Absorption spectroscopy in the analysis of biological molecules

Fluorescence methods in life sciences

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Overview

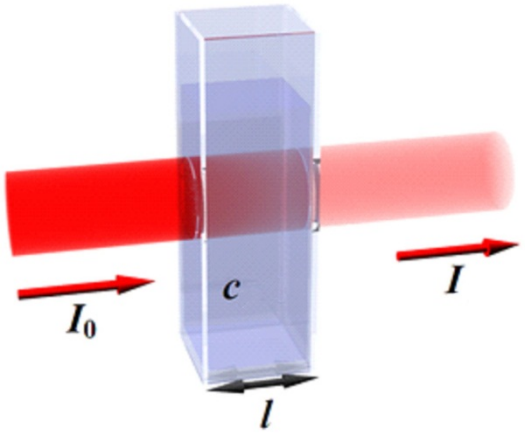
- Determination of DNA concentration
- Determination of protein concentration
- Circular dichroism in determination of biomolecular structure

UV spectroscopy of DNA and proteins

- All atoms absorb in the UV region of the spectrum, because this radiation has sufficient energy to excite the outer electrons.
- UV spectroscopy is used to determine the concentration of DNA and proteins and to define the ratio of the DNA / protein in solution
- Beer-Lambert law is used to determine the concentration of biological molecules

Beer-Lambert law

- Light is absorbed in material
- For the absorption of mono-chromatic light
- **Beer-Lambert law:**
  The absorbance is directly proportional to the concentration and thickness of the solution layer

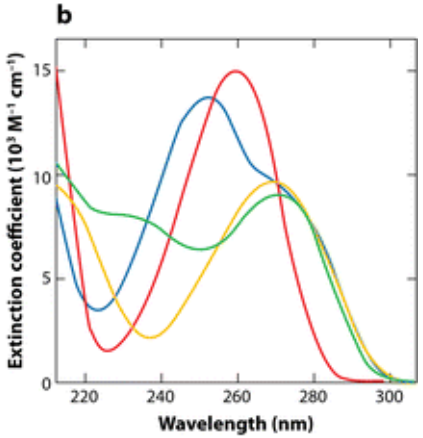


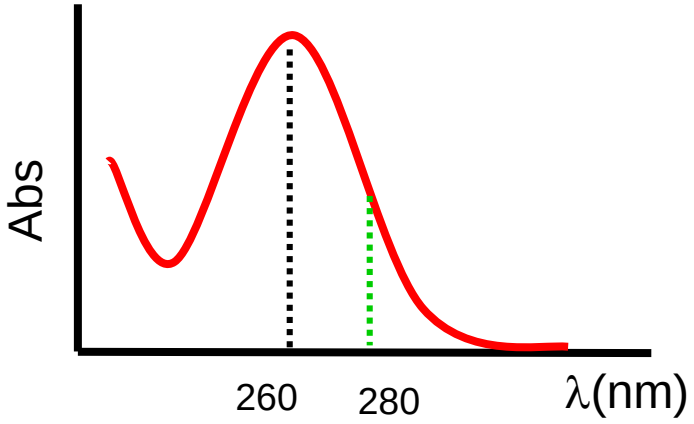
$$I = I_0 \cdot 10^{-\varepsilon \cdot c \cdot l} \quad A = \varepsilon \cdot c \cdot l = \log_{10} \frac{I_0}{I}$$

ε = molar extinction coefficient, c - concentration, l - optical path length

Absorption spectrum of DNA

- DNA spectrum is composed of contributions of individual bases
- Heterocyclic purines A, G absorb the most, C, T less
- Absorbance of DNA is measured at the maximum i.e. 260 nm
- The ratio A_{260}/A_{280} is for pure DNA 1.8
- The ratio less than 1.8 indicates the presence of proteins or contaminants.





Determination of the approximate concentration of nucleic acids (NA)

- If the solution of NA has Abs $_{260}$=1 in 1 cm cuvette, then concentration of:
- double-stranded **dsDNA** **50 μg/ml**
- single-stranded **ssDNA** **30 μg/ml**
- single-stranded **RNA** **40 μg/ml**
- From this we can calculate the molar concentration using the average Mr of nucleotides (320)
- Thus calculated concentration refers to 1 nucleotide!
- For the conversion of concentrations applies:

320 μg/ml ~ 1 mM

- **It is the molar concentration of nucleotides in solution!**

Determination of the extinction coefficient of oligonucleotides

$$A = \varepsilon \cdot c \cdot l$$

- **by measuring** – Analytical determination
  phosphor analysis

- **by calculating** – the most accurate is based on the sequence considering the influence of neighboring base

