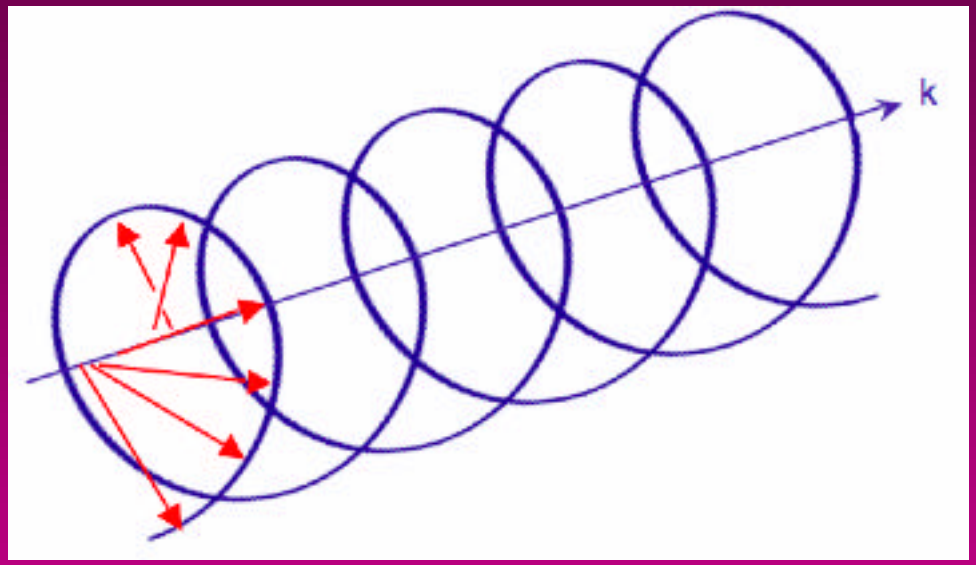
Part A.6
Circular Dichroism Spectroscopy I

Circular Dichroism Spectroscopy I



Introduction
Polarised light
Optical rotation dispersion (ORD)
Circular dichroism (CD)
Optically active chromophores
Instrumentation and practicalities

Circular dichroism: Introduction

- Circular dichroism (CD) spectroscopy measures the difference between the absorption of left and right -handed circularly polarised light.

- CD can provide information on the structures of many types of biological macromolecules, including proteins.

- Typical applications for CD:
  * Protein structure determination
  * Induced structural changes by, for example, pH, heat and solvent
  * Protein folding/unfolding
  * Ligand binding
  * Structural aspects of nucleic acids, polysaccharides, peptides, hormones and other small molecules

- Advantages:
  * Simple and quick experiments
  * No extensive preparation
  * Measurements on solution phase
  * Relatively low concentrations/amounts of sample
  * Any size of macromolecule

Circular dichroism: Introduction

Applications

- Elucidation of secondary structure of proteins

- Investigation of the effect of ligand binding on protein secondary structure (conformational changes)

- Dynamic processes, e.g. protein folding

- Studies of the effects of environment on protein structure

- Secondary structure and super-secondary structure of membrane proteins

- Carbohydrate conformation

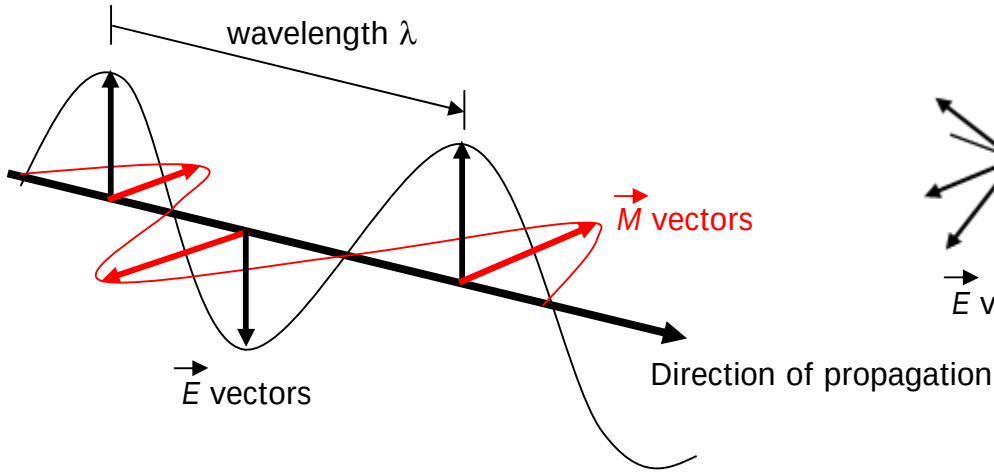
- Investigations of protein-protein and protein-nucleic acid interactions

- Analysis of coordination compounds

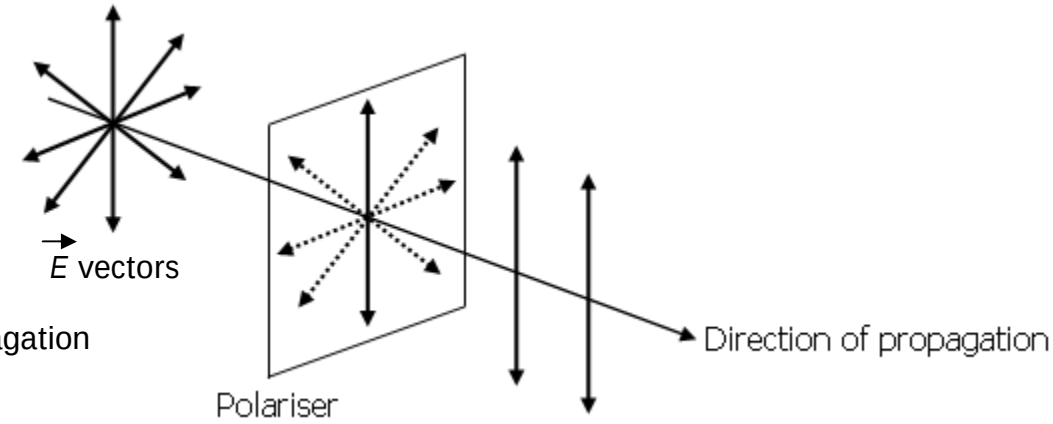
- New materials, sensor molecules, molecular basis of disease

