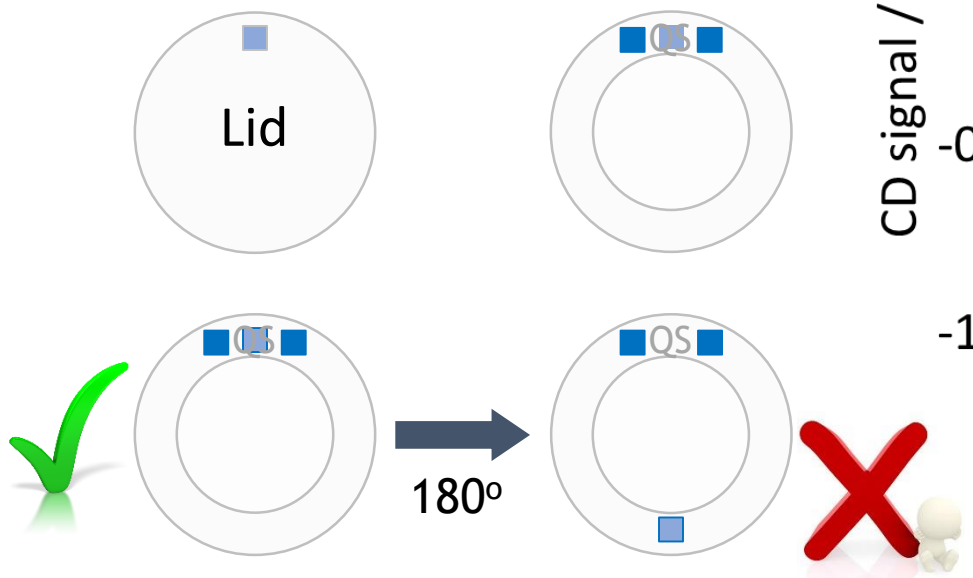
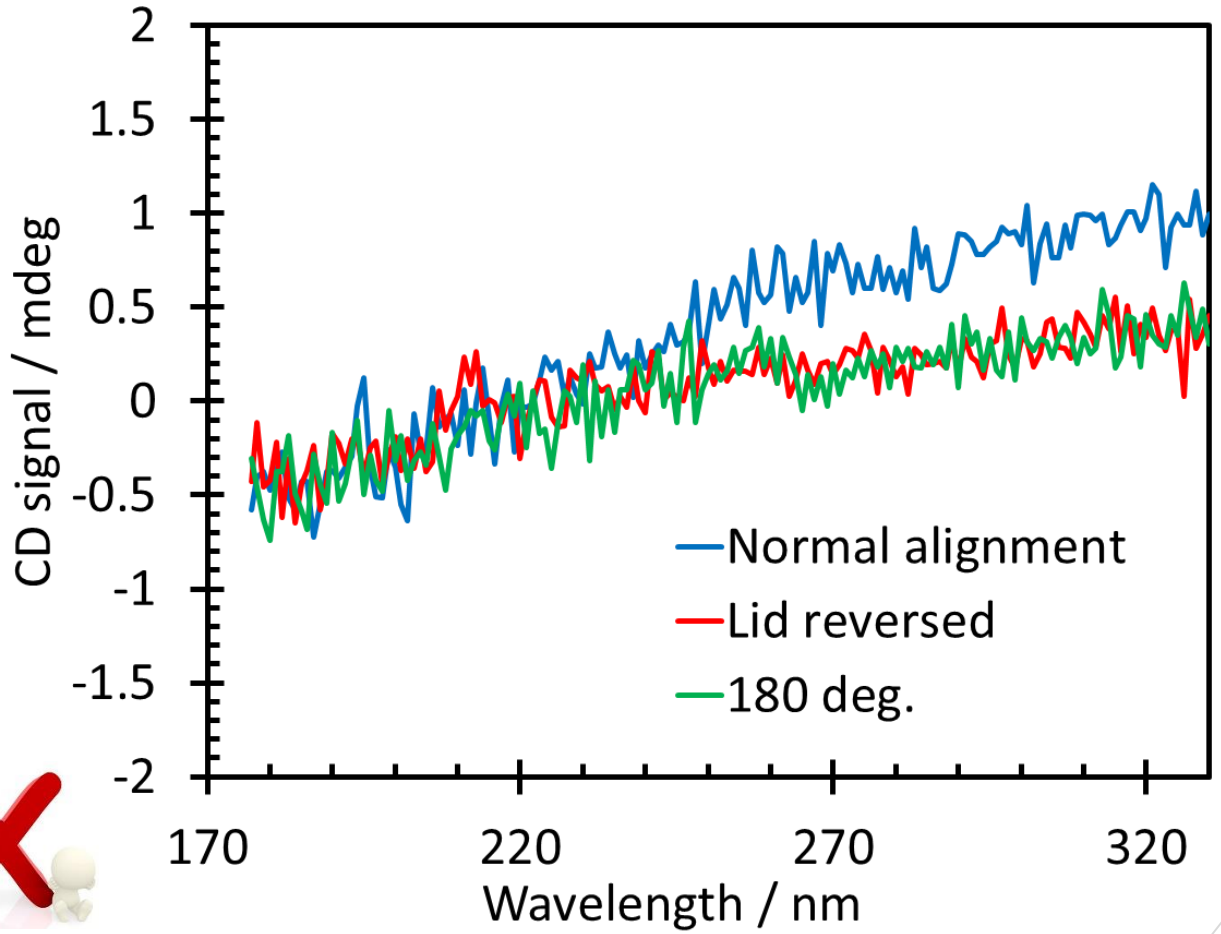
Baselines – what can go wrong?

Factors affecting the baseline shape/stability of the system

- Cell alignment





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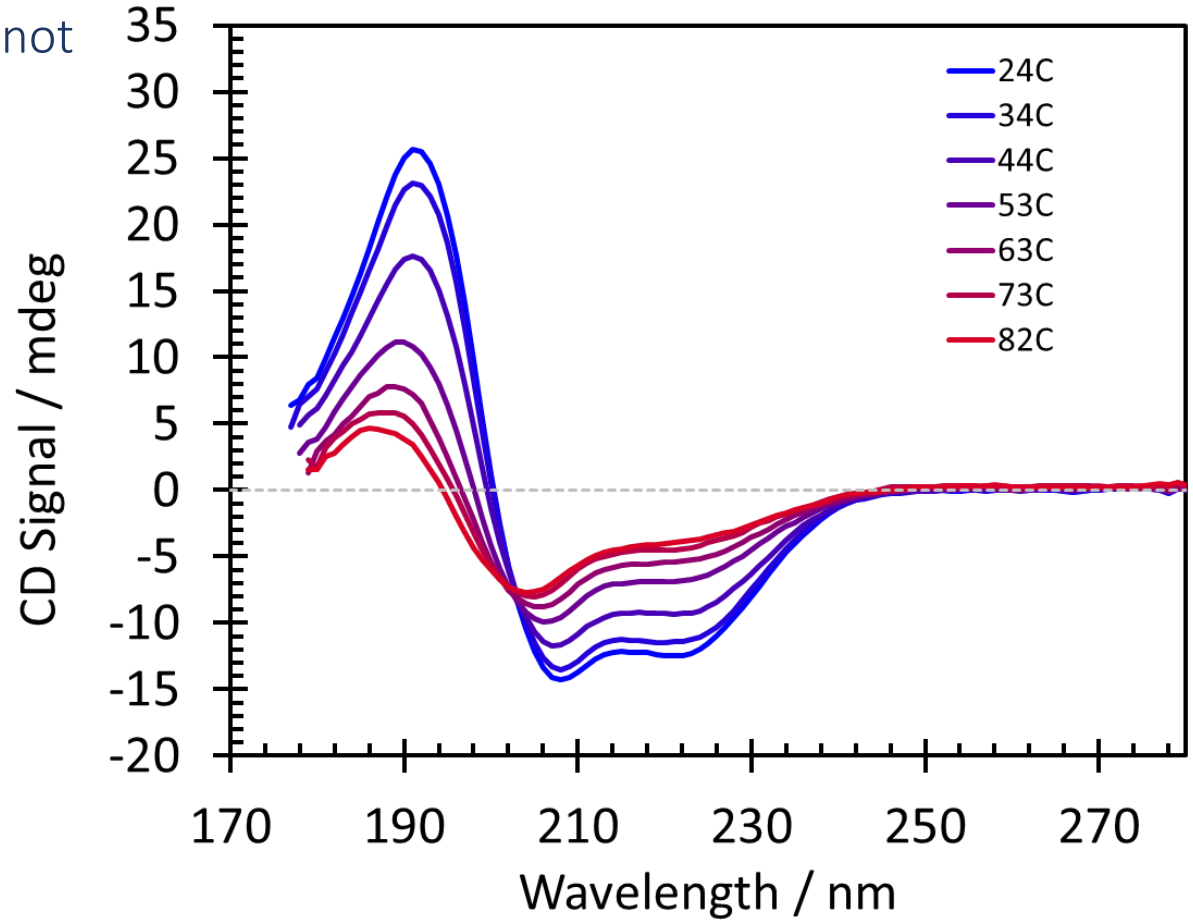
Baselines – what can go wrong?

Factors affecting the baseline shape/stability of the system

These data are baseline subtracted, not zeroed at long WL



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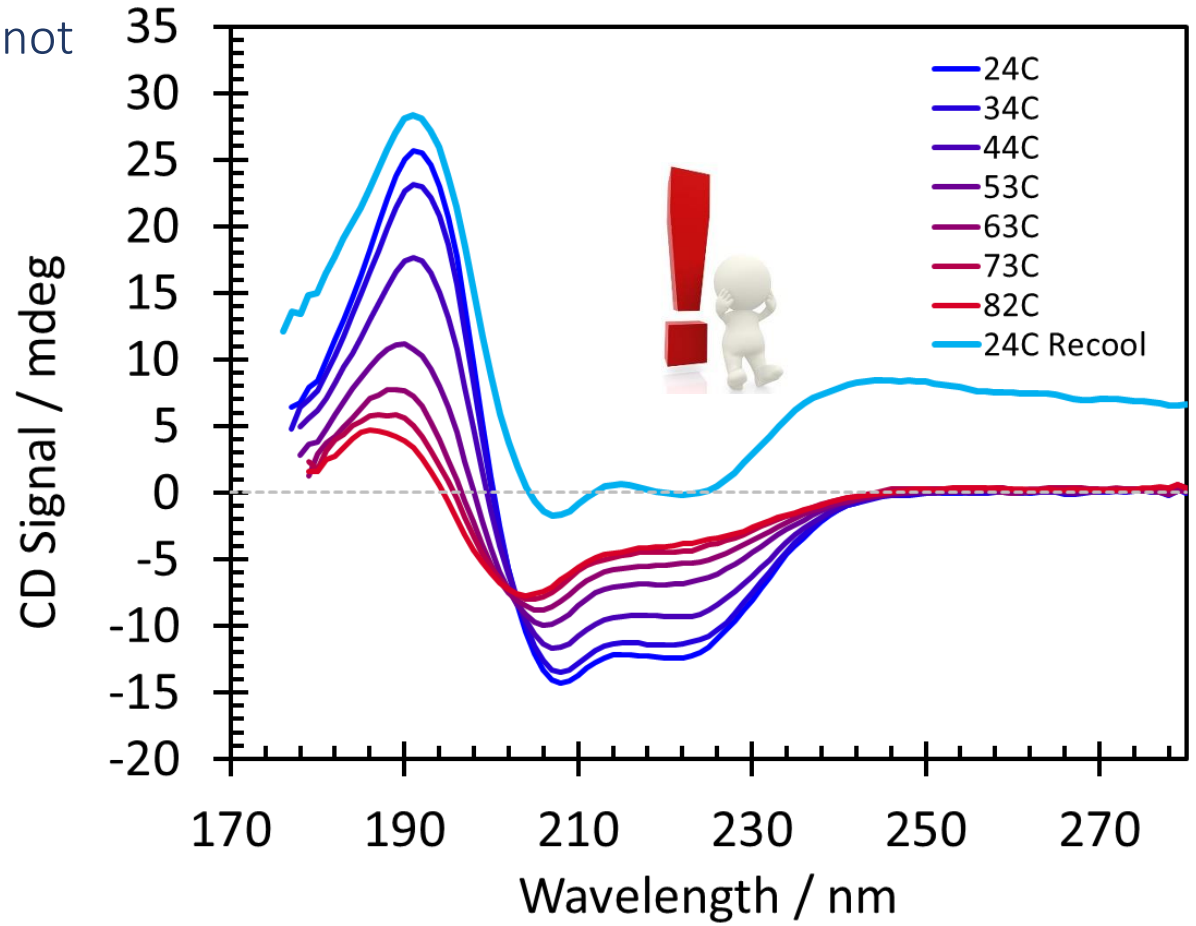
Baselines – what can go wrong?