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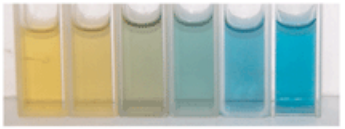
Phosphorous analysis in the determination of DNA concentration



- Precise analytical method
- Determines the concentration of phosphate groups
- Before the analysis it is necessary to cleave DNA
- It enables to determine ε also for DNA analogues and in modification of DNA e.g. fluorescent labels, which affect significantly ε
- I uses colorimetry – the degree of coloration of the solution is directly proportional to the amount of PO_4 (i.e. the amount of DNA)

Murphy, J.H. and Trapane, T.L.,1996, Analytical Biochemistry, 240, 273-282.

Calculation of extinction coefficient of the DNA

- The extinction coefficients for the individual bases contribute to the resulting ε of the entire DNA according to rule of nearest neighbor
- Interaction of neighboring bases influence the level of absorption
- Calculation of extinction coefficient of the oligonucleotide with a length of n nucleotides

$$\varepsilon_{260} = \sum_{n-1}^{1} (\varepsilon_{\text{nearest neighbor}}) - \sum_{n-1}^{2} (\varepsilon_{\text{individual}})$$

5'→3'	dA	dC	dG	dT
dA	27,400	21,200	25,000	22,800
dC	21,200	14,600	18,000	15,200
dG	25,200	17,600	21,600	20,000
dT	23,400	16,200	19,000	16,800

dA = 15,400, dC = 7,400, dG = 11,500, dT = 8,700

Warshaw, M.M. and Tinoco, I. (1966) Optical properties of sixteen dinucleoside phosphates. J. Mol., Biol., 20: 29-38.
Cantor, C.R. and Warshaw, M.M. (1970) Oligonucleotide interactions. Biopolymers, 9:1059-1103

Example of calculation

- M13 sequencing primer 5'- gTA AAA CgA Cgg CCA gTg -3,

Individual bases

dA = 15,400, dC = 7,400, dG = 11,500, dT = 8,700

nearest neighbor

5'→3'	dA	dC	dG	dT
dA	27,400	21,200	25,000	22,800
dC	21,200	14,600	18,000	15,200
dG	25,200	17,600	21,600	20,000
dT	23,400	16,200	19,000	16,800

$$\varepsilon_{260} = (\varepsilon_{GT} + \varepsilon_{TA} + \varepsilon_{AA} + \varepsilon_{AA} + \varepsilon_{AA} + \varepsilon_{AC} + \varepsilon_{CG} + \varepsilon_{GA} + \varepsilon_{AC} + \varepsilon_{CG} + \varepsilon_{GG} + \varepsilon_{GC} + \varepsilon_{CC} + \varepsilon_{CA} + \varepsilon_{AG} + \varepsilon_{GT} + \varepsilon_{TG}) - (\varepsilon_{T} + \varepsilon_{A} + \varepsilon_{A} + \varepsilon_{A} + \varepsilon_{A} + \varepsilon_{C} + \varepsilon_{G} + \varepsilon_{A} + \varepsilon_{C} + \varepsilon_{G} + \varepsilon_{G} + \varepsilon_{C} + \varepsilon_{C} + \varepsilon_{A} + \varepsilon_{G} + \varepsilon_{T})$$

$$\varepsilon_{260} = (20{,}000 + 23{,}400 + 27{,}400 + 27{,}400 + 27{,}400 + 21{,}200 + 18{,}000 + 25{,}200 + 21{,}200 + 18{,}000 + 21{,}600 + 17{,}600 + 14{,}600 + 21{,}200 + 25{,}000 + 20{,}000 + 19{,}000) - (8{,}700 + 15{,}400 + 15{,}400 + 15{,}400 + 15{,}400 + 7{,}400 + 11{,}500 + 15{,}400 + 7{,}400 + 11{,}500 + 11{,}500 + 7{,}400 + 7{,}400 + 15{,}400 + 11{,}500 + 8{,}700)$$