Polarised light

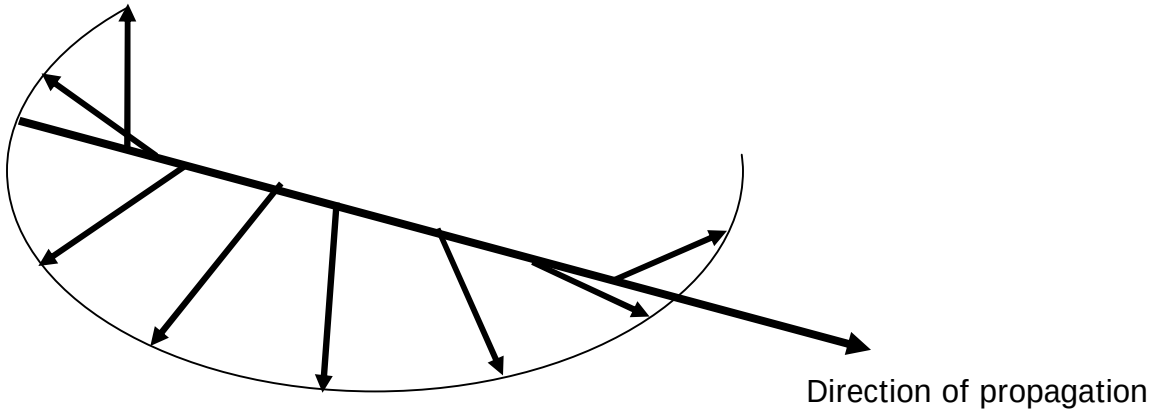
Non-polarised light



Linearly (plane) polarised light

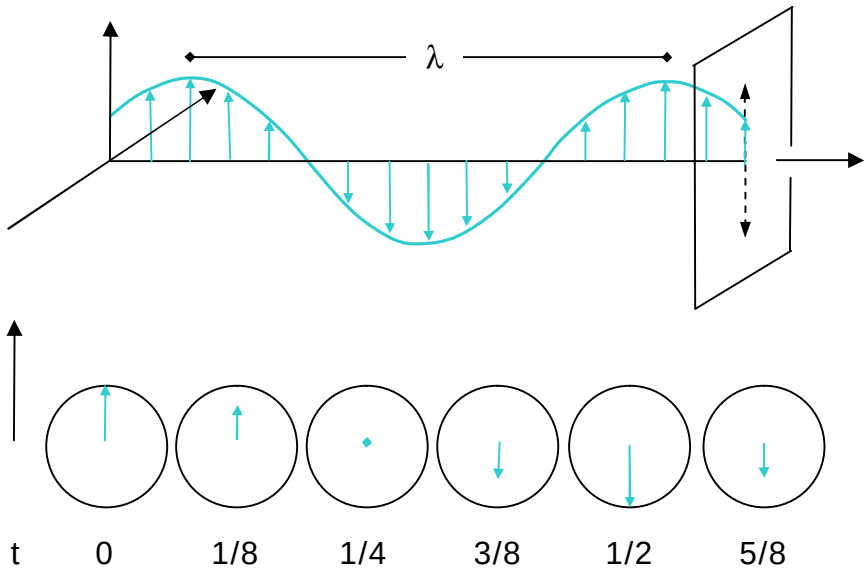


Circularly polarised light



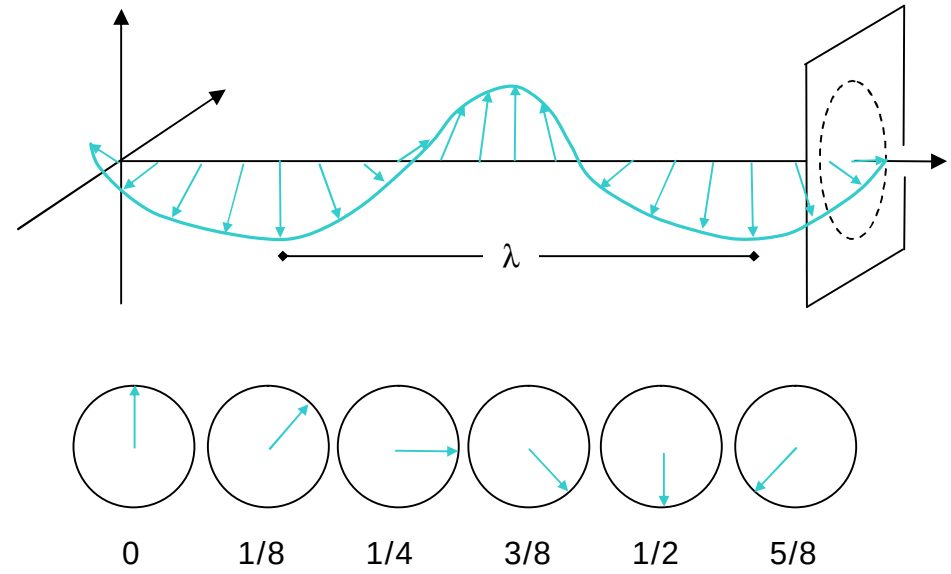
Polarised light

Linearly (plane) polarised light



Direction of electric vector is constant.
Magnitude of electric vector varies.

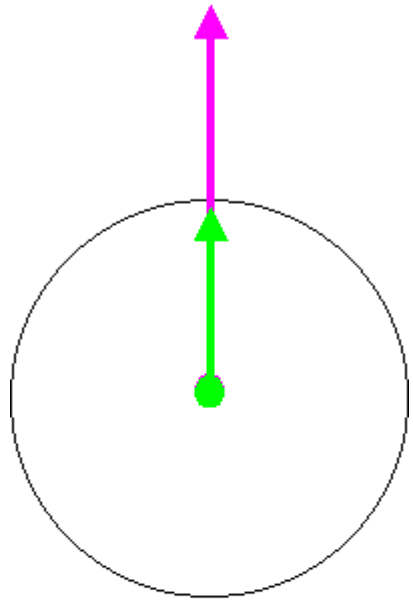
Circularly polarised light



Direction of electric vector varies.
Magnitude of electric vector is constant.

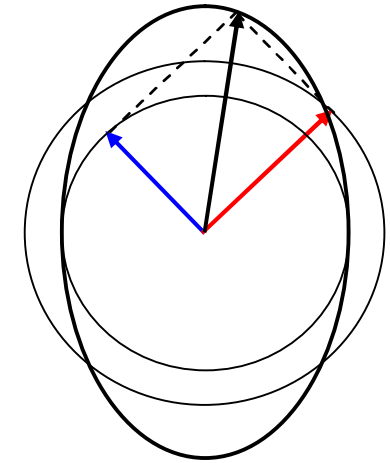
Vector decomposition: Linearly and elliptically polarised light

Linearly polarised light



Two circularly polarised light waves with the same amplitude combine to yield linearly polarised light.

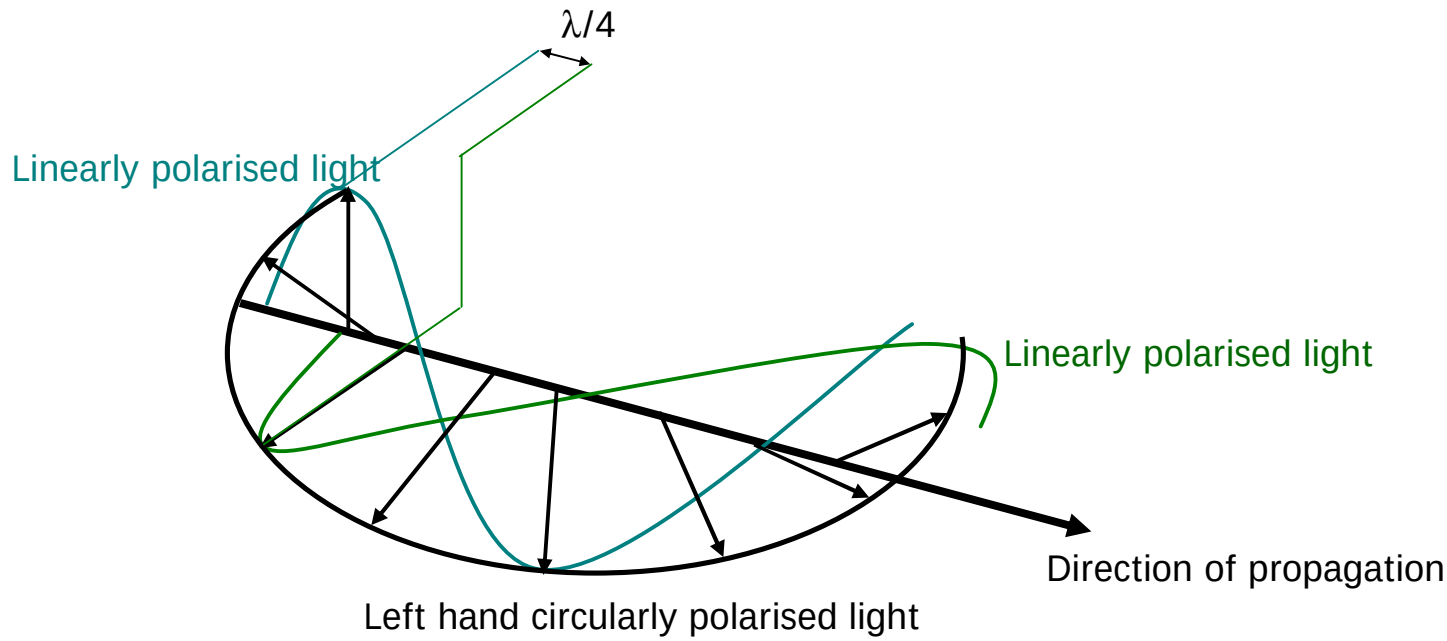
Elliptically polarised light



If two orthogonal linearly polarised components possess different amplitudes, the resulting light is elliptically polarised.

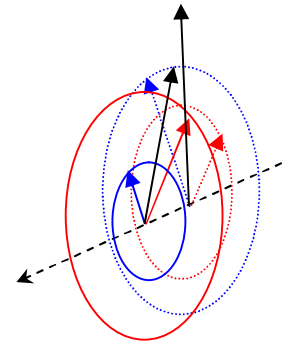
Polarised light

How to produce circularly polarised light: Superposition of two orthogonal linearly polarised light beams

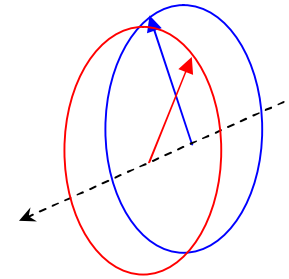


Polarised light

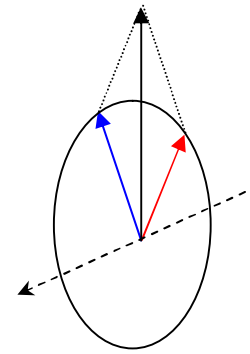
Elliptically polarised light is the most general state of polarisation. Amplitude and phase of both orthogonal linearly polarised beams are not restricted.



A special case is circularly polarised light, where both orthogonal linearly polarised beams have the same amplitude and a phase difference of $\lambda/4$.

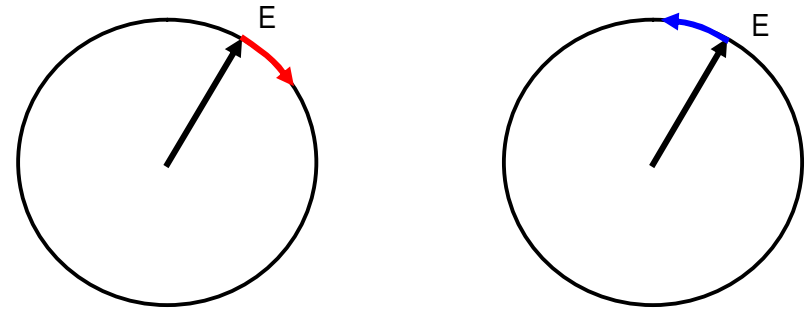


Linearly polarised light is also a special case; here, both orthogonal linearly polarised beams have the same amplitude and the same phase.