Factors affecting the baseline shape/stability of the system

These data are baseline subtracted, not zeroed at long WL

- Stress on the cell

  The cell was stuck in the holder after the temperature ramp



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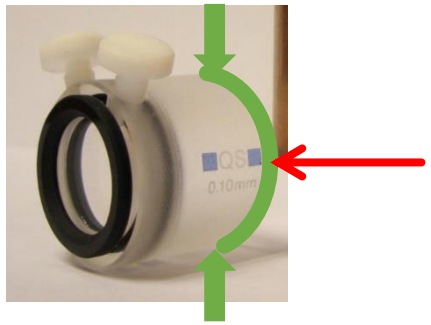
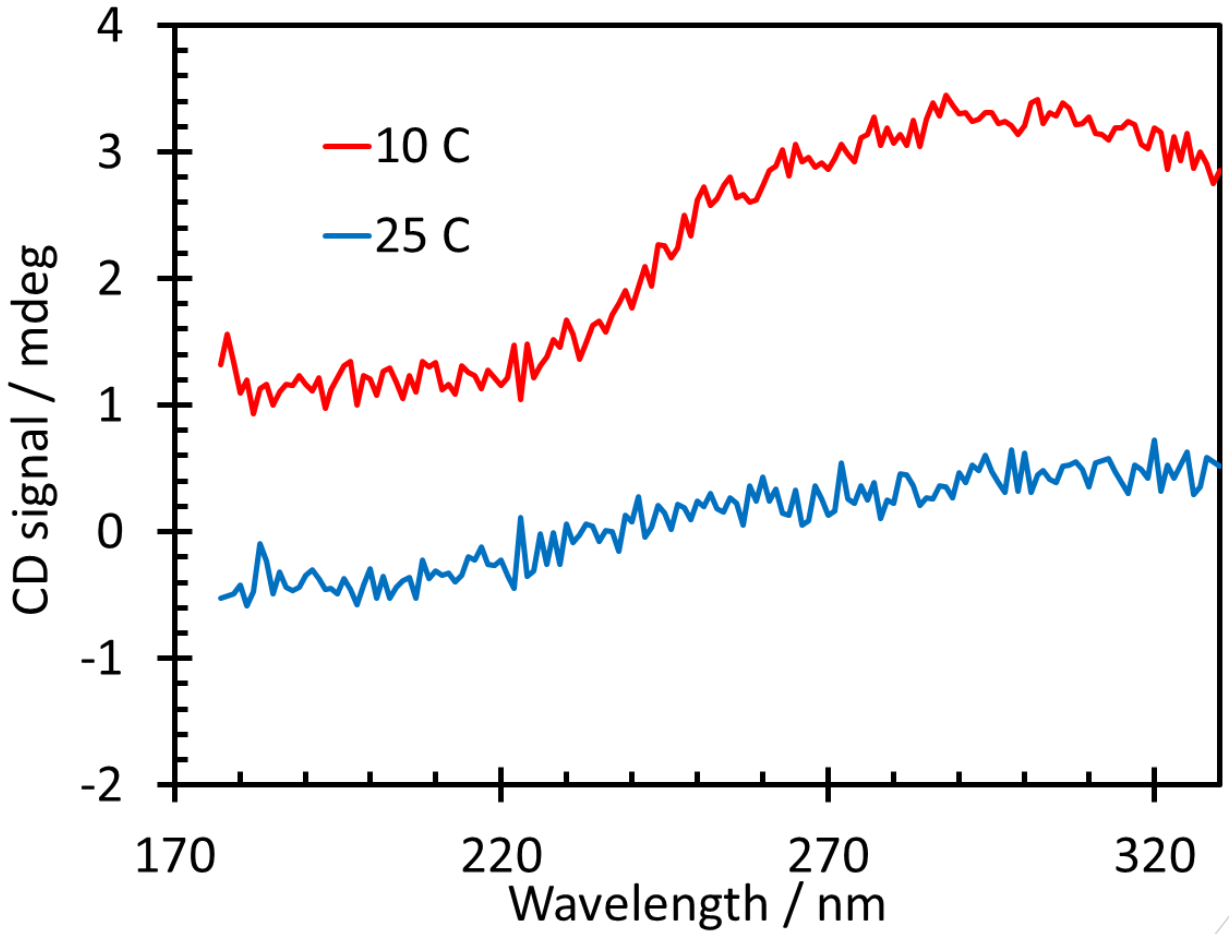
Baselines – what can go wrong?

Factors affecting the baseline shape/stability of the system

- Stress on the cell

  Can also be seen at lower temperatures





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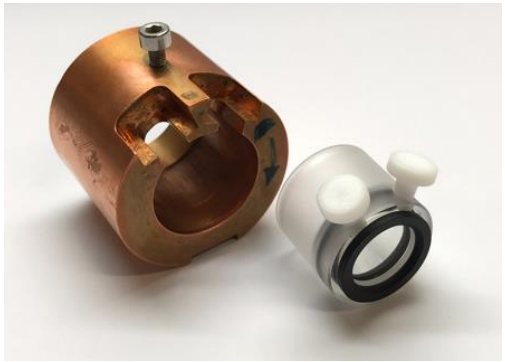
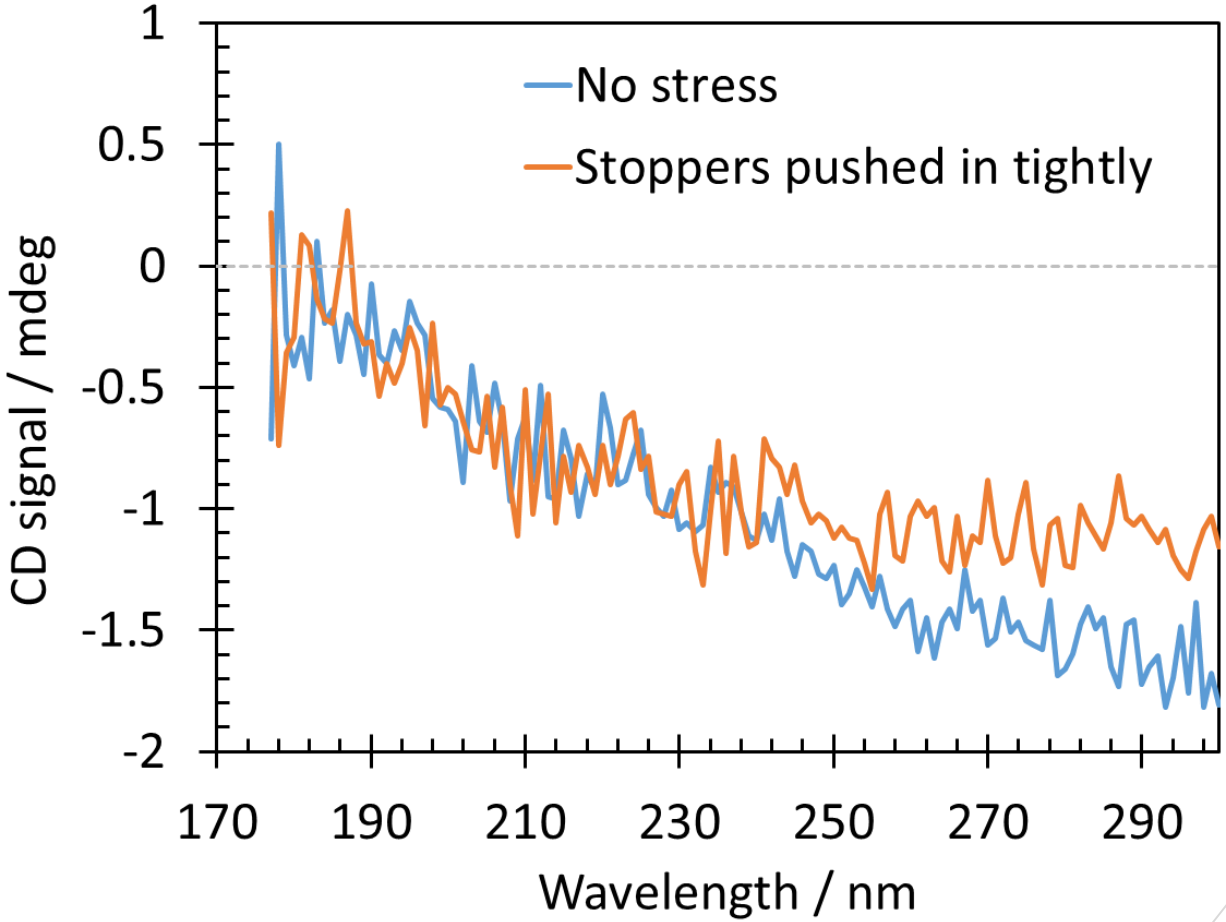
Baselines – what can go wrong?

Factors affecting the baseline shape/stability of the system

- Stress on the cell

  Using too much force putting the stoppers into the cell





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Baselines – what can go wrong?

- The examples given here show **some of the ways** a cell baseline can be affected.
- It is **difficult to reproduce** the exact "error" which causes the cell baseline to change e.g. the amount of stress or wrong alignment, so it is unlikely that the same conditions can be found to measure the corresponding buffer baseline.
- Some of the cell baselines are close to just being an offset of the "normal" one, while others have **significant shape changes**, which when subtracted can have an effect on the resulting sample spectrum.

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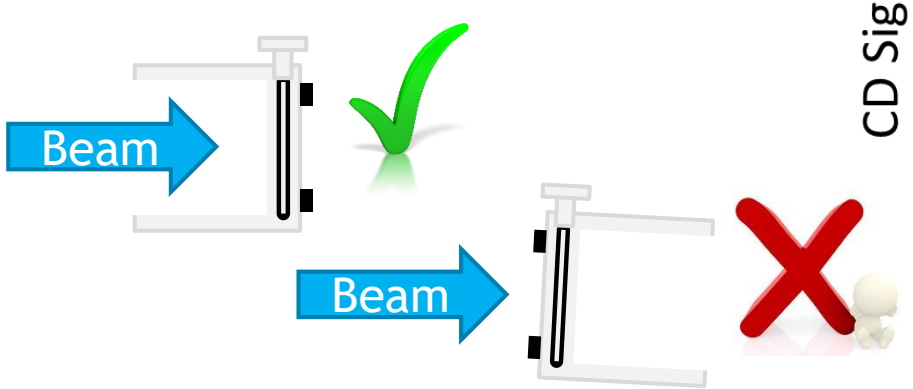
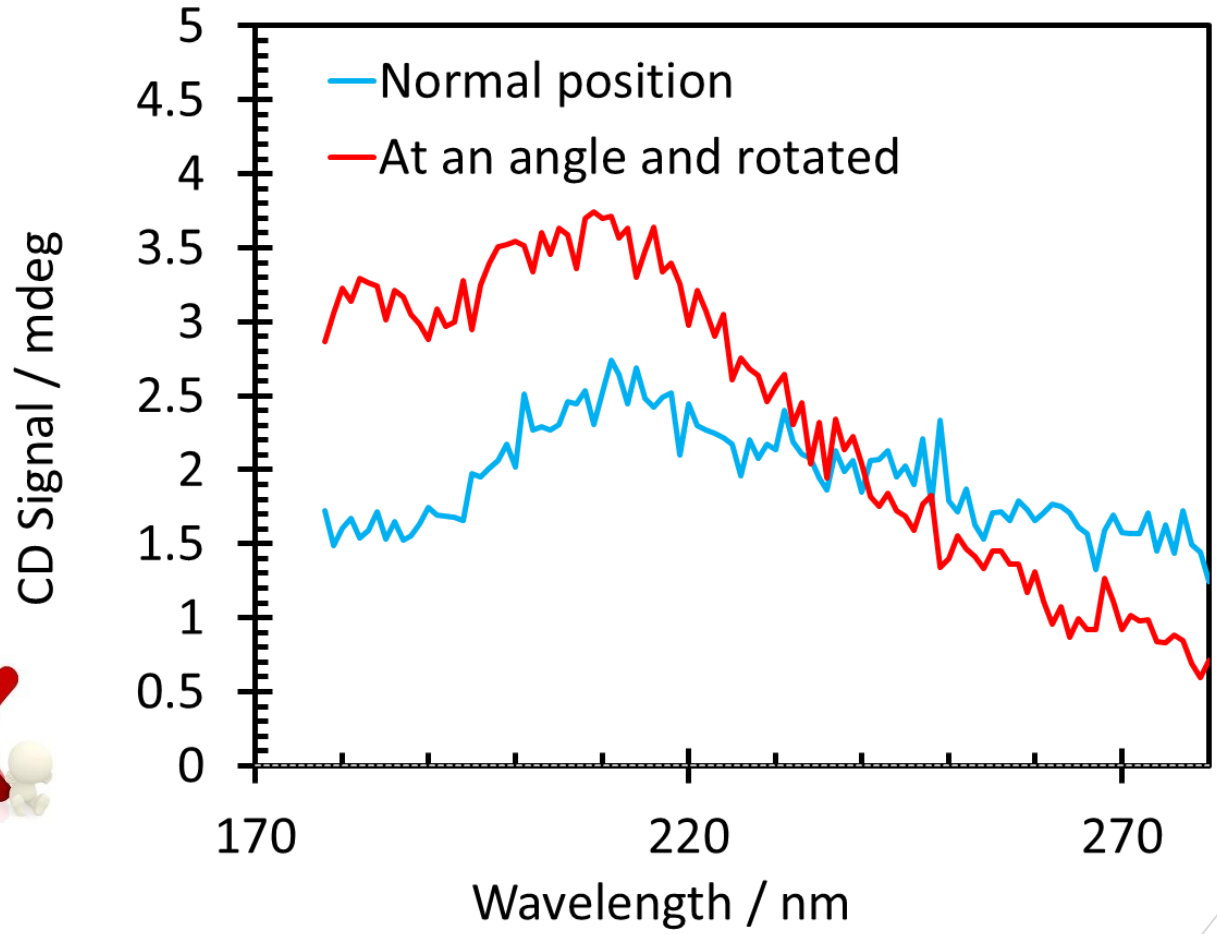
Baselines – what can go wrong?