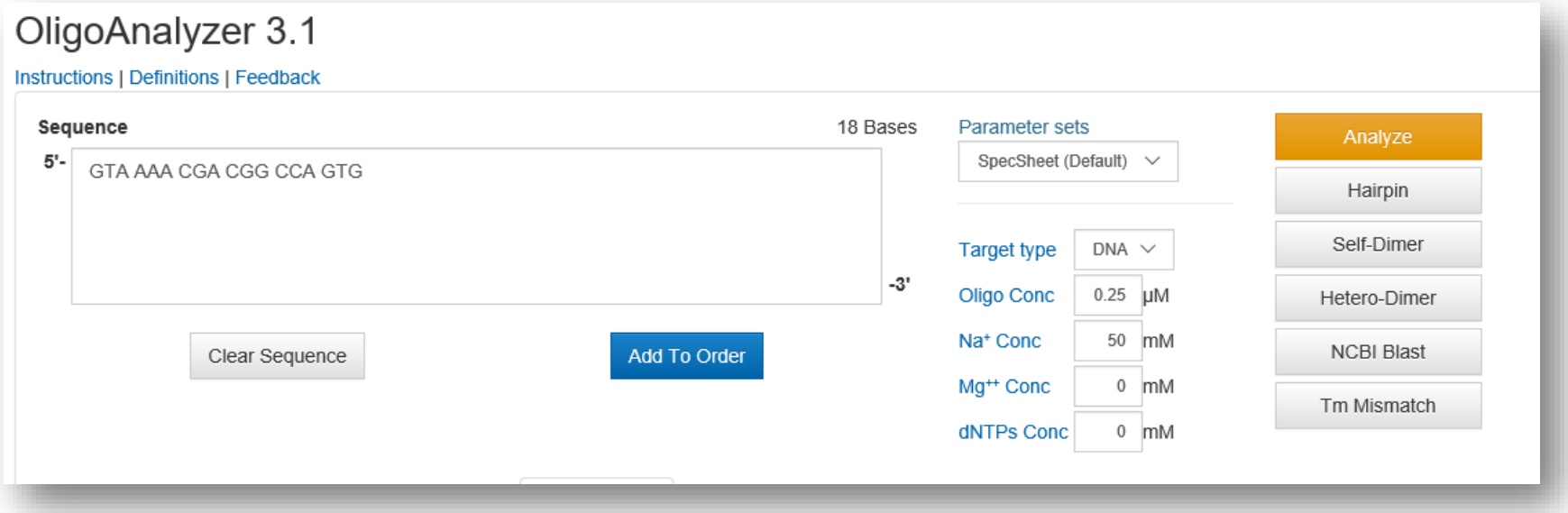
= (368 000) – (185 400)

= **182 800 $M^{-1}cm^{-1}$**

Calculators of the extinction coefficients for DNA / RNA oligonucleotides

Calculation of ε

https://eu.idtdna.com/calc/analyzer



Unit of optical density OD

OD is optical density unit

1 OD is the amount of DNA or protein which, when is dissolved in one milliliter, has absorbance 1 when is measured in a cuvette with optical paths of 1 cm.

Measurement of optical density OD is frequently used in biology as a simple method for determining the concentration, because in the range of 0..1 there is an approximately linear relationship between the concentration of the biological material and the value of absorbance.

Example of calculating DNA concentration

$\varepsilon = 182\,800\ M^{-1}cm^{-1}$

Total after synthesis 8,5 OD_{260}
Add 500 ul H_2O
What is the molar concentration of DNA?

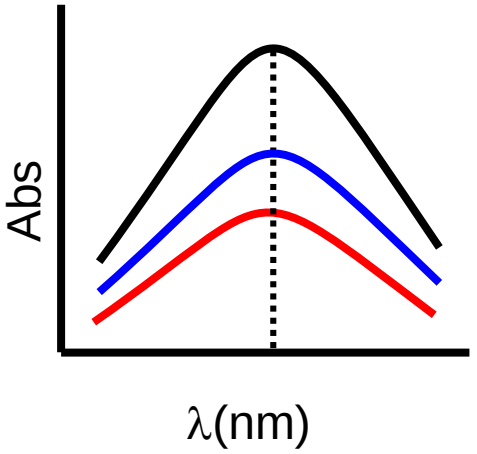
c = 93 μM

The molar concentration of the whole strands!