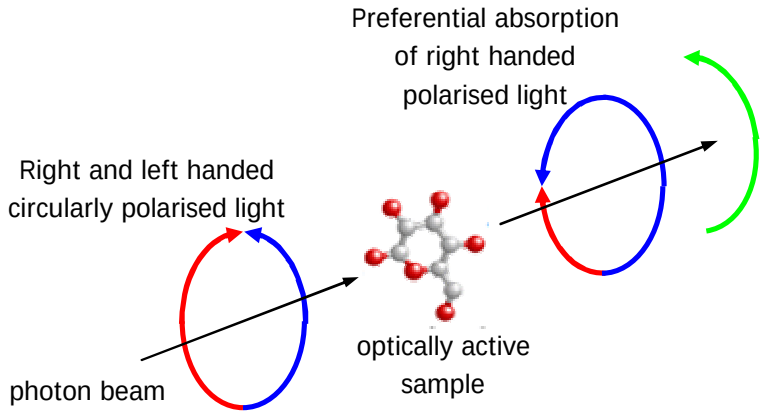


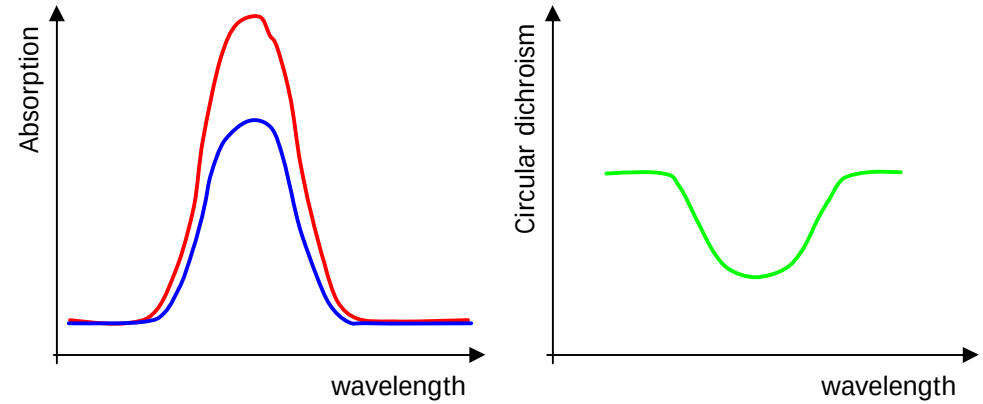
Circularly polarised light

- Magnitude of electric vector is constant, but the direction varies circularly.
  => Right handed and left handed polarisation.



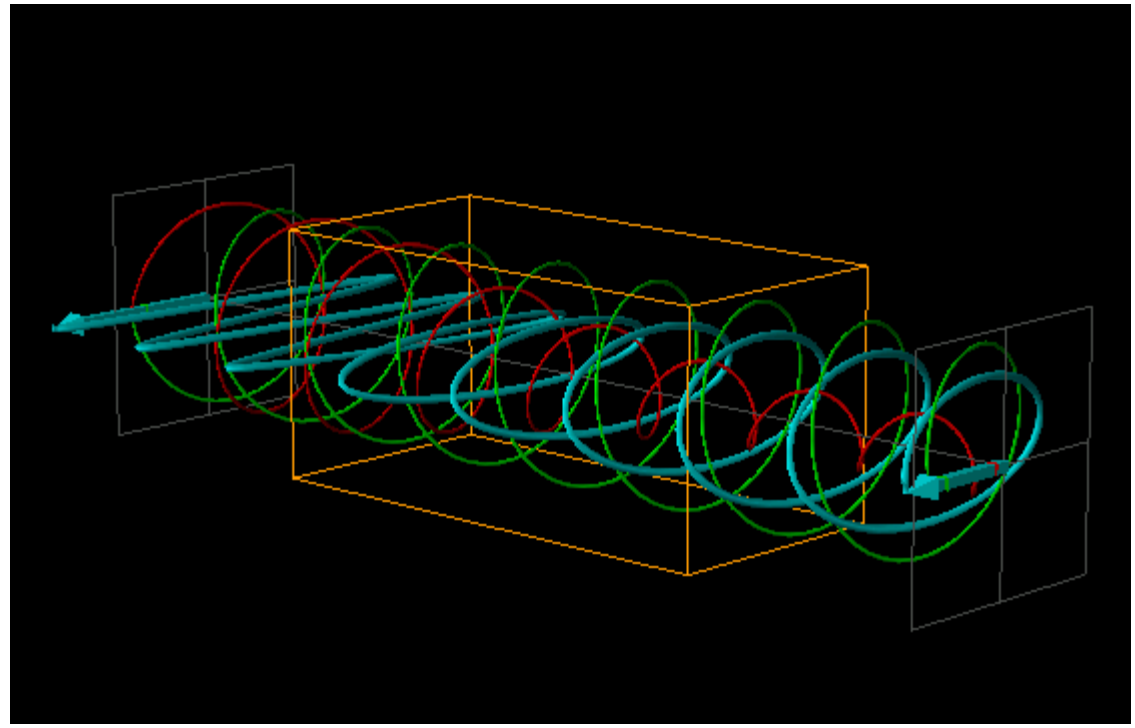
- CD measures the difference between the absorption of left and right handed circularly polarised light.



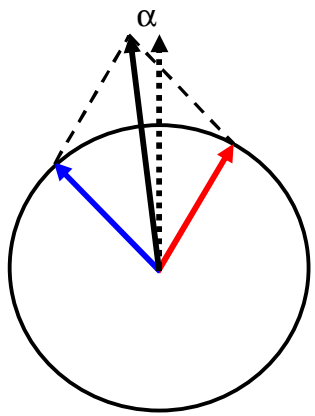


Circular dichroic samples: linear -> elliptic polarisation

Linearly polarised light, when passed through a circular dichroic sample, will become elliptically polarised. This is because the circular polarised components of the original linear polarised light are no longer of equal magnitudes due to differential absorbance (circular dichroism).



Optical rotatory dispersion (ORD)



When an optically active sample is transilluminated with linearly polarised light, the **velocities of both circular components differ after passing the sample**.

The ORD is the angle of the resulting linearly polarised light against the plane of the linearly polarised light of the incident beam:

$$\alpha_\lambda = \frac{180° \cdot d}{\lambda} (n_{left} - n_{right}) \qquad [\alpha_\lambda] = 1°$$

n is the refractive index: $n = \frac{c_0}{c_{matter}}$

c_0 is the speed of light *in vacuo* and c_{matter} the speed of light in matter.
d is the sample thickness (cuvette) and λ the wavelength.

The specific ORD is normalised with respect to sample thickness and concentration:

$$[\alpha]_\lambda = \frac{\alpha_\lambda}{\rho^* \cdot d} \qquad [[\alpha]_\lambda] = 1° \frac{cm^2}{g}$$

Circular dichroism (CD)

Both polarised components are absorbed differently by the sample, which yields a **difference in the absorption coefficients**. This is called circular dichroism.

$$\Delta A = A_{left} - A_{right} \qquad \Rightarrow \qquad \Delta\epsilon = \frac{\Delta A}{c \cdot d} \qquad [\epsilon] = 1\frac{l}{mol \cdot cm}$$

ΔA represents the difference in absorption of left and right handed circularly polarised light. $\Delta\varepsilon$ is the **difference in molar absorption coefficients**.
In practical CD spectroscopy, however, the data given is the **ellipticity** Q, which is proportional to $\Delta\varepsilon$ (and ΔA). It is obtained as per:

$$\Theta_\lambda = \ln 10 \cdot \frac{1}{4} \cdot \frac{360^\circ}{2\pi} \cdot \Delta A \qquad [\Theta_\lambda] = 1^\circ$$

ORD, CD and ellipticity

Circular dichroism (CD) is the difference in absorption of left and right handed circularly polarised light.

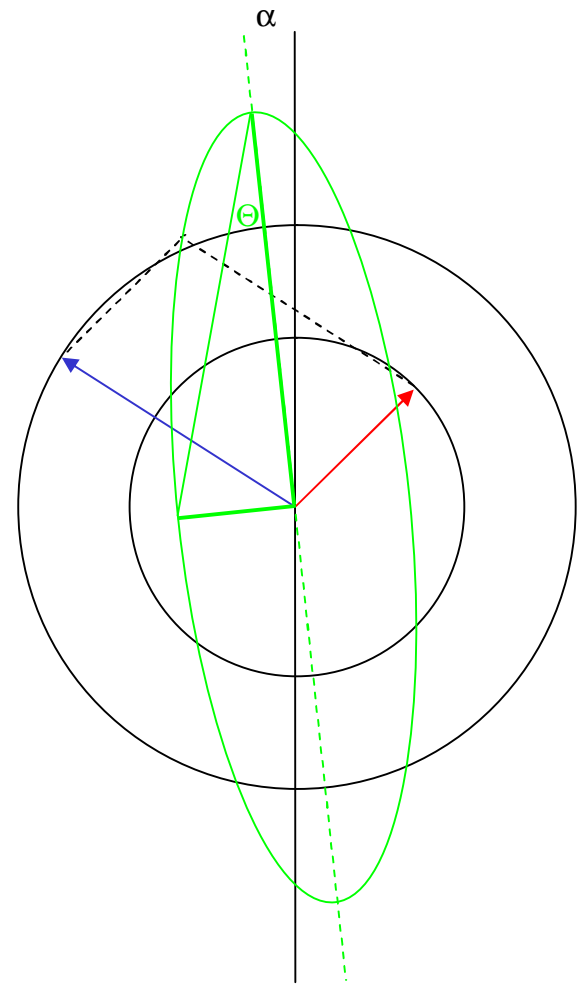
$$\Delta A = A_{left} - A_{right}$$

Ellipticity Θ is a direct consequence of CD.

$$\Theta_\lambda = \ln 10 \cdot \frac{1}{4} \cdot \frac{360^\circ}{2\pi} \cdot \Delta A$$

The ORD (α) is also associated with the CD.

$$\alpha_\lambda = \frac{180^\circ \cdot d}{\lambda} \left(n_{left} - n_{right}\right)$$