Factors affecting the baseline shape/stability of the system

- Cell **position**/**angle** with respect to the beam



Cell at an angle and rotated



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Baselines – what can go wrong?

- The examples given here show **some of the ways** a cell baseline can be affected.
- It is **difficult to reproduce** the exact "error" which causes the cell baseline to change e.g. the amount of stress or wrong alignment, so it is unlikely that the same conditions can be found to measure the corresponding reference baseline.
- Some of the cell baselines are close to just being an offset of the "normal" one, while others have **significant shape changes**, which when subtracted can have an effect on the resulting sample spectrum.
- The larger the difference of a cell baseline from the baseline of the system, the **more sensitive it is to position changes and stress**, therefore causing more significant changes in the baseline.

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Baselines – take home message

- Want to do everything possible that you are reproducing the same underlying CD baseline spectrum for a sample scan as there is for a reference baseline scan.
- There are a variety of ways that the underlying baseline can be affected, so try to be consistent with how you load and mount the cell for every measurement.
  - Same person loading a cell for both sample and baseline
  - Avoid putting any stress on the cell
  - For demountable cells, keep matched pairs together
- **Scan to high enough wavelength** to have a large enough region where there is no CD signal from the sample – check that the sample and baseline scans agree in this region, both **level and shape**.

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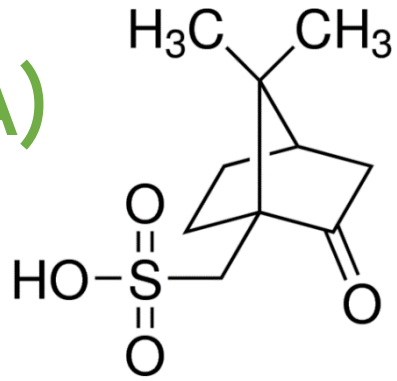
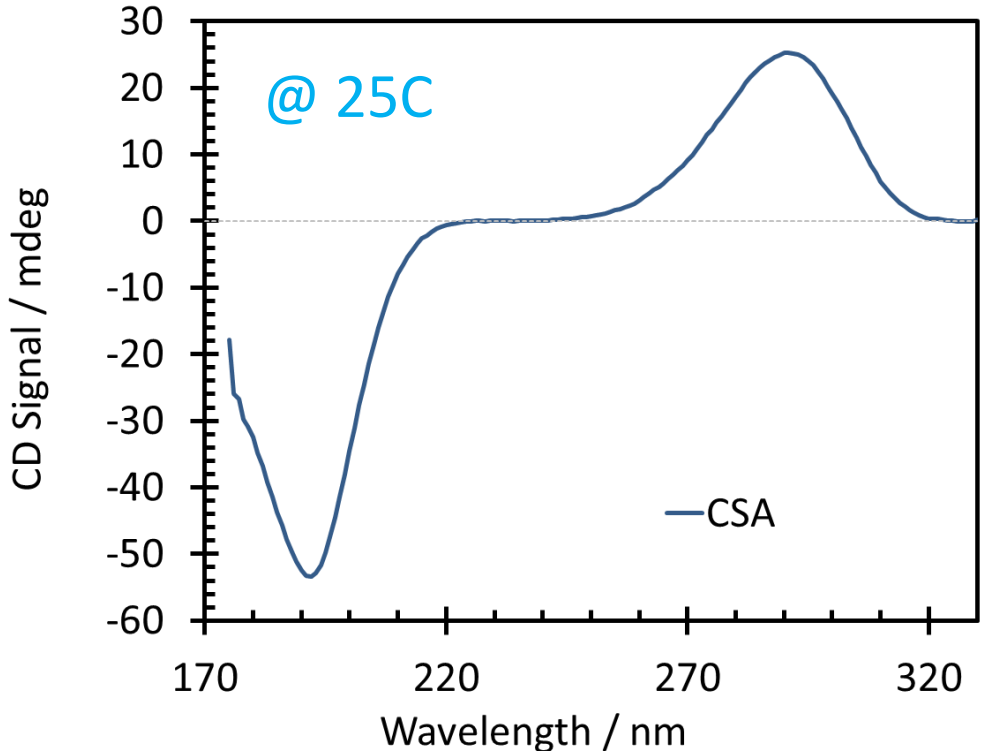
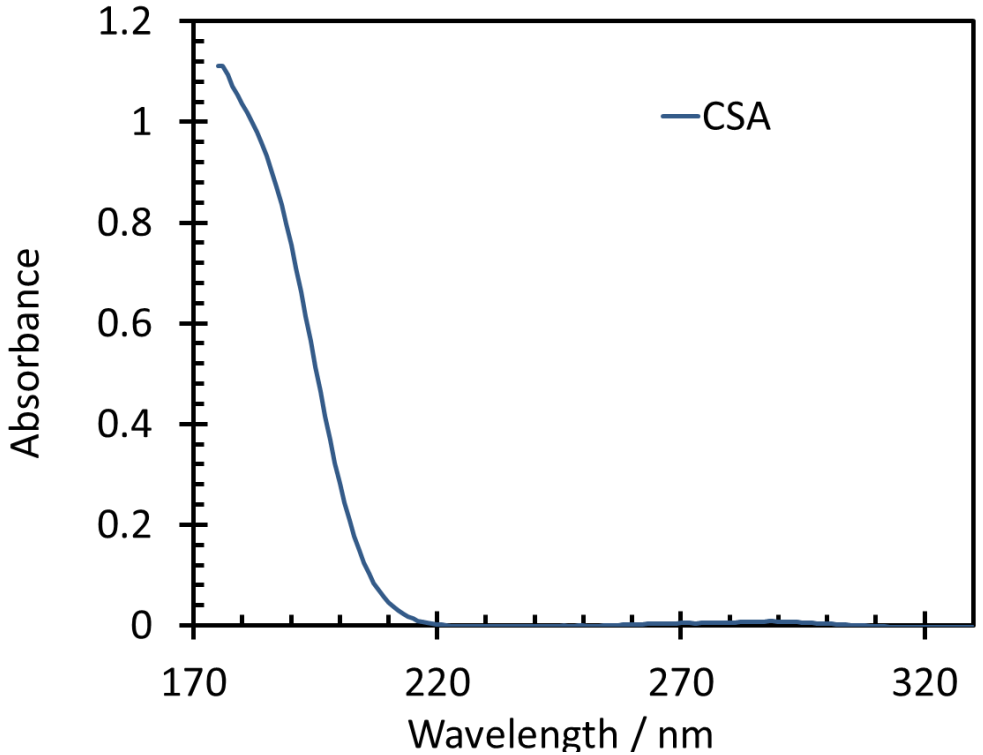
(1S)-(+)-10-Camphorsulfonic acid (CSA)



- Ratio of the peaks ~2.1
  - The peak intensities and also the ratio are temperature dependent

This spectrum was taken in a 0.1 mm cell with a sample concentration of 7.2 mg/ml.





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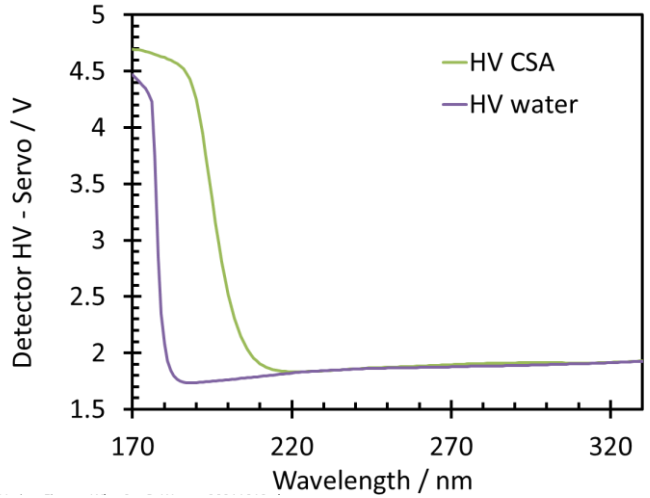
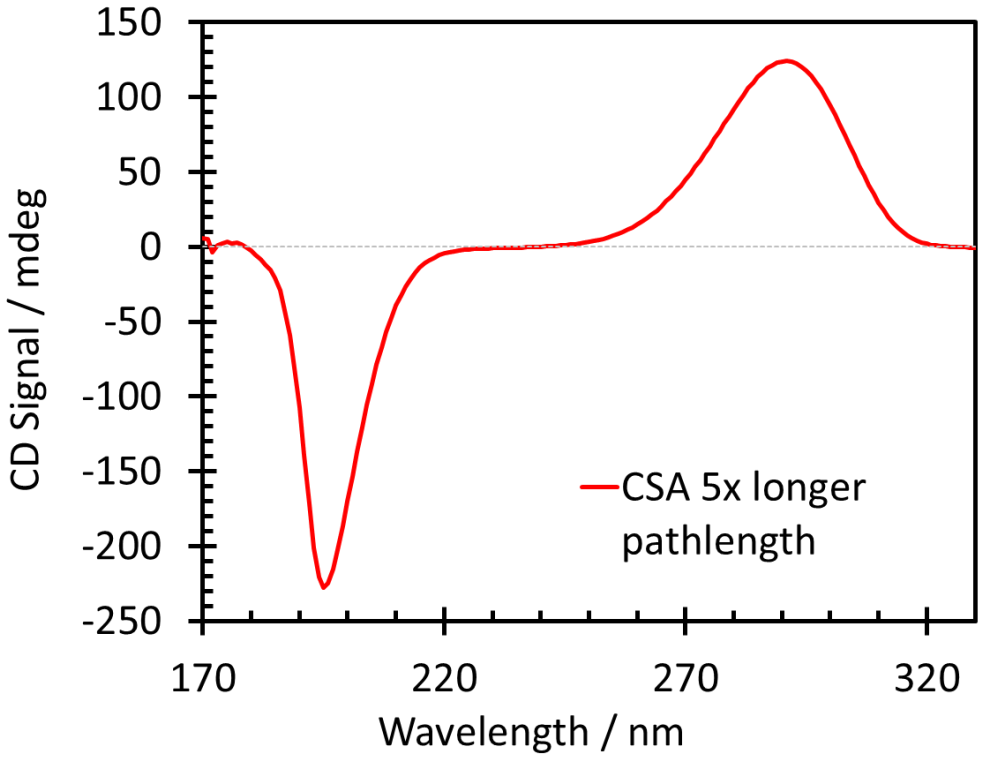
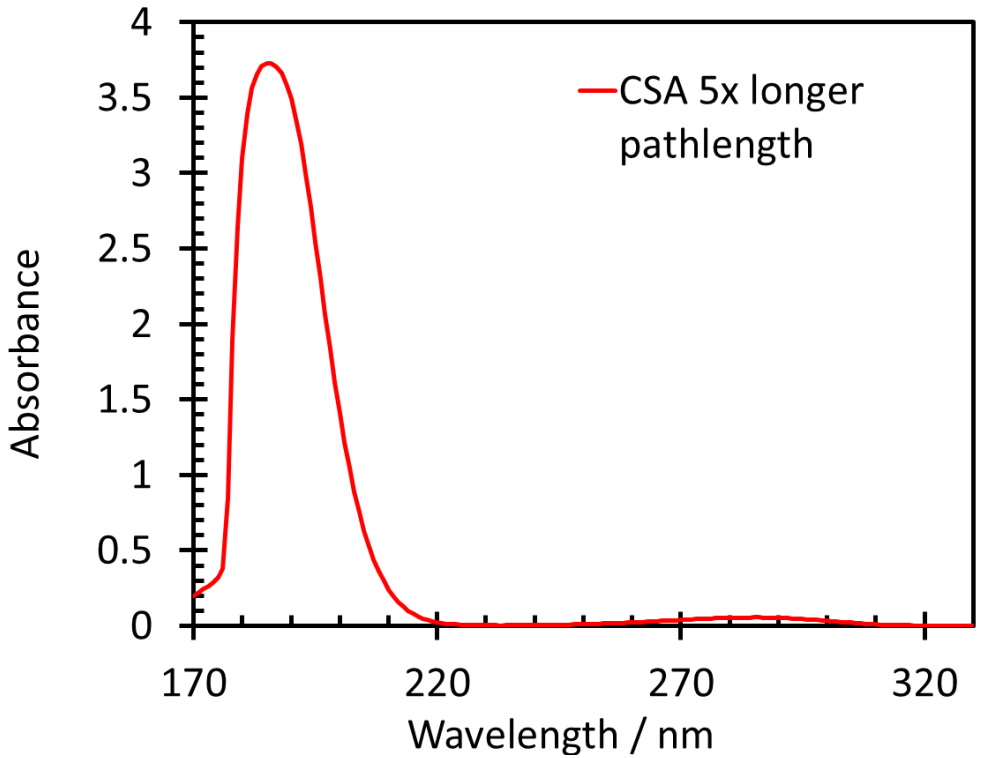
Too high concentration

- What happens when the absorbance is too high?