Hypochromic effect in the formation of DNA

Reduction of absorbance :

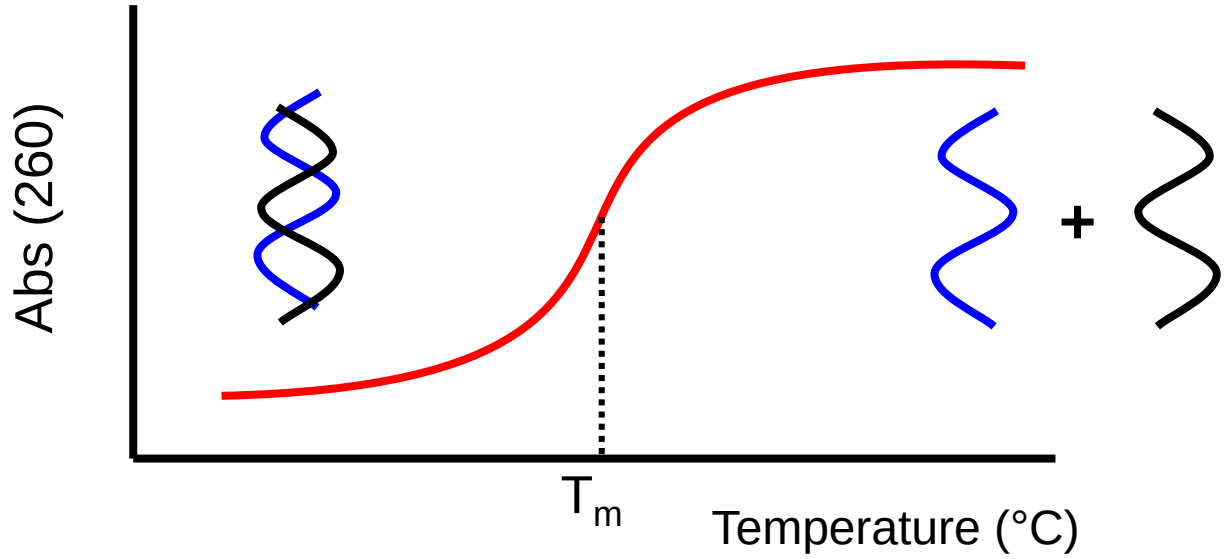
Abs (nucleotides) >Abs (ssDNA) >Abs (dsDNA)



- Double-stranded DNA absorbs less DNA than single-stranded, and that less than individual nucleotides
- Use: monitor melting-dissociation of complementary strands

Monitoring of DNA melting

- DNA denaturation curve - dependence of absorbance (260nm) on temperature
- Melting temperature T_m - the temperature at which half of the molecules is denatured



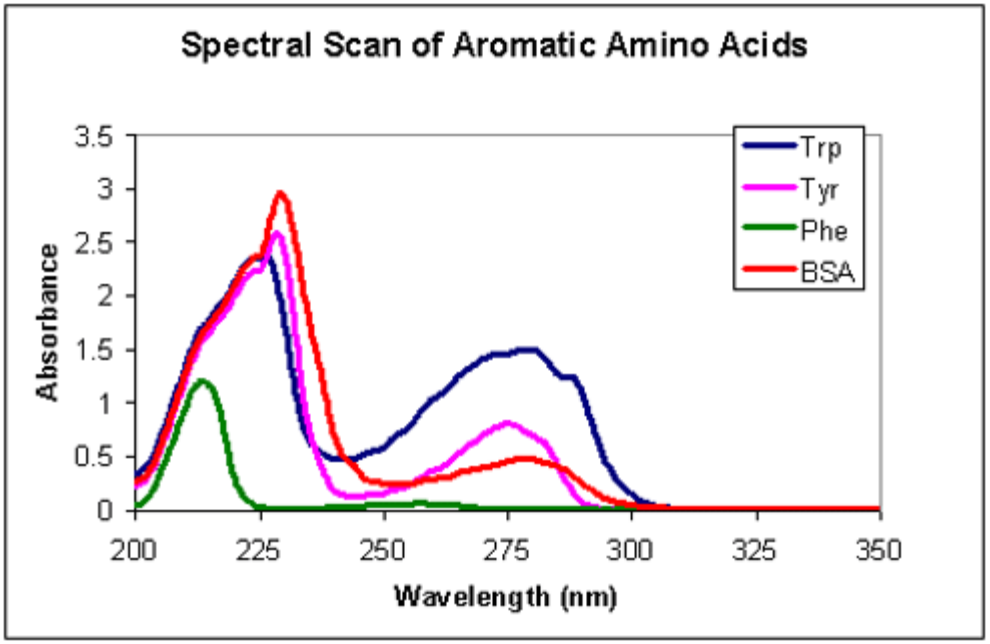
Calculator of DNA thermal stability

Calculation of melting temperature and its dependence on the concentration of oligonucleotide and salts

http://www.basic.northwestern.edu/biotools/oligocalc.html

Absorption spectrum of proteins

- Spectrum of proteins is composed of contributions of individual amino acids
- Tryptophan, tyrosine, and cysteine absorb the most
- Absorbance of proteins is measured at 280 nm