Circular dichroism (CD)

In practical CD spectroscopy, the reported data are given as ellipticity Θ:

$$\Theta_\lambda = \ln 10 \cdot \frac{1}{4} \cdot \frac{360^\circ}{2\pi} \cdot \Delta A \qquad [\Theta_\lambda] = 1^\circ$$

After normalisation (and substitution of the molar concentration *c* with the mass concentration ρ*), the molar ellipticity θ is obtained:

$$\theta_\lambda = \frac{M \cdot \Theta_\lambda}{10 \cdot \rho^* \cdot d} = \frac{\ln 10}{10} \cdot \frac{1}{4} \cdot \frac{360^\circ}{2\pi} \cdot \Delta\epsilon \qquad [\theta_\lambda] = 1^\circ \frac{cm^2}{dmol}$$

Historically, most published data refer to 1 dmol = 0.1 mol, rather than 1 mol:

$$0.1^\circ \frac{cm^2}{dmol} = 1^\circ \frac{cm^2}{mol}$$

Circular dichroism (CD)

While typical values of ε are 20000 – 35000 l mol^{-1} cm^{-1}, the difference in molar absorption coefficients for left and right handed circularly polarised light has much smaller values:

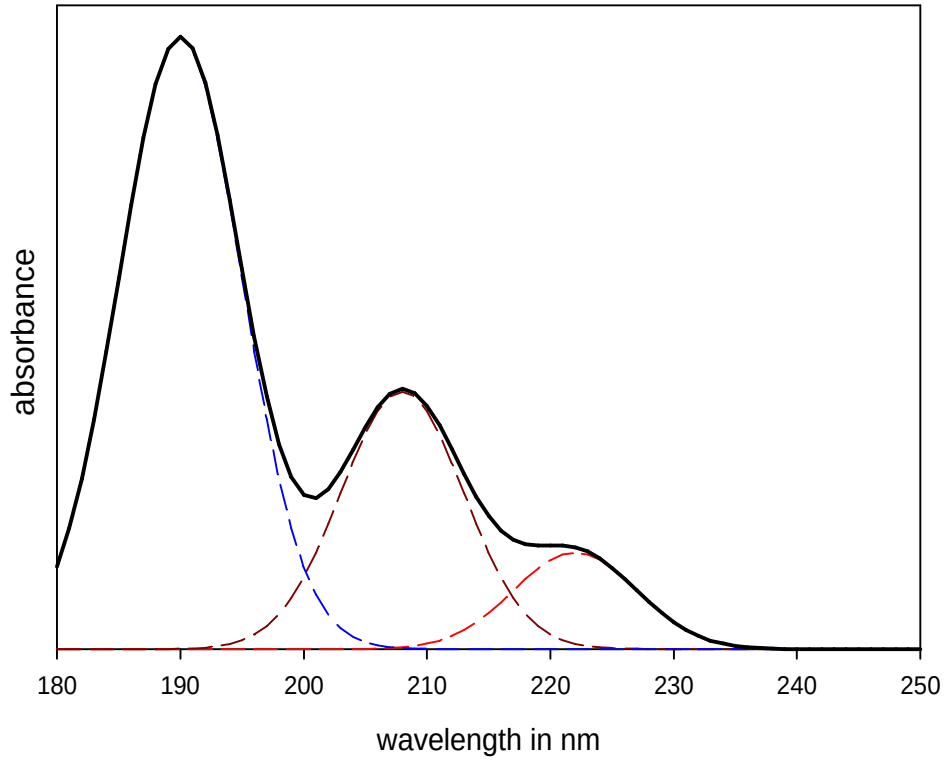
$$\Delta\varepsilon < 10 \text{ l mol}^{-1} \text{ cm}^{-1} \approx 1/1000\ \varepsilon$$

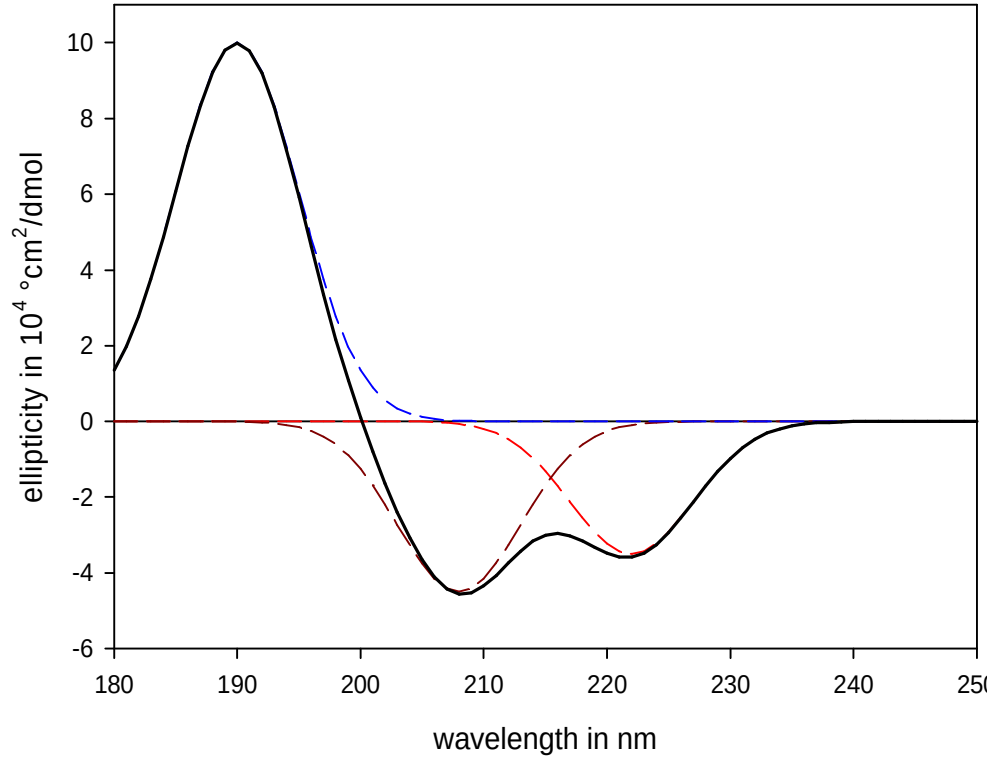
=> The CD signal is a very small difference between two large originals, which poses technical challenges to cope with poor signal-to-noise ratios.

CD is only observed at wavelengths where absorbances of right and left handed circularly polarised components are not zero, i.e. in absorption bands.

Circular dichroism: CD is only observed in absorption bands

Absorption and CD spectra of α-helical poly-(γ-methyl-L-glutamate)





Holtzwarth & Doty (1965) *JACS* **87**, 218-228.

π ? π* (perpendicularly polarised)

π ? π* (parallel polarised)

n ? π*

Optically active chromophores

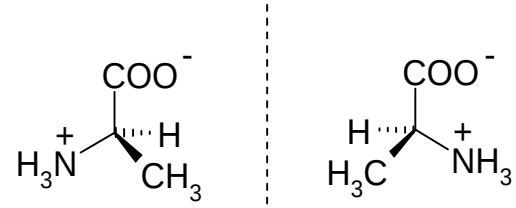
Stereo isomerism: Diastereo isomerism (geometric isomerism), e.g. *cis-trans* isomerism
Enantiomerism (optical isomerism)

Enantiomers:
Despite identical constitution, molecules or ions might not be congruent, i.e. they do not coincide but behave like mirror images. Such objects are called chiral, the individual mirror objects are called enantiomers.

Enantiomers have identical physical properties with exception of absorption of polarised light; they are optically active.

An equi-molar mixture of two enantiomeric forms of a molecule is called a racemat and is optically inactive.

The most important optically active centre in peptides is the Cα atom of amino acids, which has four different binding substituents:

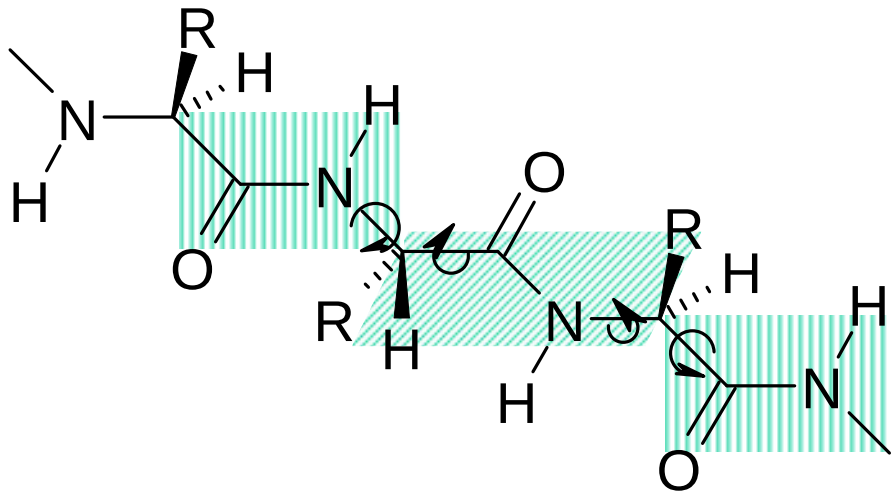


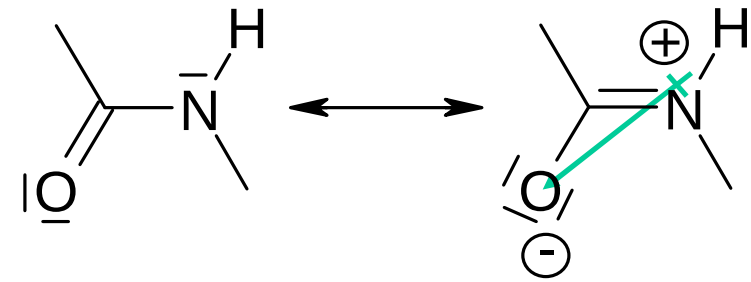
R-Ala
(D-Ala)