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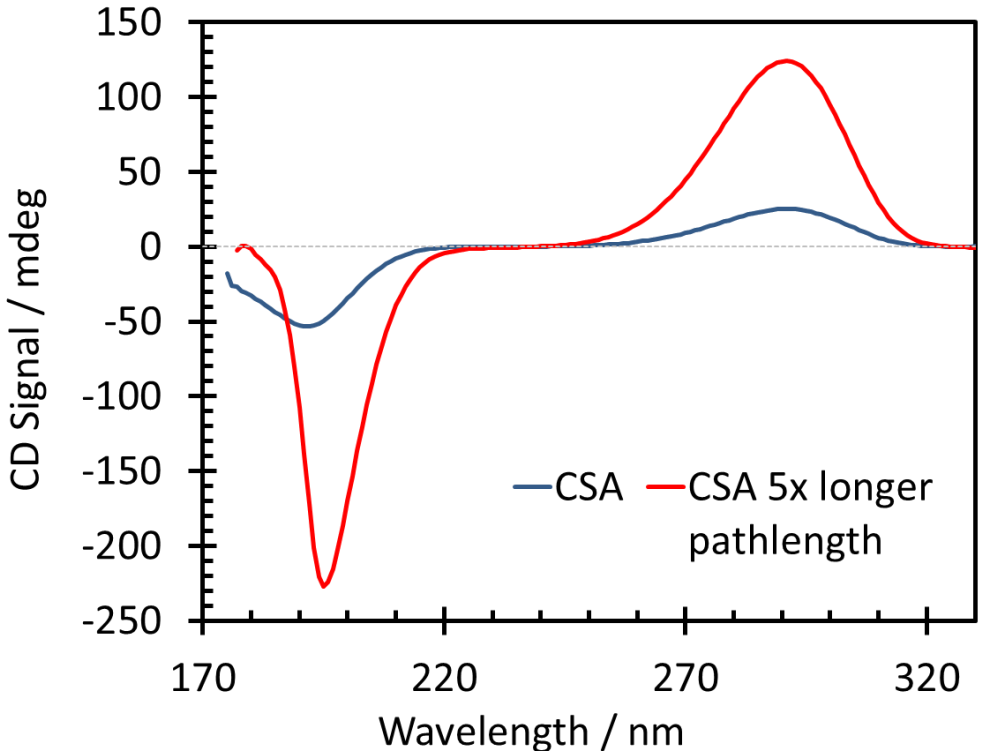

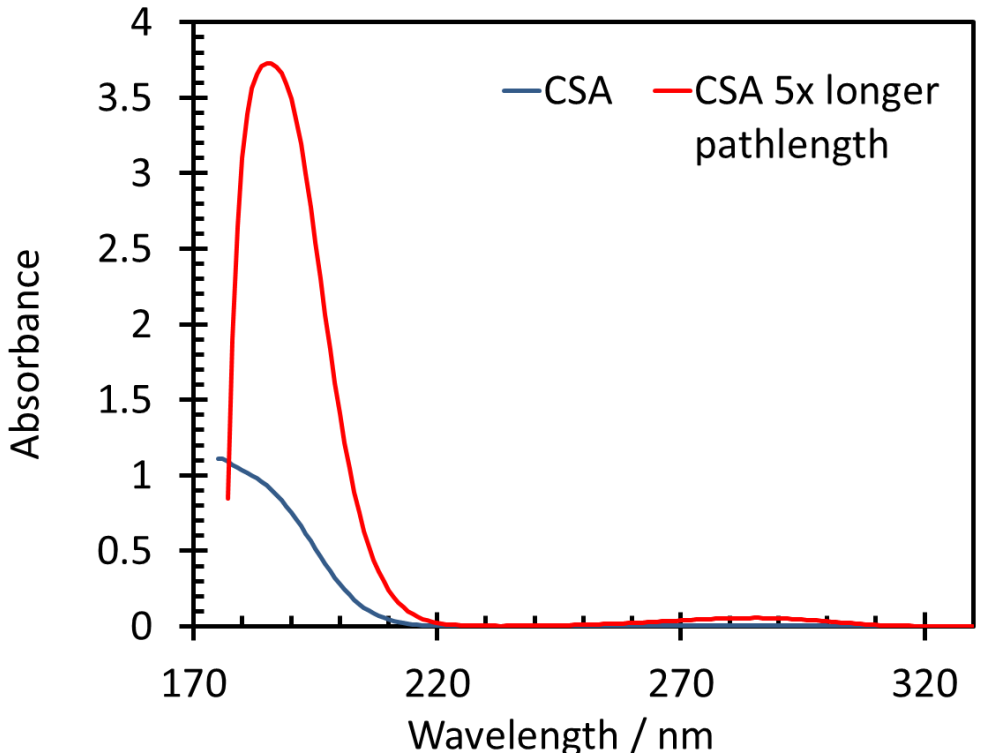

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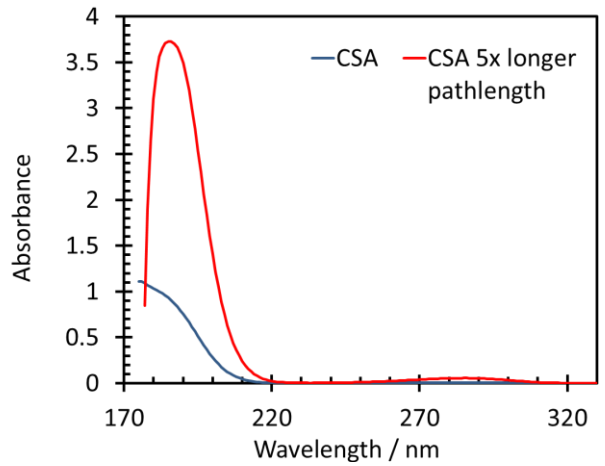
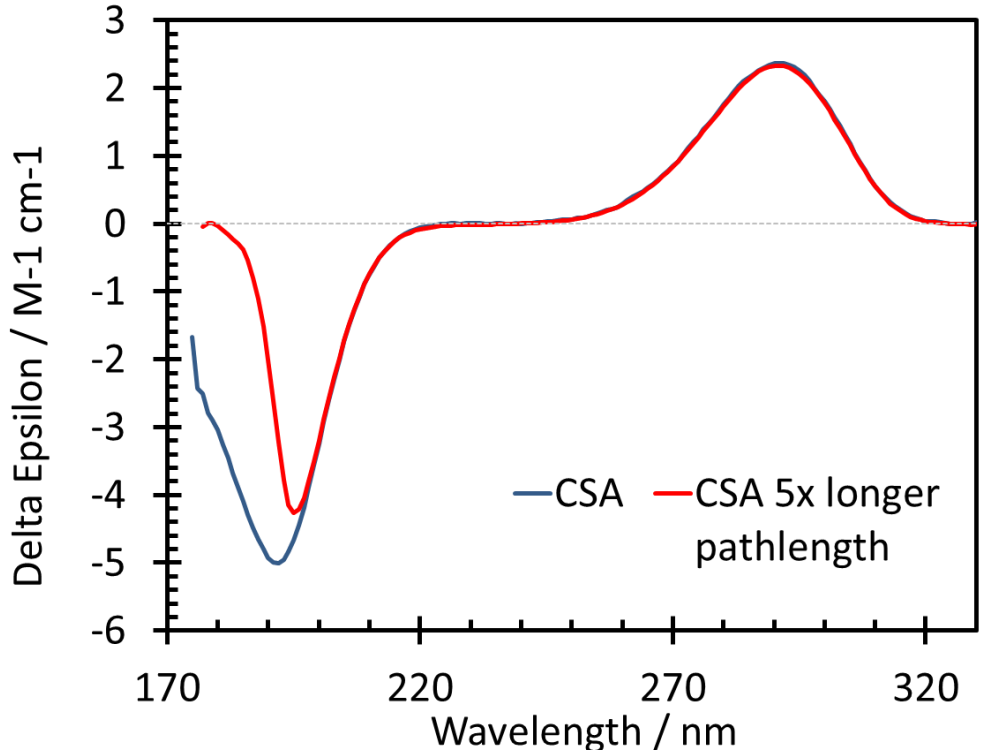
Too high concentration

Clear effect on the measurement of the CD peak where the absorbance is high, resulting in incorrect measurement of:

- Intensity
- Peak width
- Maximum intensity wavelength position



A good test of a spectrometer to find out what the highest absorbance (high voltage) should be



@ 200 nm the absorbance is 1.4

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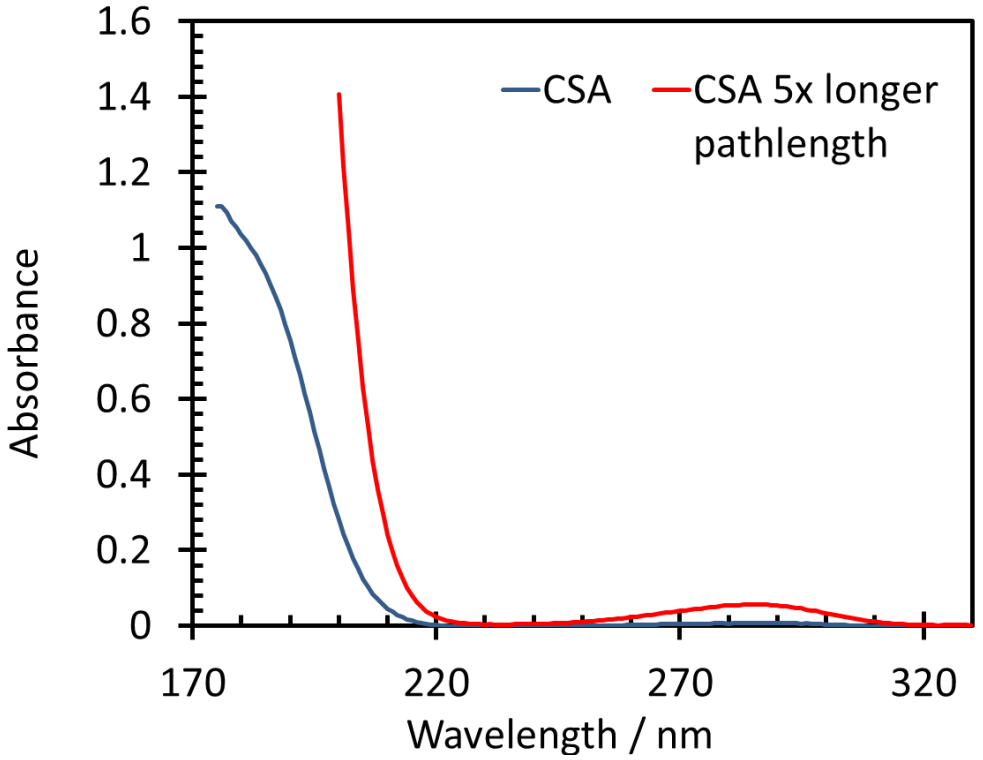
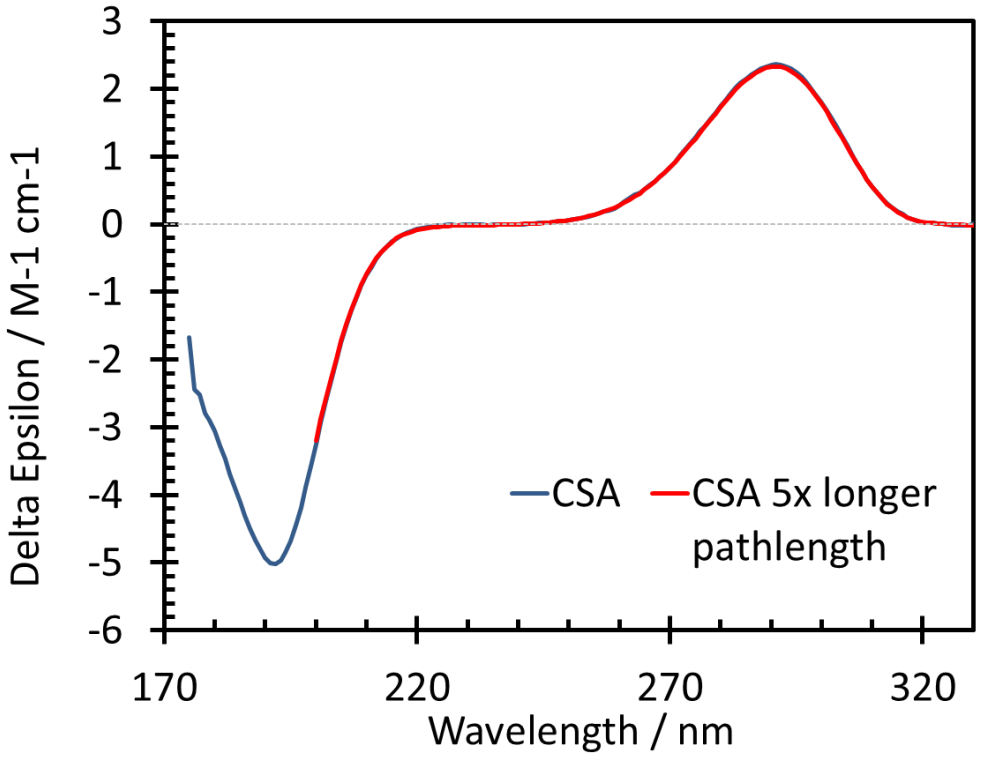
Too high concentration

- The high concentration data is only valid to 200 nm where the absorbance is 1.4

This does not mean that it is always okay to measure on a sample with an absorbance of 1.4! – Need to consider the absorbance of the whole system.