Spectroscopic determination of protein concentration

Analytical determination

Bradford method - change of the absorption maximum in the presence of protein

Calculation of extinction coefficient

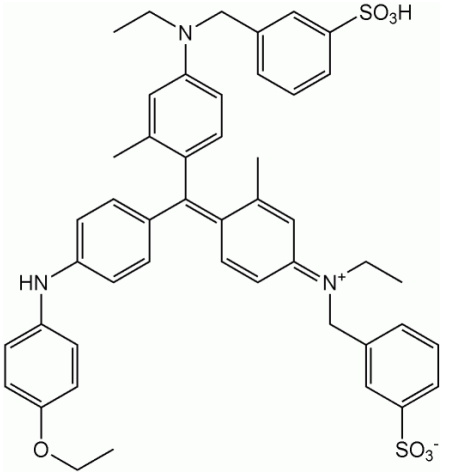
based on the amino acid sequence

Bradford method to determine the protein concentration

- The method is based on the shift of the absorption maximum of acidic solution Coomassie Brilliant Blue G-250 from 465 nm to 595 nm upon binding to the protein. The change is caused by ionic and hydrophobic interactions with the protein, that stabilize the negatively charged form of dyes resulting in a color change of the solution.
- Range of application of this method is 0.1 – 1.25 mg/ml
- In this range there is no significant change in the extinction coefficient of the protein/Coomassie complex

Bradford, MM. *Analytical Biochemistry 72:* 248-254. 1976.
Stoscheck, CM. Quantitation of Protein. *Methods in Enzymology 182:* 50-69 (1990).

Coomassie

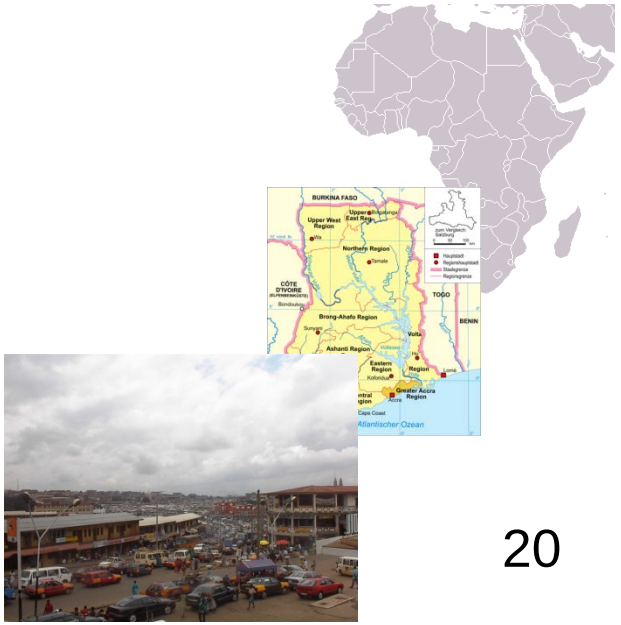


Other names:

Coomassie Blue, Brilliant Blue, Brilliant Blue G, Acid Blue 90, C.I. 42655, Brilliant Blue G 250, or Kunasty Blue

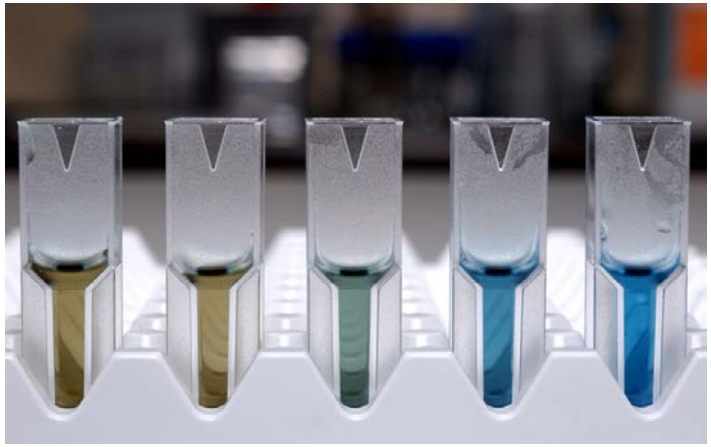
Originally, this substance was used in the textile industry for dyeing wool.