S-Ala
(L-Ala)

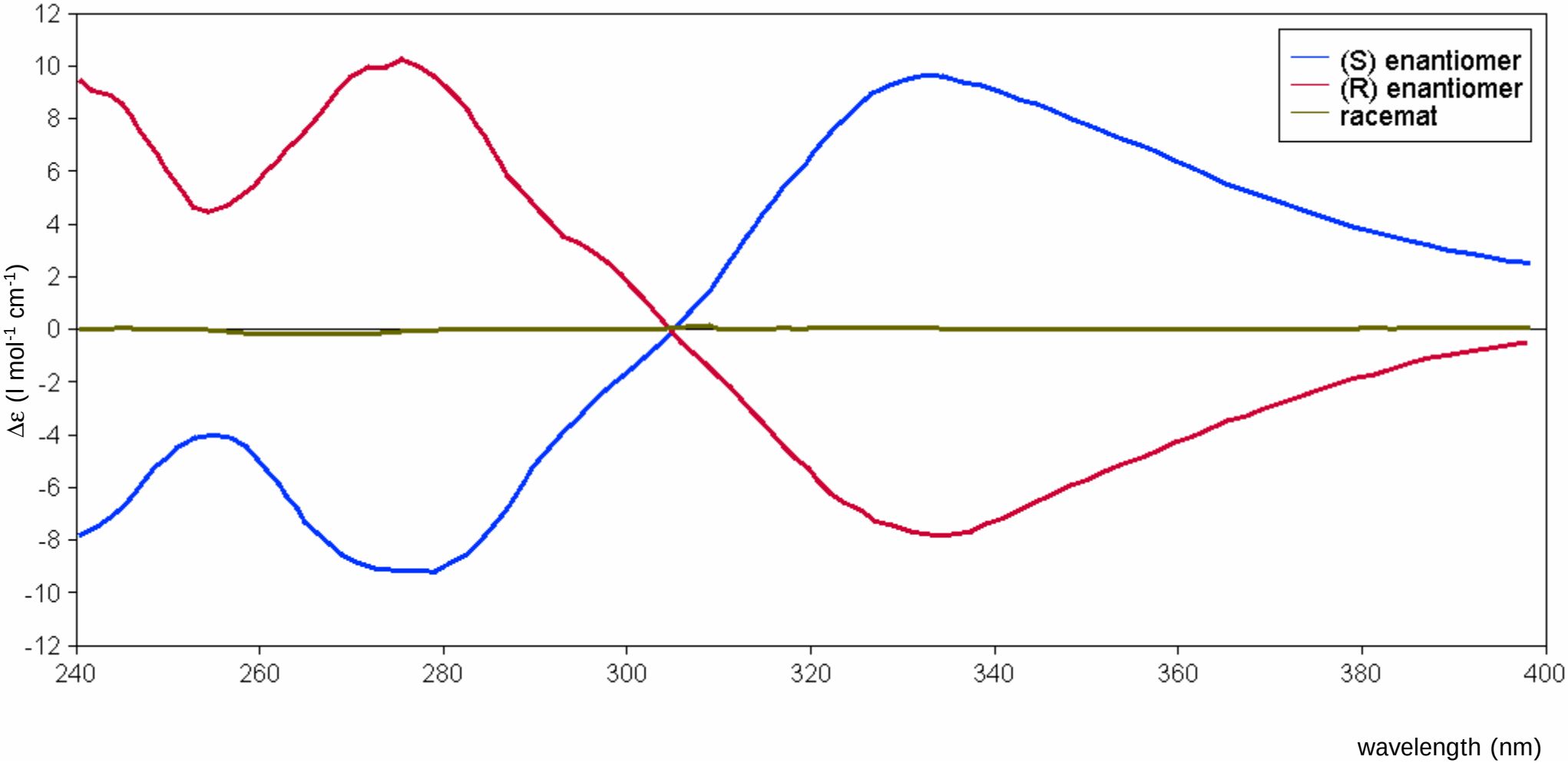
Optically active chromophores: The peptide bond

- Chromophor: CO group of peptide bond
- The vicinal C of CO (C_α) is asymmetric and **induces asymmetry** into the chromophor
- Coupling with other chromophores: secondary structure forms coupled oscillators without symmetry centre or symmetry plane. **The structure** of a polypeptide induces its chirality.





CD spectra of enantiomers are mirror images with respect to $\Delta\varepsilon = 0$



Summary: Theory of CD

Polarised light

Non-polarised light is a collection of waves with all possible orientations of $\vec{E}$ vectors. To obtain linearly polarised light, a polariser is used that only allows transmission of light with $\vec{E}$ vectors parallel to the axis of the polariser; the direction of the $\vec{E}$ vector is constant, the magnitude varies. Circularly polarised light consists of two wave populations that are out of phase by $\lambda/4$. The direction of $\vec{E}$ vector varies, but the magnitude is constant. Elliptically polarised light is the most general state of light polarisation; amplitude and phase of the $\vec{E}$ vector varies.

ORD

Upon translumination of an optically active sample the velocities of both circular components of linearly polarised light differ. The resulting linearly polarised light encloses an angle with polarisation of the incident light beam. This angle is called ORD.

CD

CD is the difference in absorption coefficients of left and right handed polarised light. Traditionally, the “observed” parameter in CD spectroscopy is the ellipticity Θ. In order to compare CD data of different samples (at different concentrations, with different amounts of optically active chromophores), Θ is normalised to yield the specific ellipticity θ_{res}. CD is only observed where absorbances of left and right handed components of circularly polarised light are not zero, i.e. in absorption bands.

Optically active chromophores

Optically active chromophores are chiral compounds (no intrinsic symmetry centre or symmetry plane). In peptides and proteins the Cα atom is chiral and responsible for optical activity of the molecule. In secondary structure elements the individual chromophores are coupled. Thus, secondary structure of proteins can be deduced from CD spectra.

The instruments

ORD spectrometer: Polarimeter

- Usually used in organic chemistry laboratories

- Check stereo selective synthesis of organic molecules:
  e.g. sugars, steroids, etc

CD spectrometer

- Costs: £80000+
- Automatic acquisition of spectra vs. λ, time, temperature, stopped flow
- λ scanning to 190 nm
- 450 W Xe bulb:
  * produces ozones (health, silver coated optics)
  * thus, provide a constant stream of N_2

The sample

Typical sample conditions

- Protein concentration: $\rho^* = 0.1$ mg/ml

- Volume: $V_L = 300$ µl for a cell pathlength of $d = 1$ mm

- Buffer:
  * rather low concentration in order to prevent detector saturation at low wavelengths
  * 5 mM or as low as possible while maintaining protein stability
  * TRIS not suitable due to high UV absorption

- Quartz cell path lengths: 0.0001 cm - 10 cm; most common: 0.1 cm, 1 cm

- Use of filtered/centrifuged solutions to avoid turbidity

Generally

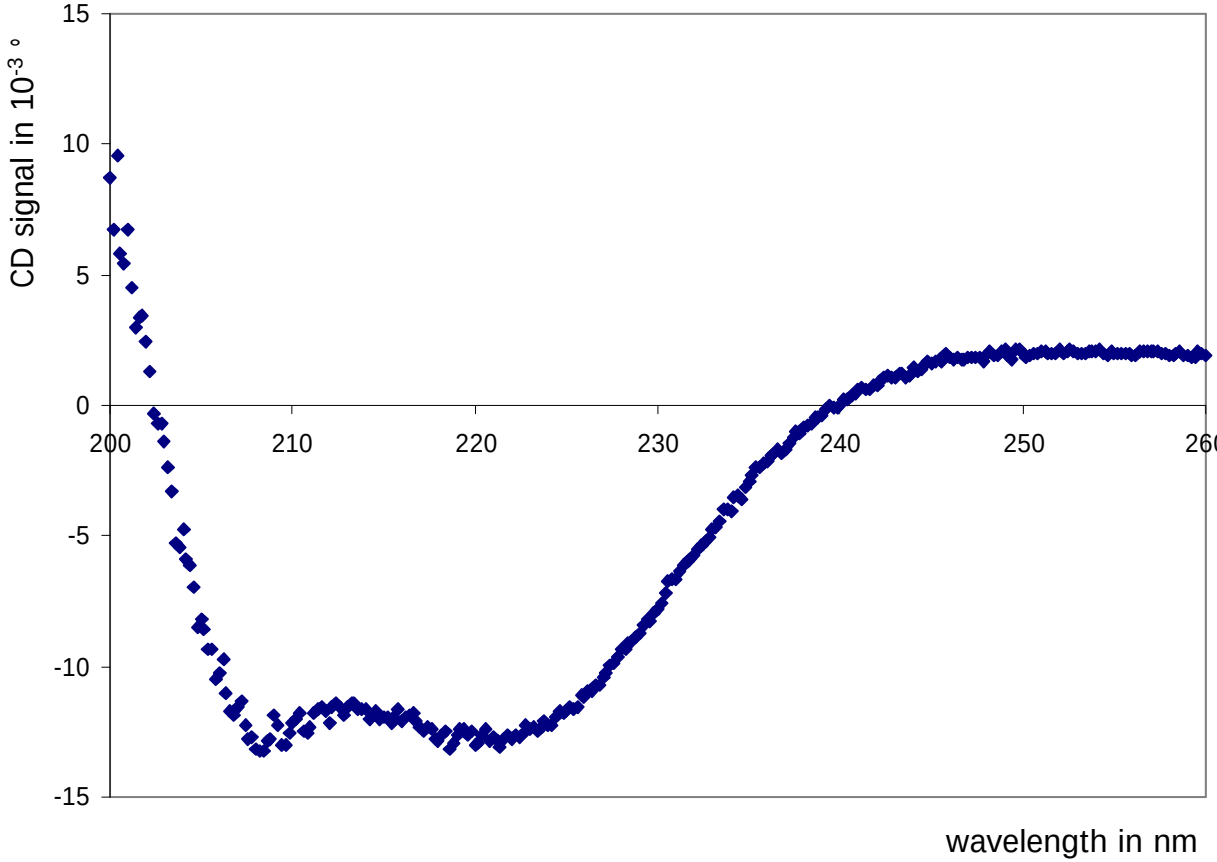
- CD is based on measuring a very small difference between two large signals: must be done carefully