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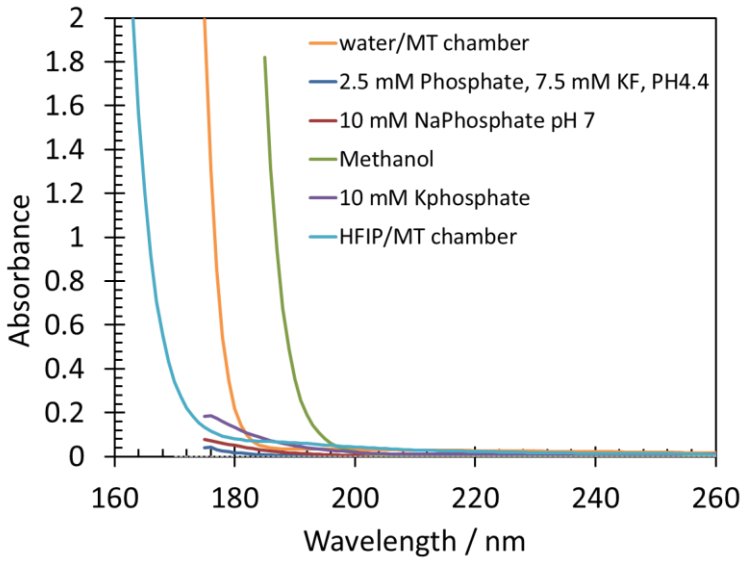
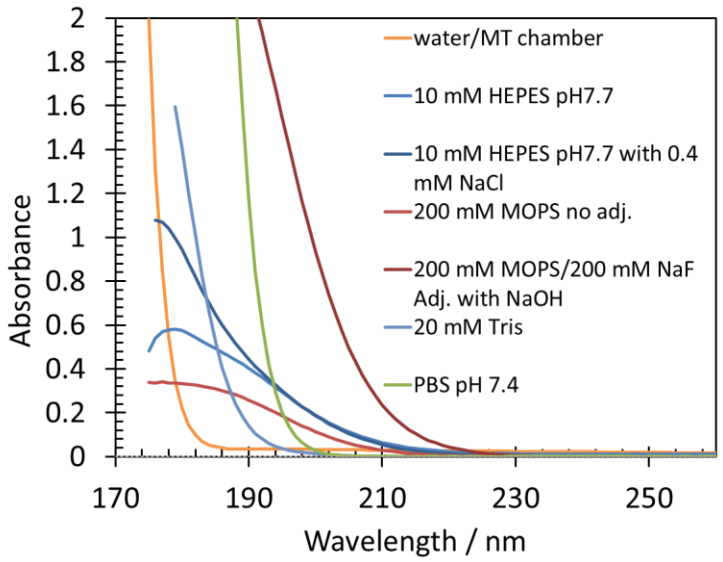
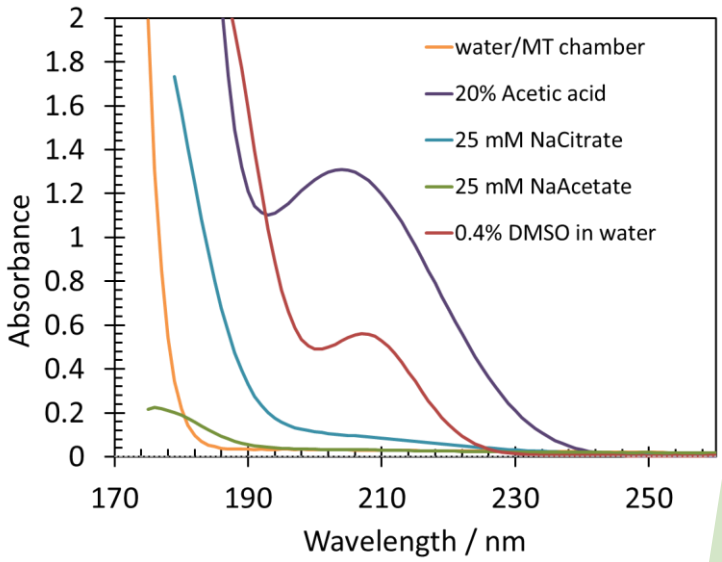
Buffer absorbance

- Need to also consider the absorbance of the buffer, some of which can be very high.
  - If you need to adjust pH avoid using HCl and NaOH.







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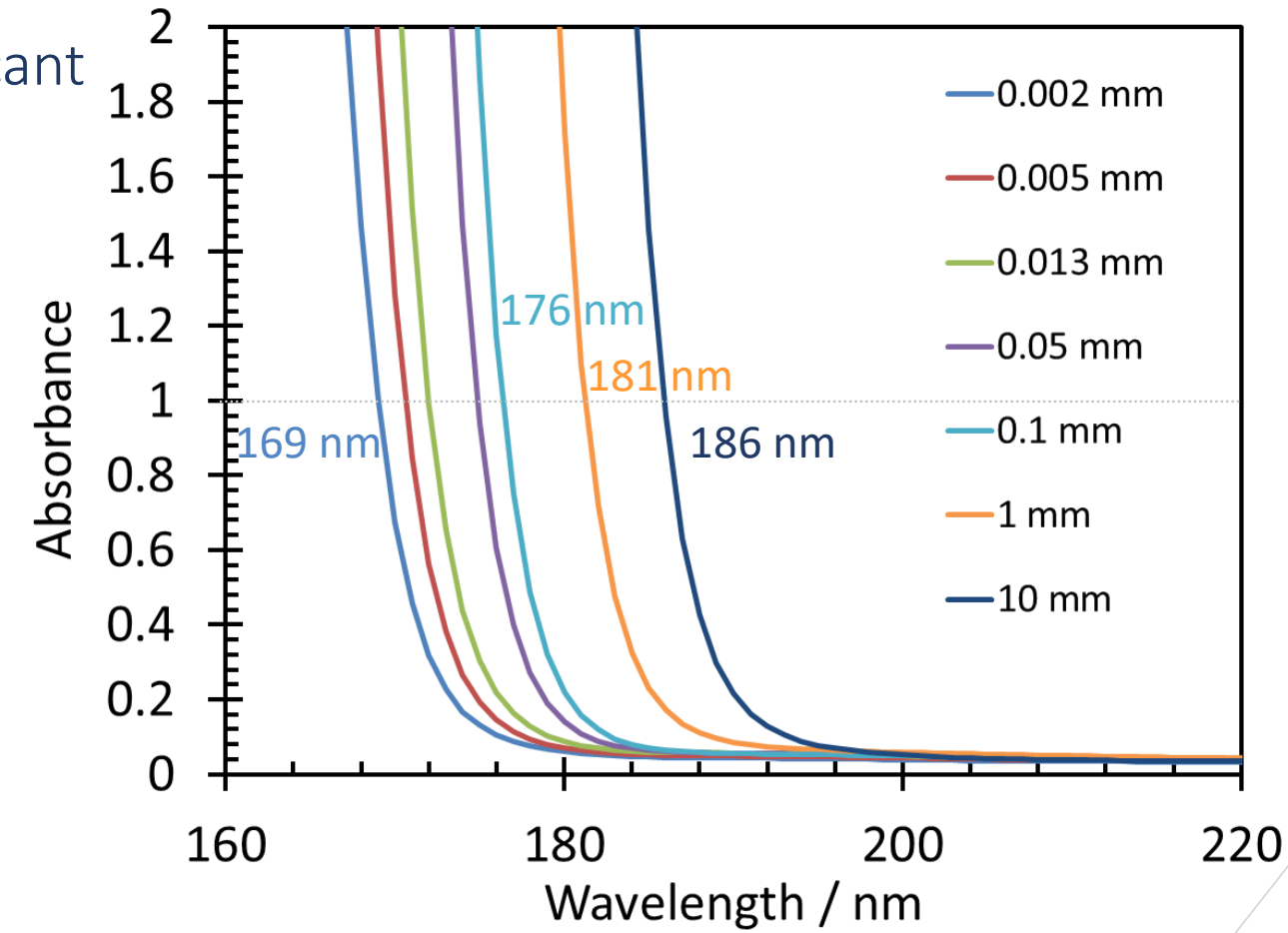
Water cut-off with changing pathlength

- Choice of cell pathlength used for measurements.
- Water alone has a significant effect on how low down in WL you can reach.



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Air bubbles

- Sometimes you can have air bubbles in your sample in the cell. There are many possible causes of this.
  - Loading demountable cells – can be very tricky to load without air bubbles
  - The cell is dirty, so when loading new sample an air bubble can form
  - Loading cold samples into a cell – air bubbles can form over time
  - Evaporation of sample can occur over time
    - Particularly in long-term temperature melts which can take many hours
- What effect can this have on the measurements?

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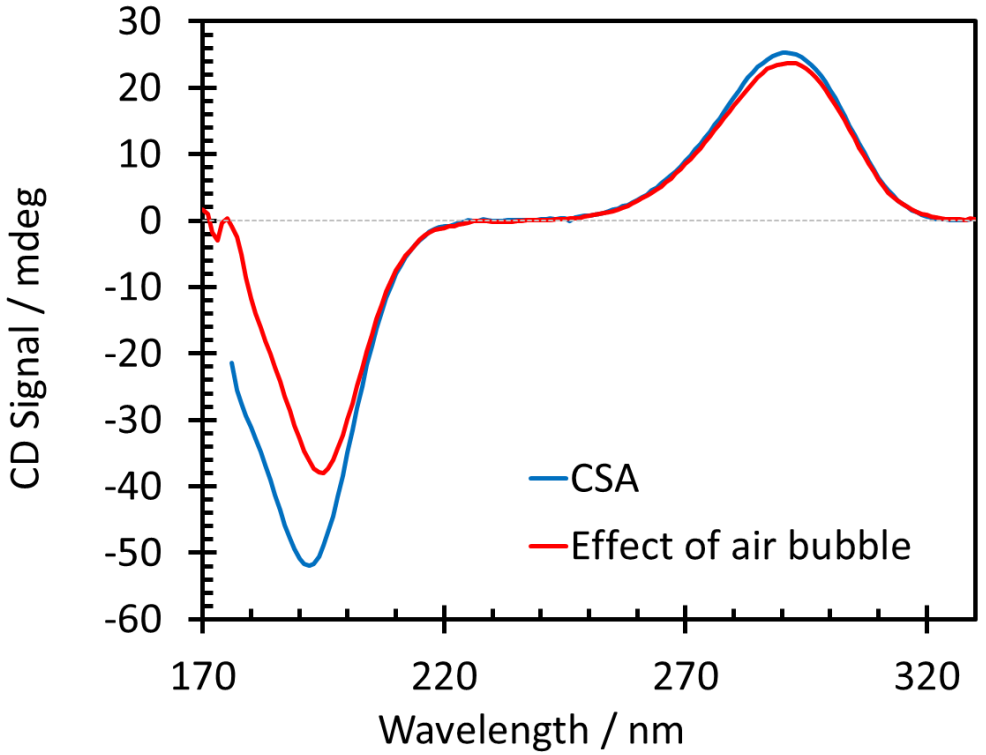
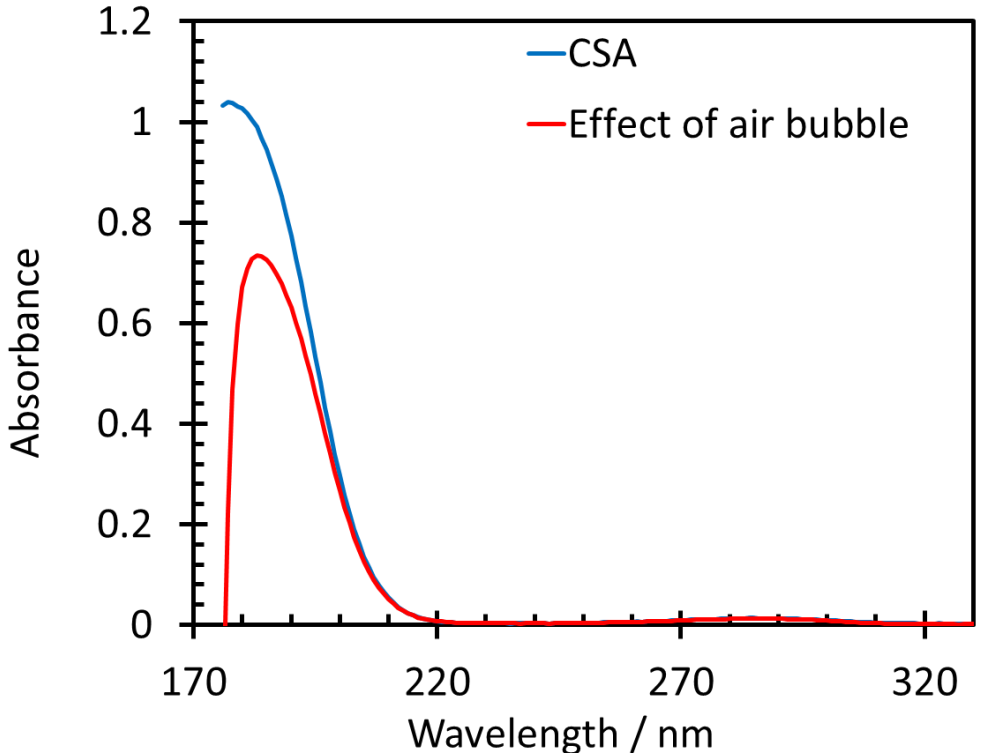
Air bubbles (or sample evaporation)

- Possible effects on the spectrum
  - Lower signal, distortion of spectrum, incorrect ratio of peak intensities.





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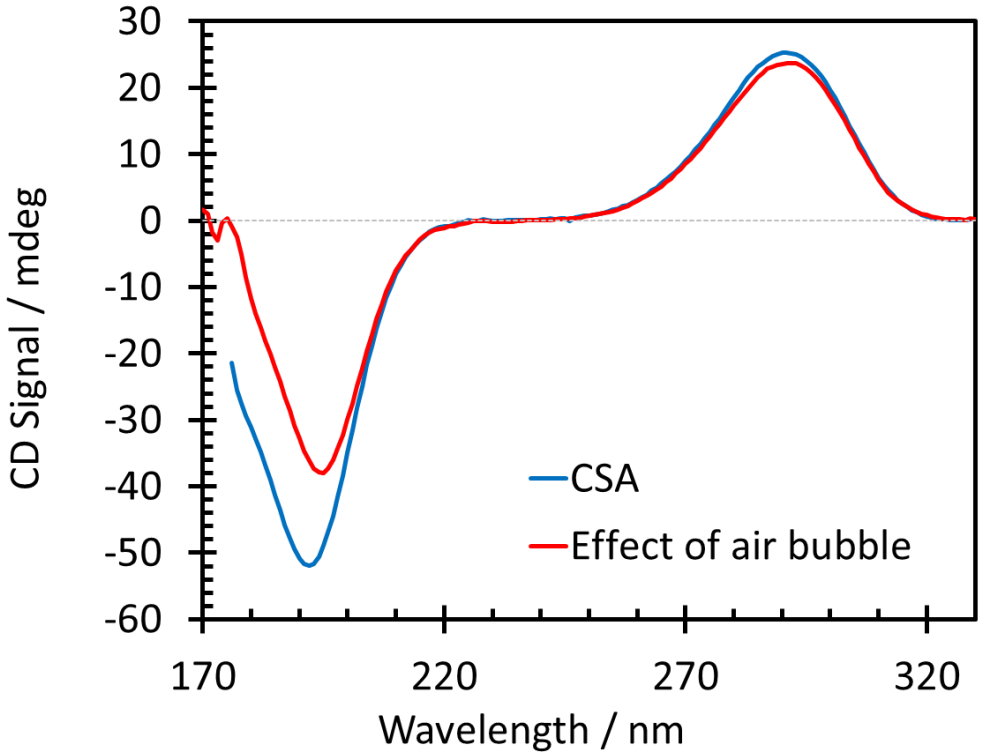
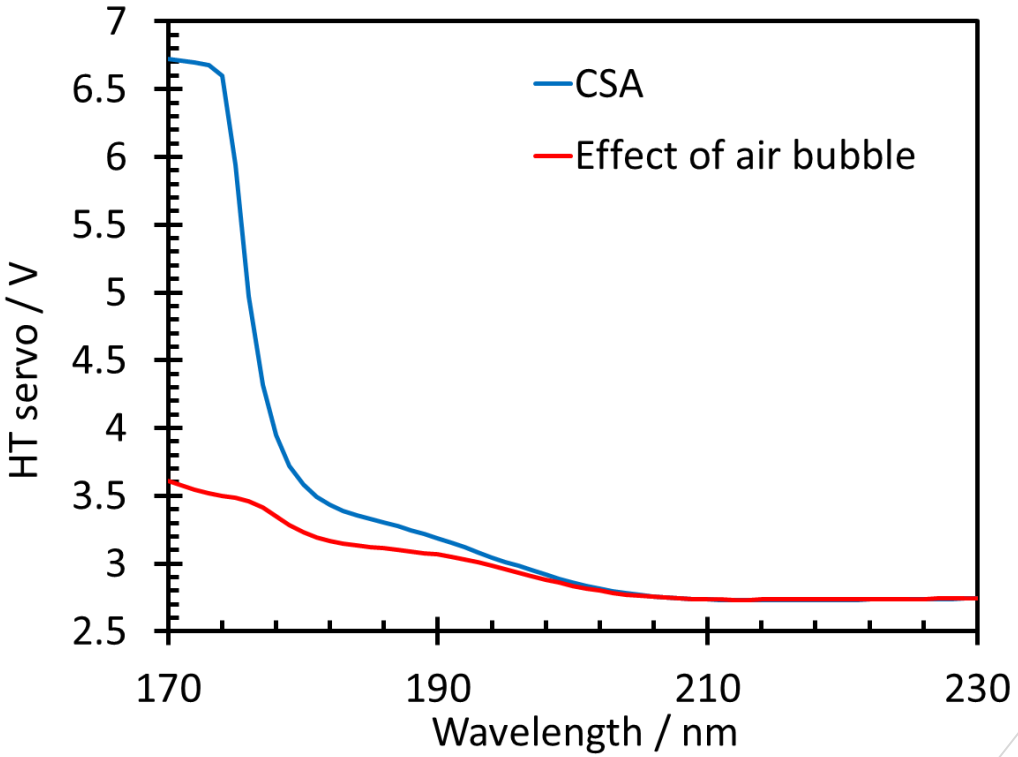
Air bubbles (or sample evaporation)

- Possible effects on the spectrum
  - Lower signal, distortion of spectrum, incorrect ratio of peak intensities.
- How to identify an air bubble?
  - If possible, measure down to wavelengths where there should be complete absorption by water/buffer





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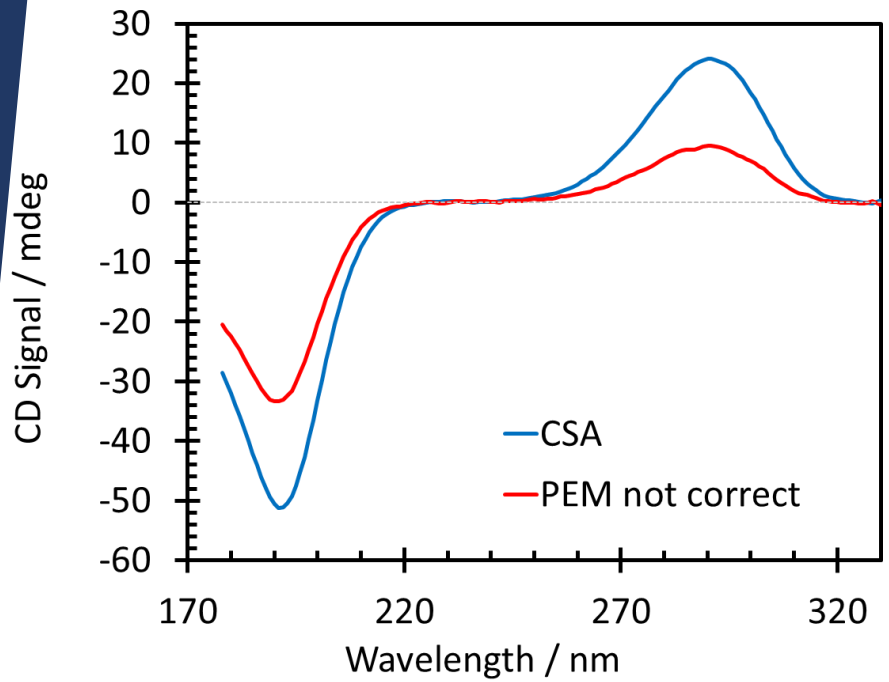
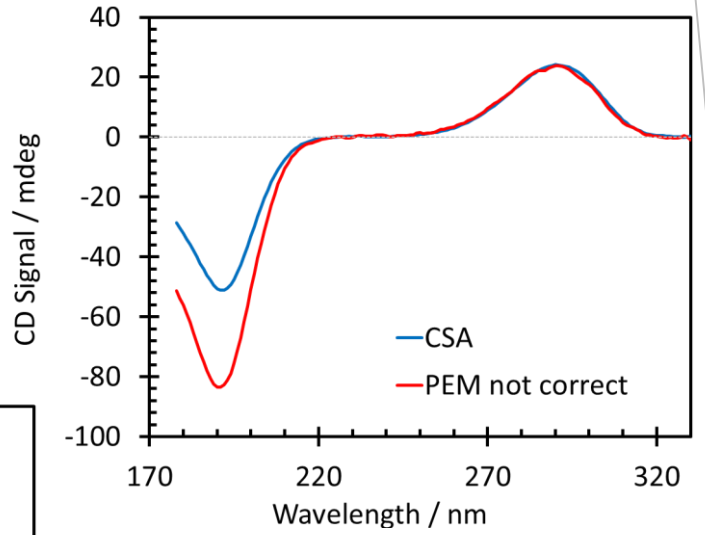
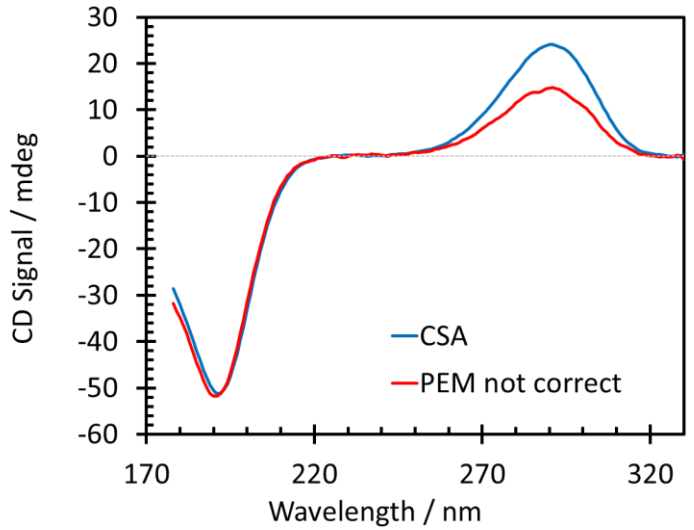
PEM incorrectly set (not changing)

- The effect is to incorrectly measure the intensity of the CD peaks, it is not the same for positive and negative signals



Scaled by 2.5





Scaled by 1.55

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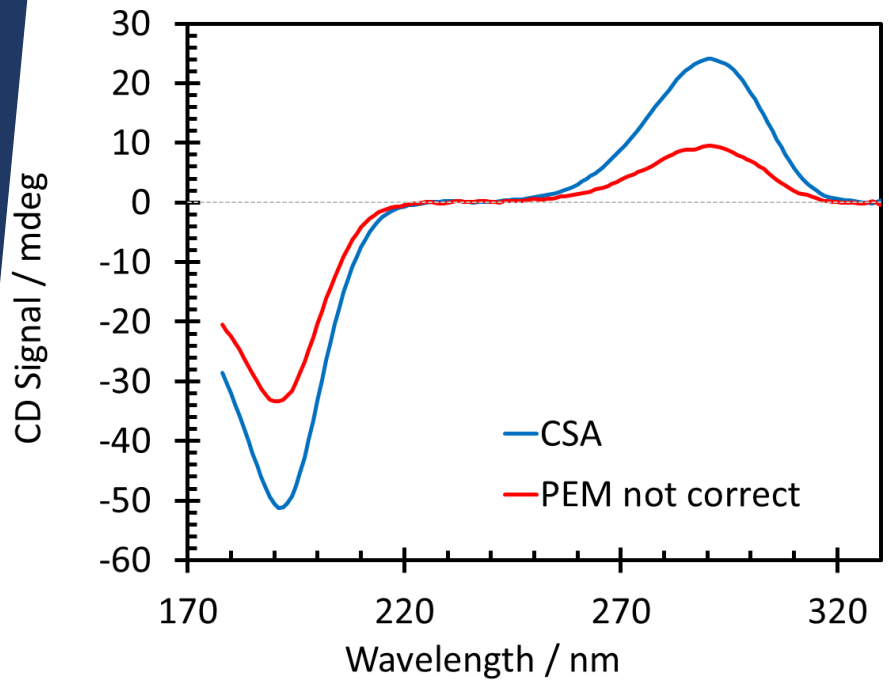
PEM incorrectly set (not changing)

- The effect is to incorrectly measure the intensity of the CD peaks, it is not the same for positive and negative signals



VERY RARE!