- Measure cell base line with solvent, then sample with same cell inserted same way around

- Ensure clean cell surface

- Concentration of sample has to be determined in order to calculate specific ellipticities and secondary structure

Example of a CD experiment: Raw CD spectrum



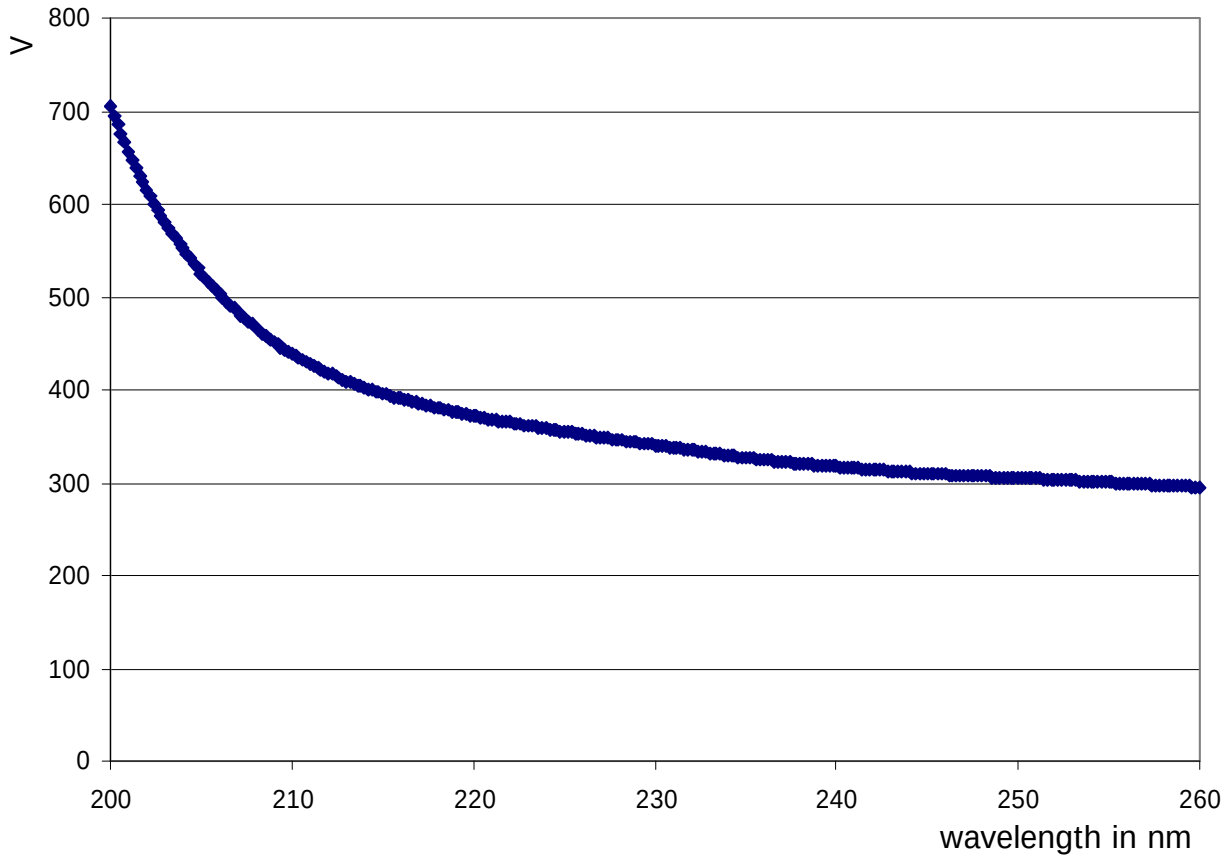
- Sample:
  Annexin 24 from bell pepper in
  100 mM NaCl, 5 mM HEPES (pH= 8.0)
  20°C (thermostatted by Peltier element)

- Raw spectra
  Data every 0.2 nm from 260 nm to 200 nm
  Scan speed: 10 nm/min
  Each data point measured 3 times, 1 s averaging

- Noise
  requires measurement of many data points

- Final spectrum
  average of 3 runs

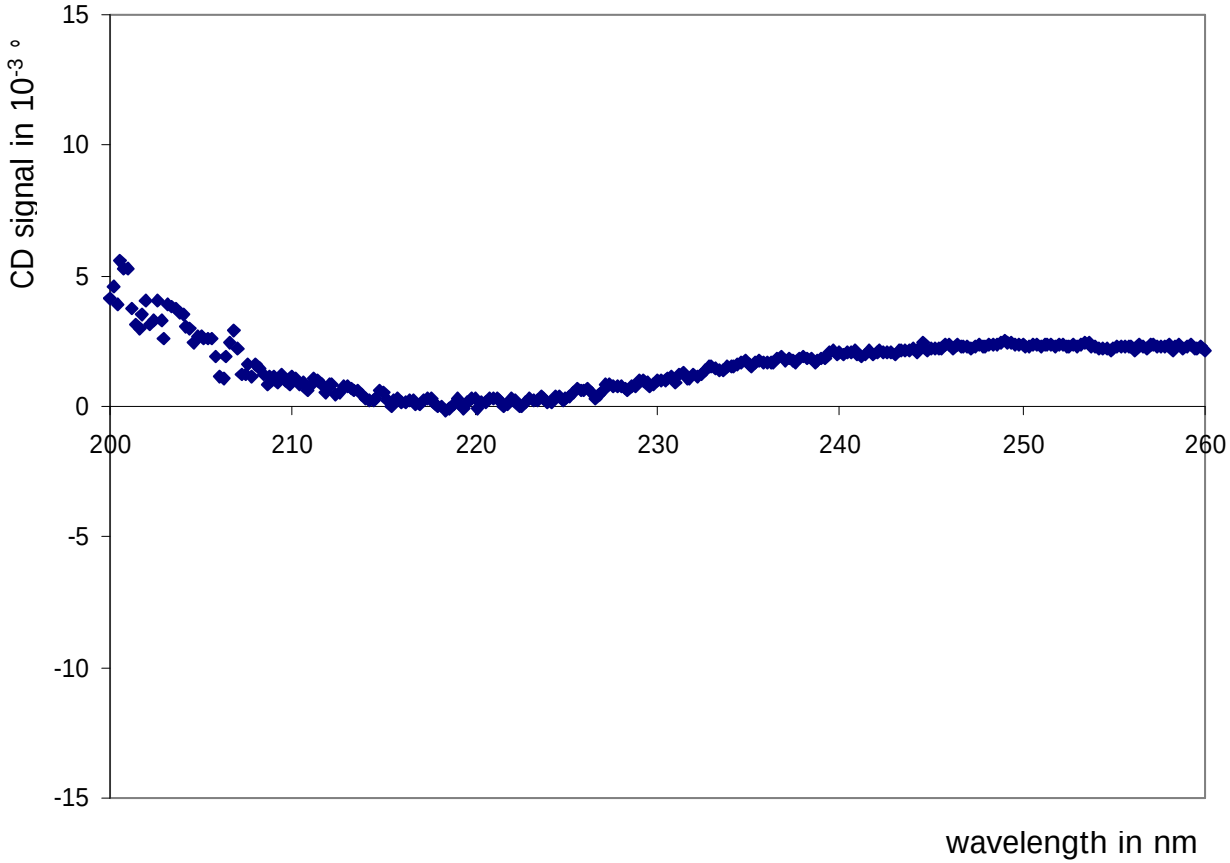
Example of a CD experiment: Voltage on the detector