But… this shows the usefulness of having an easy sample to measure and monitor the performance of a CD spectrometer.

So if in doubt, measure CSA!

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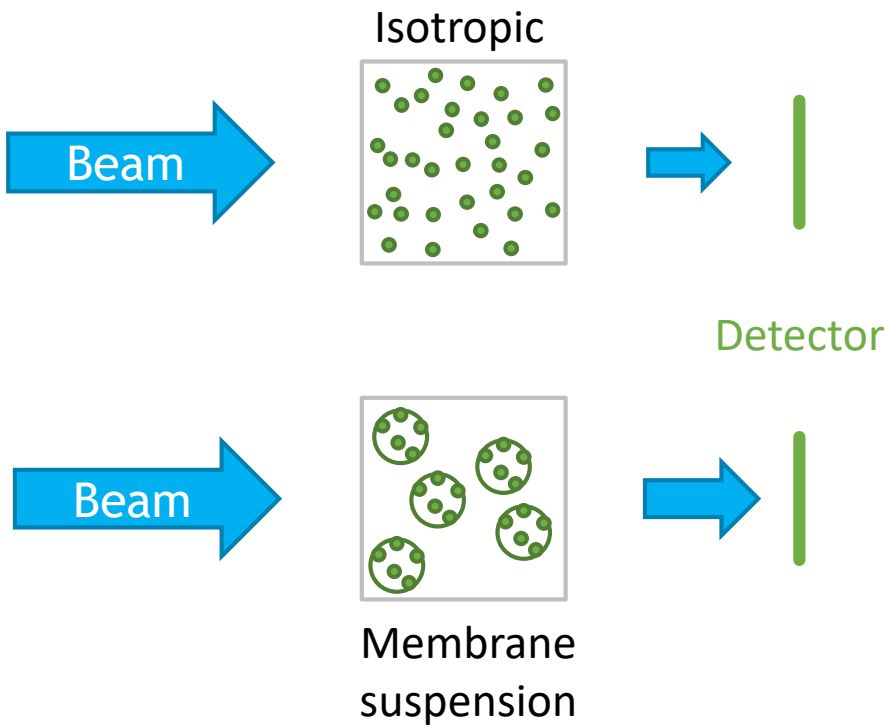
Absorbance flattening

- When there is an inhomogeneous distribution of an absorbing chromophore in the sample being measured.



Amount of flattening is proportional to extent of non-uniformity of the sample.

The higher the absorbance – the more significant effect from flattening – so not uniform across the spectral range.

Effect is to incorrectly measure CD, both distorting the shape and the intensity.

Miles, A. J & Wallace, B. A., Chem. Soc. Rev., 2016, 45, 4859

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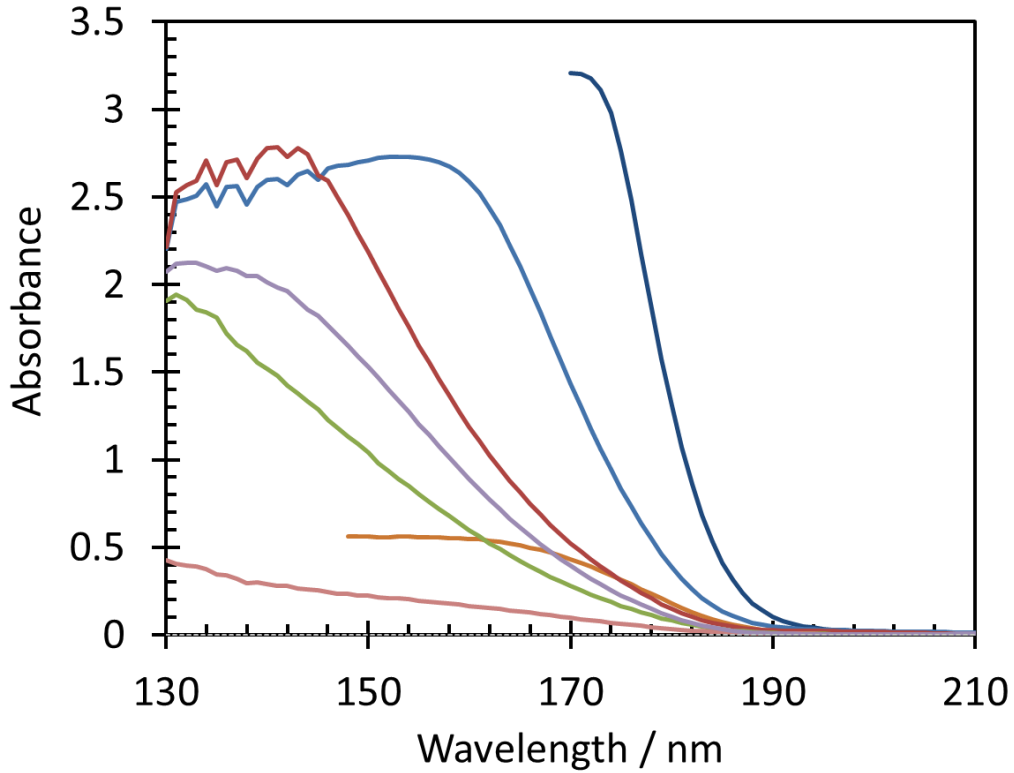
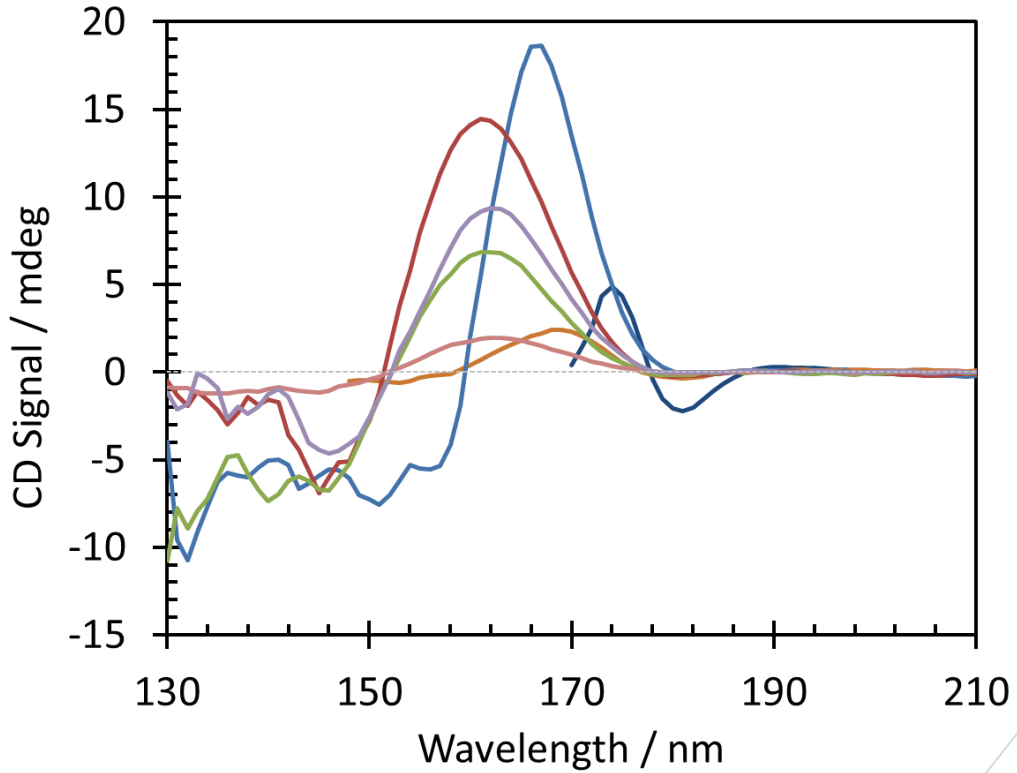
Absorbance flattening

- See a similar effect with thick non-uniform films

The spectra below are all taken on different preparations of films of the same sugar. – Quite a variety in thickness and CD spectra!





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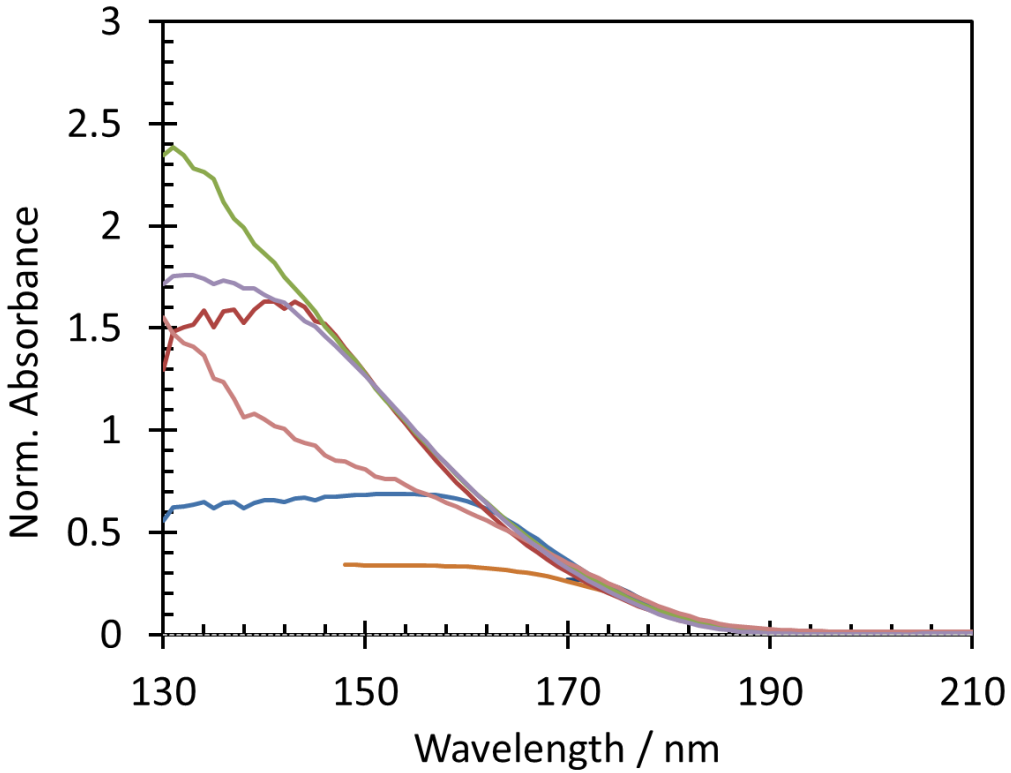
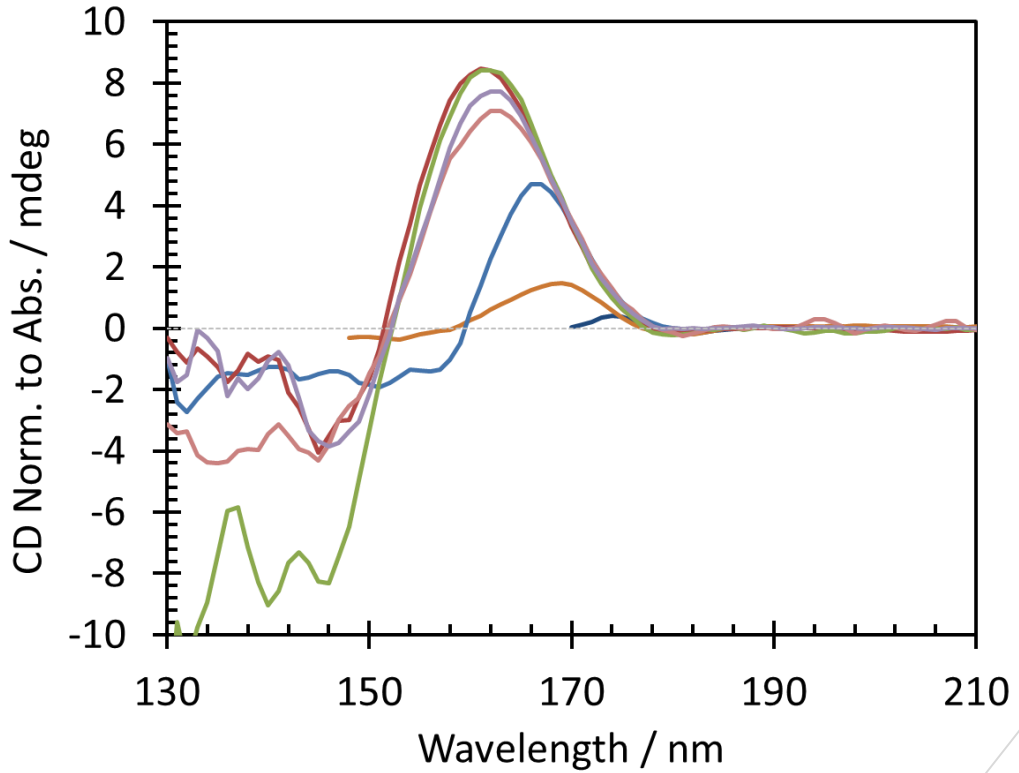
Absorbance flattening