- Detector

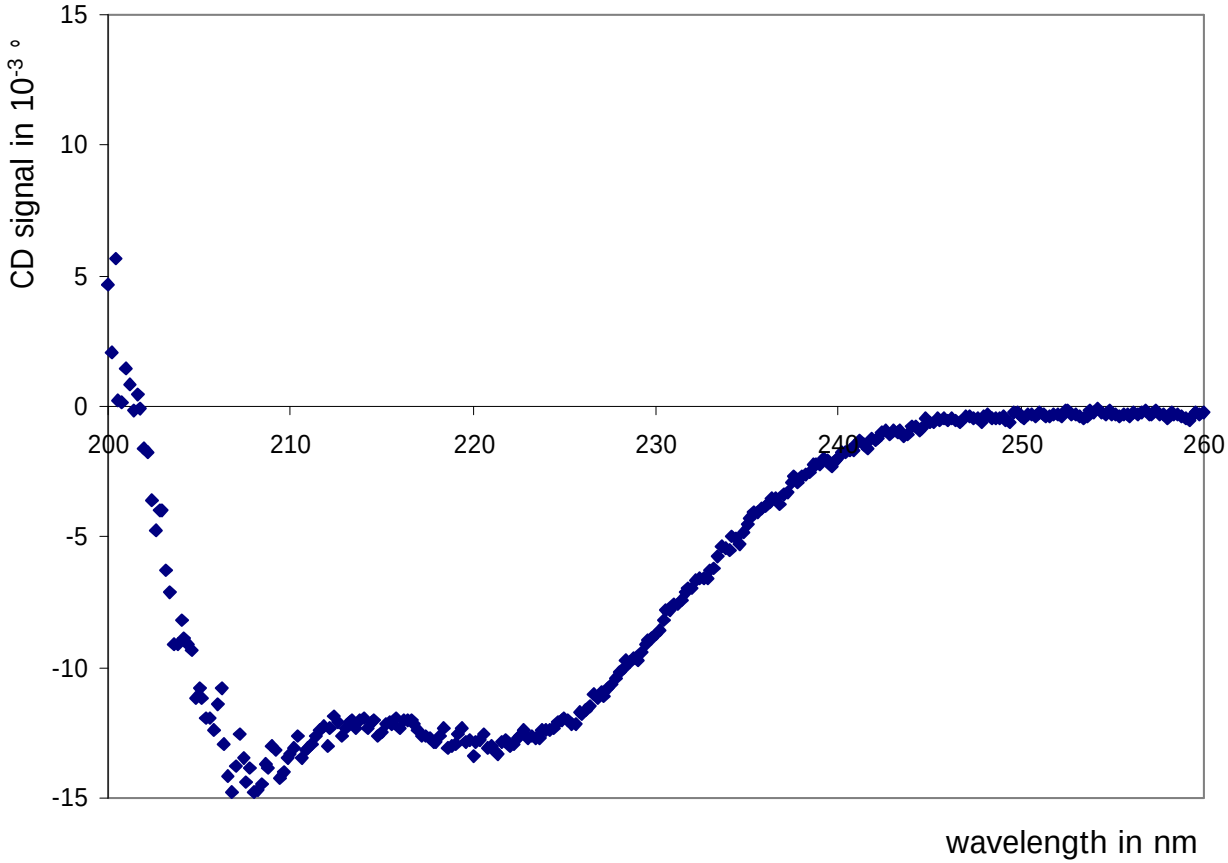
  For in-house CD spectrometers, voltage should not exceed 600 V to ensure good data quality on the blue side of the spectrum

Example of a CD experiment: Buffer spectrum



- Baselines vary:
  * from cell to cell
  * if instrument has been moved
  * new bulb
  * recalibrated

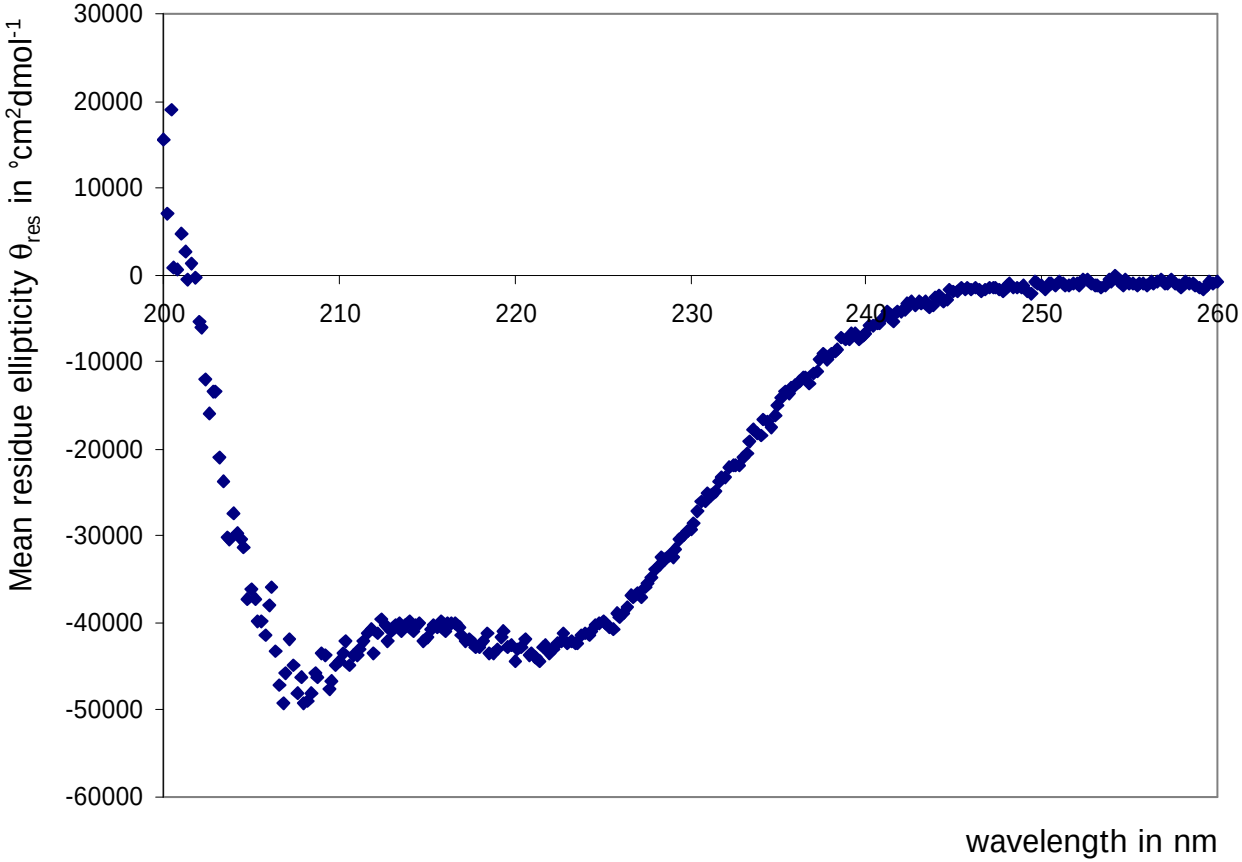
Example of a CD experiment: Corrected protein spectrum



Corrected spectrum =

Sample spectrum - Buffer spectrum

Example of a CD experiment: CD spectrum of protein



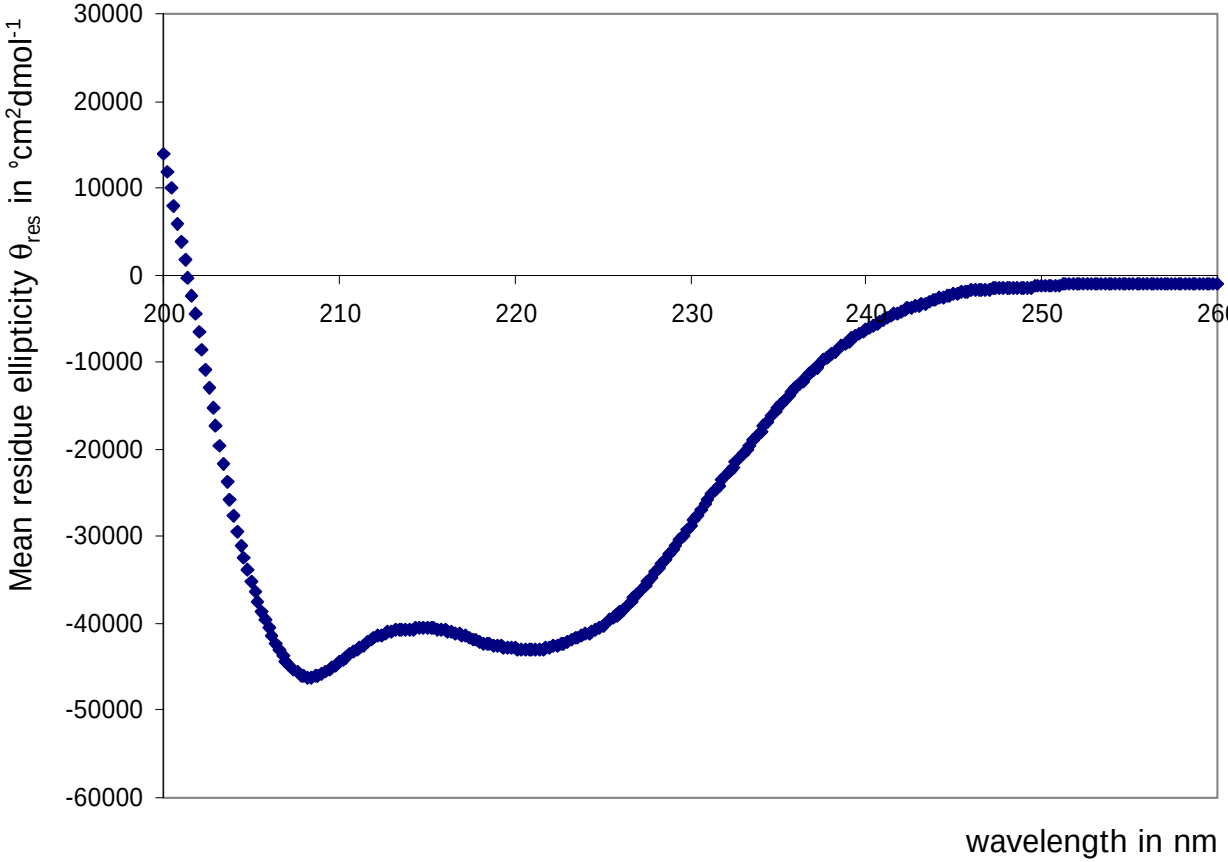
- Conversion of CD signal to mean residue ellipticity