- See a similar effect with thick non-uniform films

Normalise the absorbance spectra to be similar values in a WL region where the shape is similar





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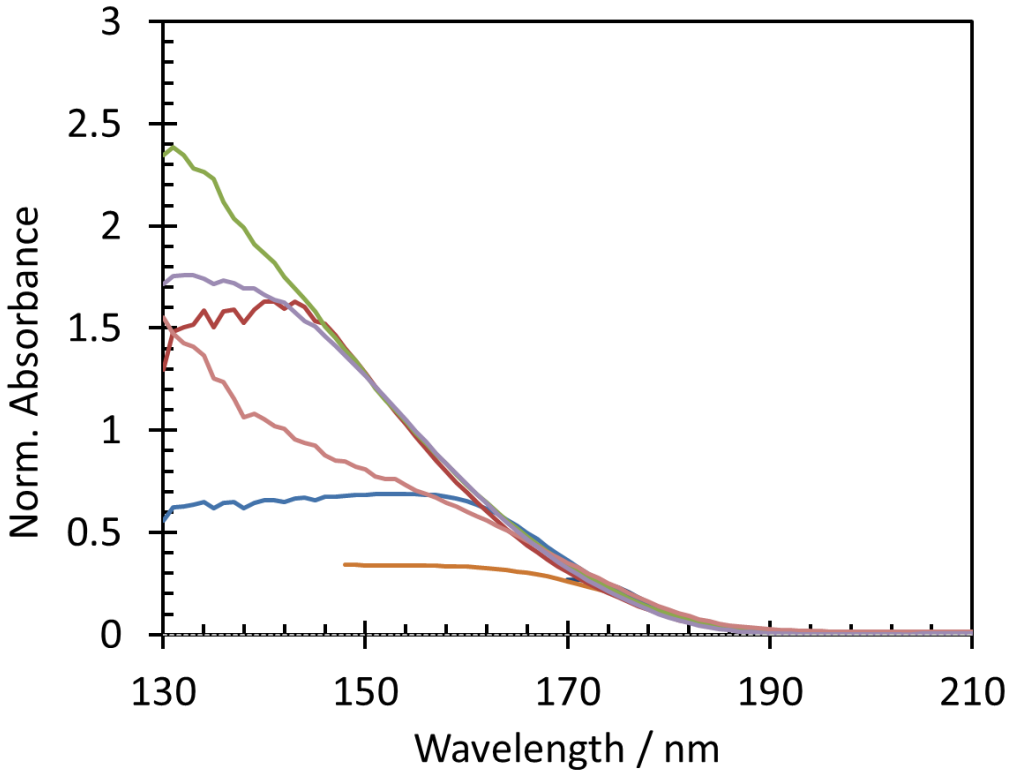
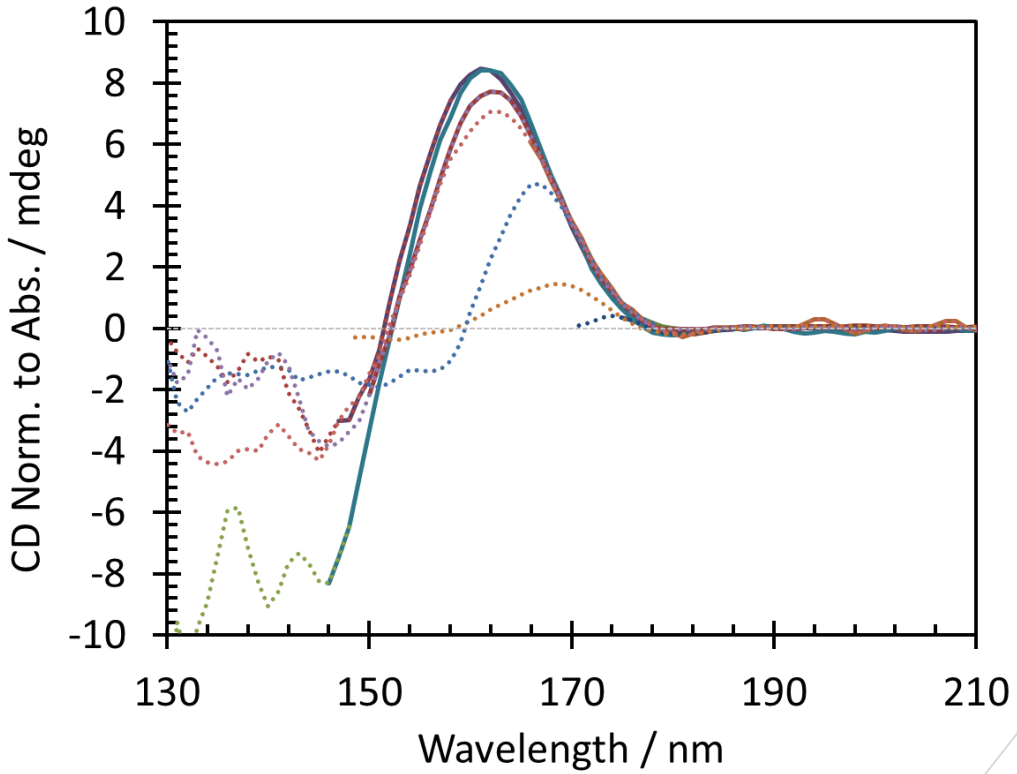
Absorbance flattening

- See a similar effect with thick non-uniform films

Dotted lines reflect the sections of the CD spectrum which has been incorrectly measured due to the effect of absorbance flattening.





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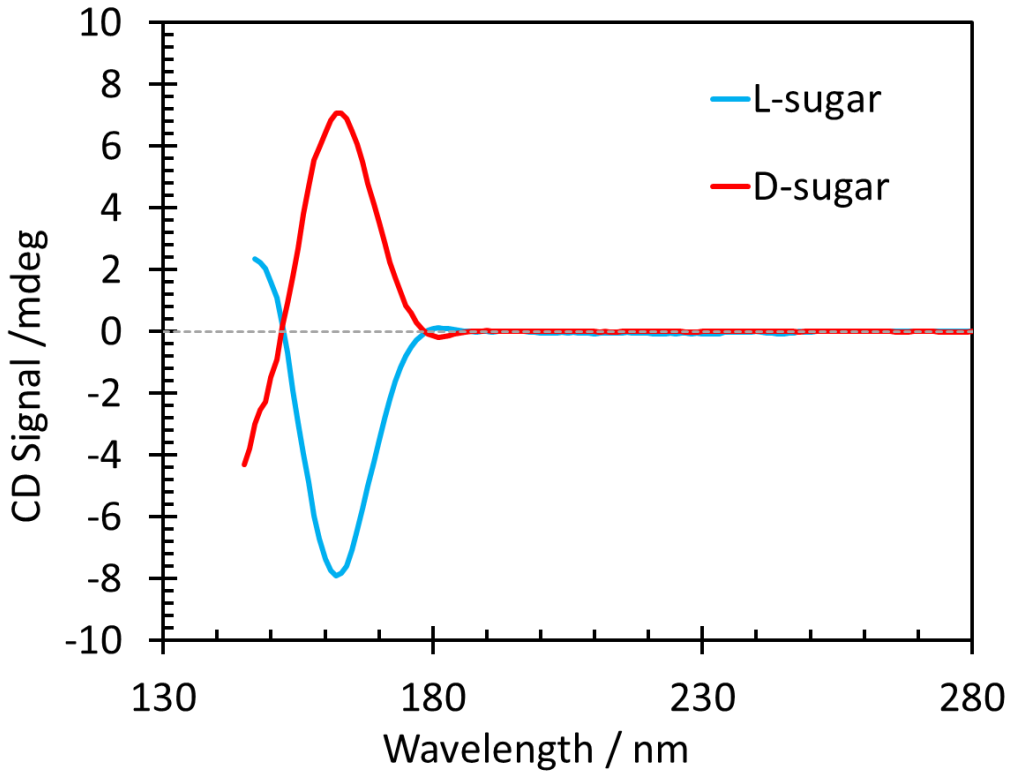
Absorbance flattening

- See a similar effect with thick non-uniform films

It requires that films are prepared and measured multiple times with varying thickness to really be sure of the result,

There still remains some uncertainty of the “true” spectrum at the lowest wavelengths.

However for these types of measurements the other enantiomer can also be measured.



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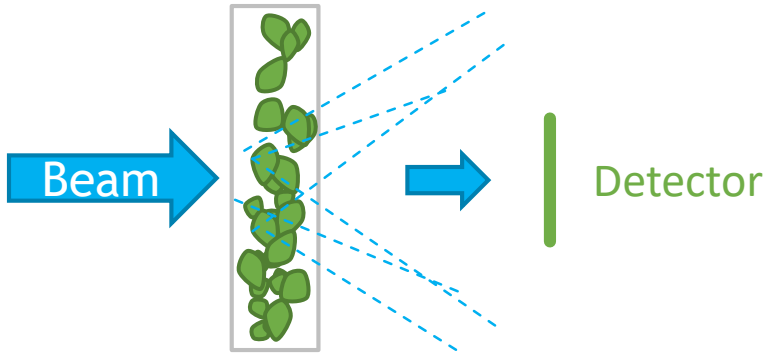
Light scattering effect

- When particles with a size similar or large compared to the wavelength of the incident light are suspended in solution → scattering
- This effect increases the apparent absorbance of the sample.
- Wavelength dependent effect.
- More significant effect in CD than UV spectra, as the refractive indices of an optically active sample are different for left- and right-circularly polarised light.



- Can cause distortion of CD spectrum – both peak position and relative intensity.
- More on this in lecture 5!

Miles, A. J & Wallace, B. A., Chem. Soc. Rev., 2016, 45, 4859

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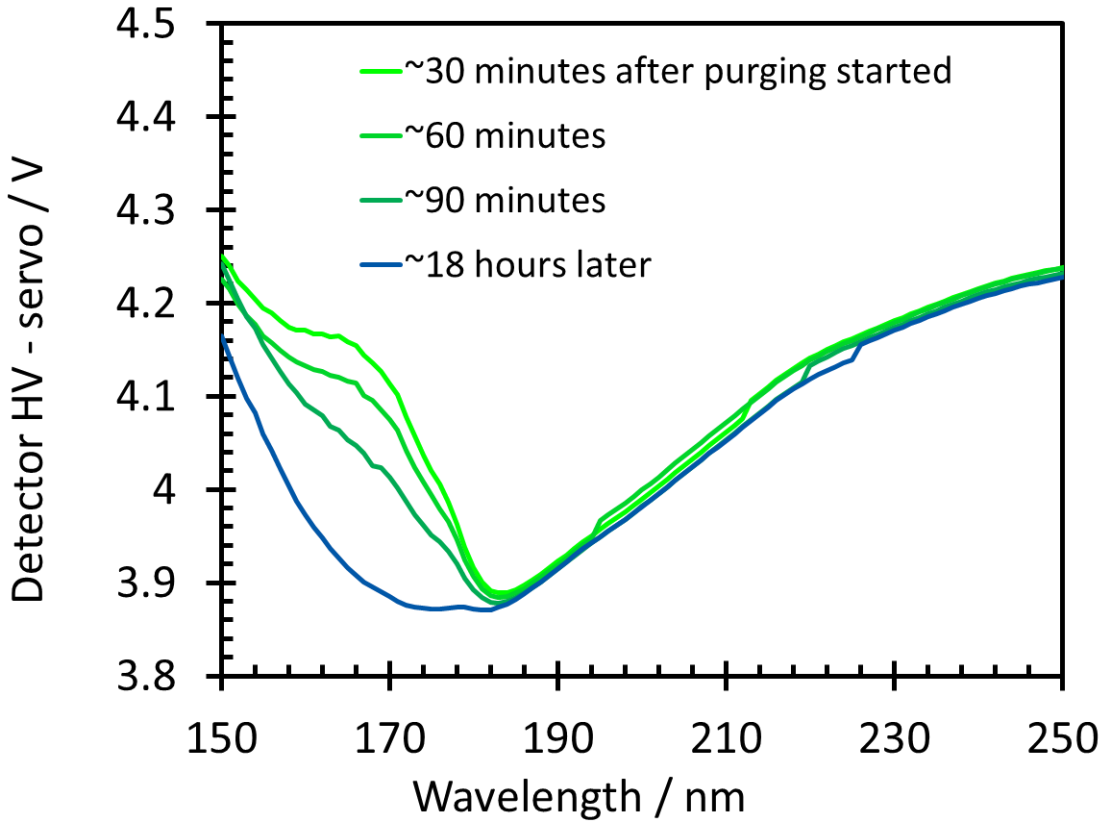
Effect of nitrogen purging on light intensity

- It can take some time for a spectrometer to be fully purged with nitrogen to remove the oxygen and water from the system. Several hours in our case… Effect on intensity really only seen at wavelengths less than 185 nm.



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