  * Allows comparison with other samples/proteins

  * Normalised with respect to concentration and number of chiral chromophores

  * Required for secondary structure prediction

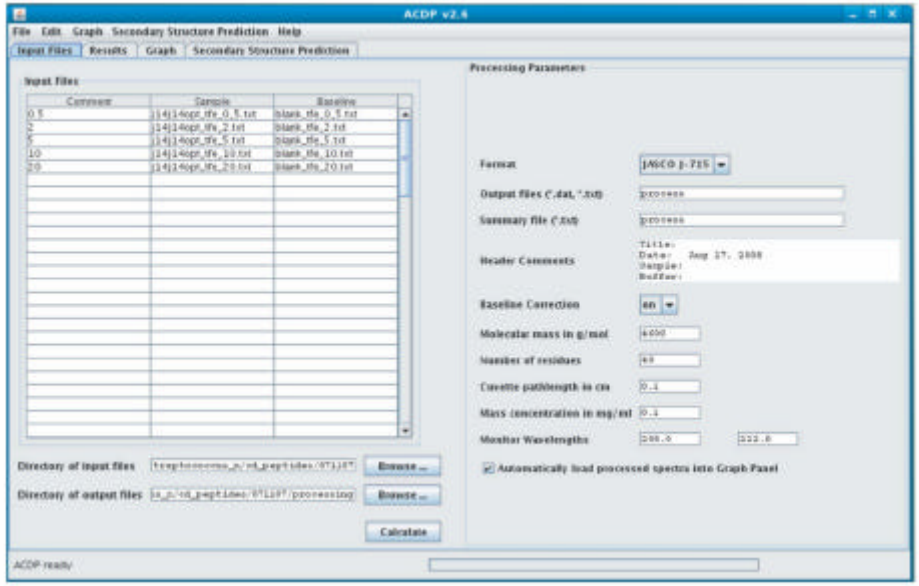
Example of a CD experiment: Smoothed CD spectrum of protein

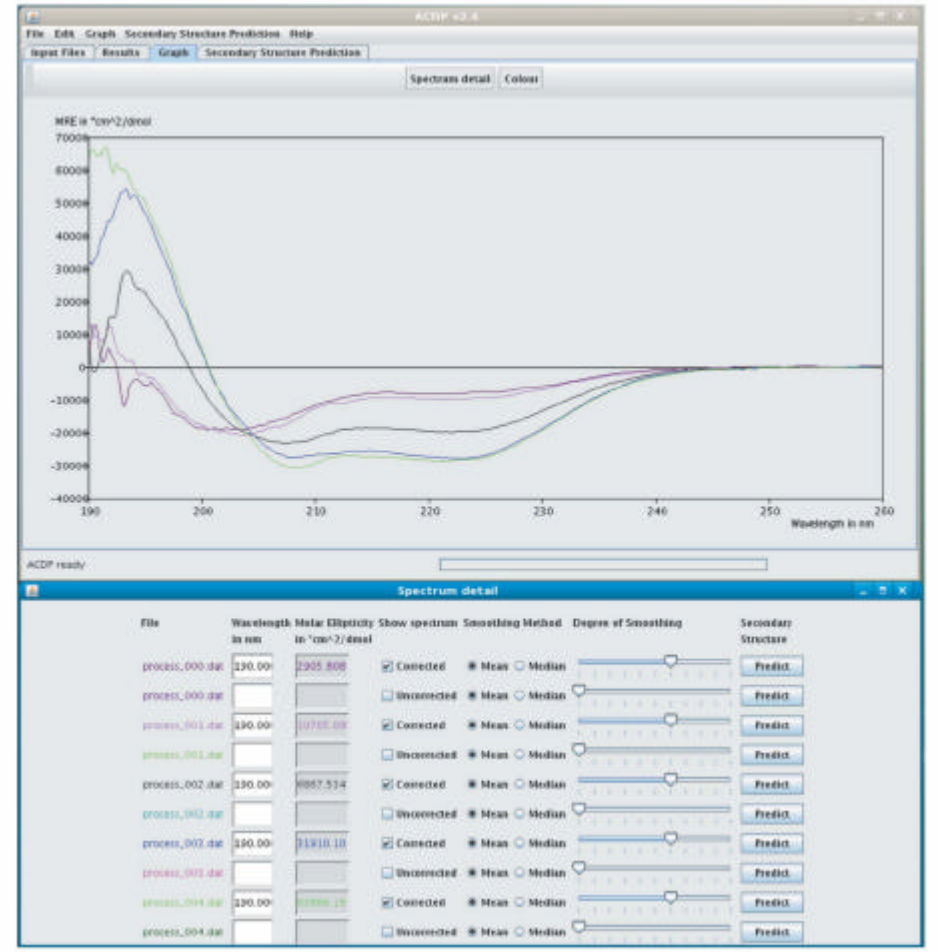


- Curve smoothing; different algorithms available:
Mean value in sliding window
Loess filtering
etc.

Software to analyse CD spectra

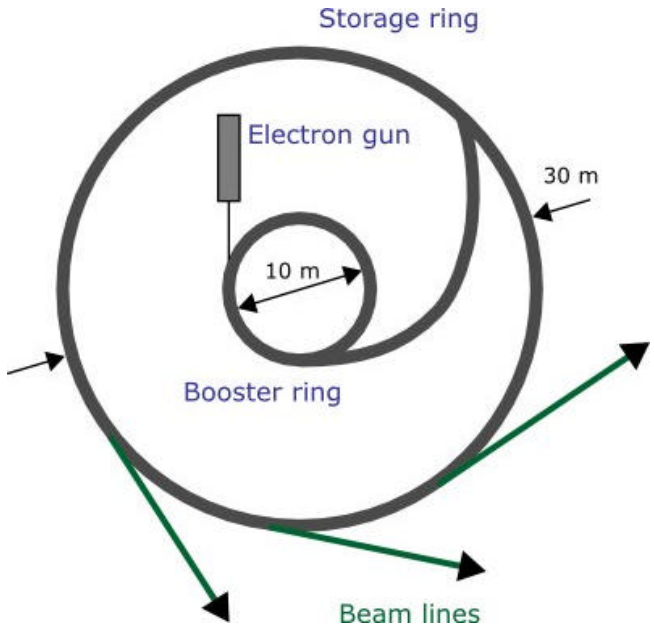
ACDP - a Java application for data processing and analysis of protein circular dichroism spectra

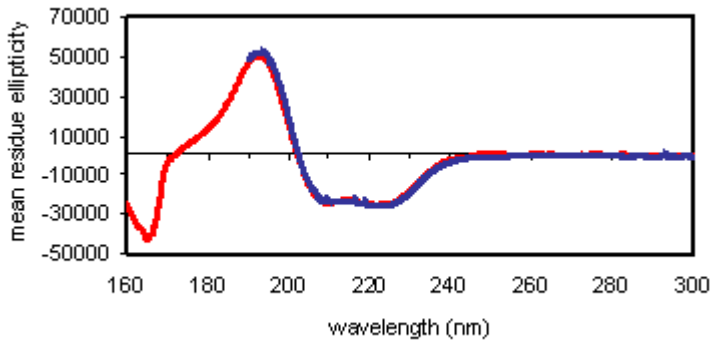




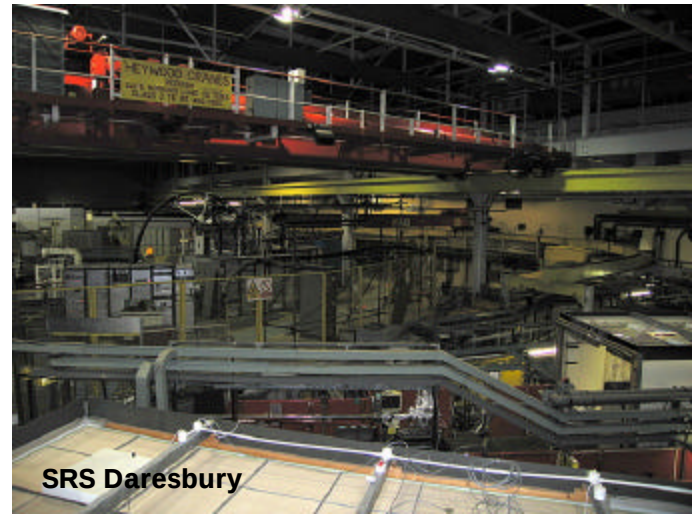
Hofmann, A. (2009) *J. Appl. Crystallogr.* **42**, 137-139.

Synchrotron-based CD spectroscopy





- Synchrotron - whiz electrons around a ring
- Can be used to produce very intense radiation by wiggling beam
- Commonly used to produce X–rays (λ around 0.1 nm)
- But can be used to acquire at wavelengths of 160 nm and below
- Great for fast stopped-flow to see rapid changes
- UK: CD beamline at Diamond Light Source


SRS Daresbury

Further Reading

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CD links

Daresbury: http://www.srs.dl.ac.uk/VUV/CD/
Dichroweb (online CD analysis tool): http://www.cryst.bbk.ac.uk/cdweb/html/