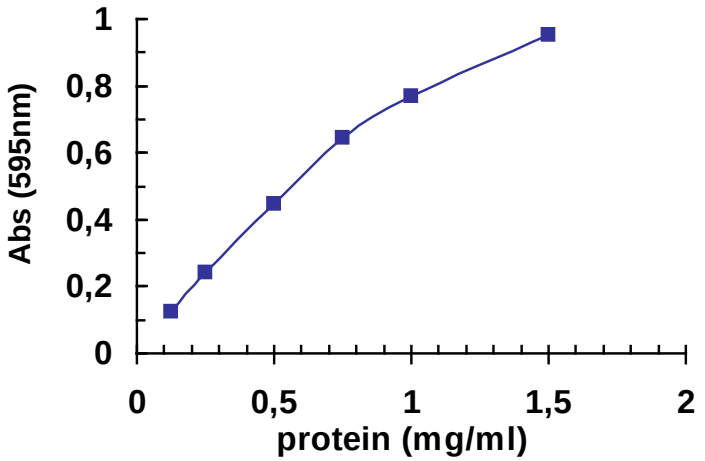
Named after the African city of Kumasi in Ghana



Practical example of the determination of protein concentration

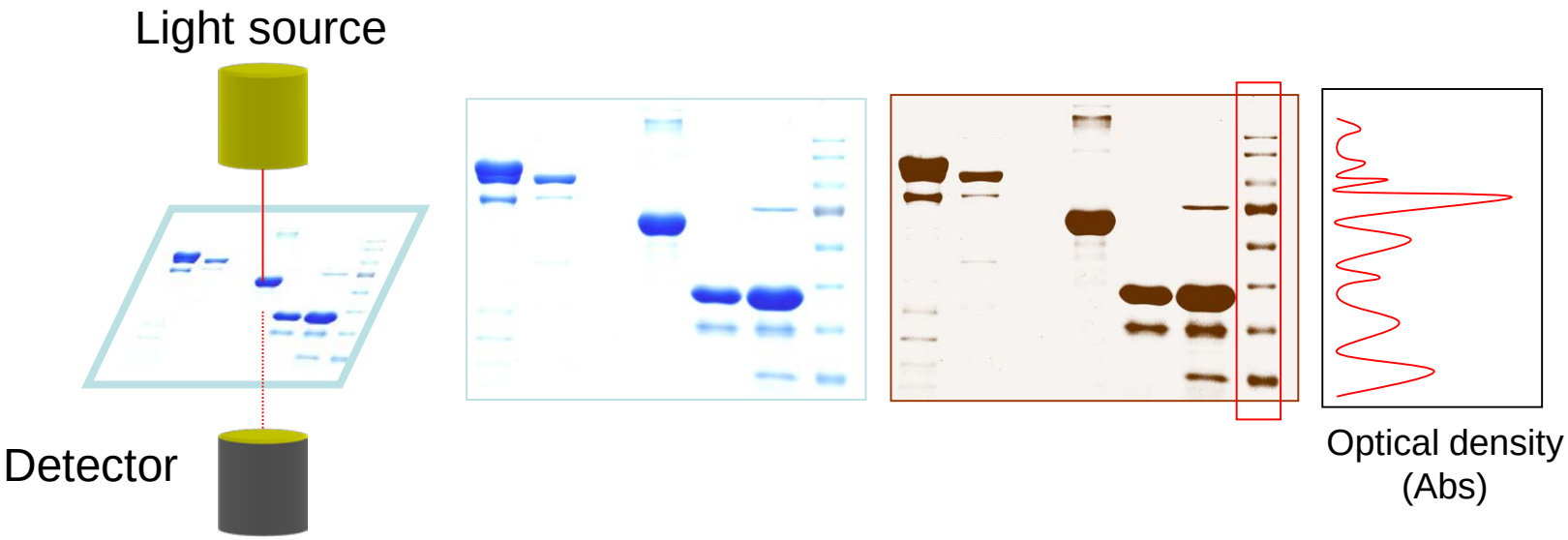
- Standards of BSA (bovine serum albumin or immunoglobulin G) were prepared in the range of 0.125…1.5 mg/ml
- After addition of Coomassie solution (e.g. 980 μl + 20 μl of protein solution) and a short incubation (5 min) Abs at 595 nm is measured
- Based on the calibration curve, the concentration of the sample is defined
- The most accurate determination is in the range of 0.2 – 0.7 mg/ml





Absorption densitometry of gels

- **In the analysis of molecules in the gel, the information about the quality and quantity can be obtained simultaneously**
- **Amount of absorbed light depending on 2D position is measured**
- **Determination of DNA and protein concentration after staining the gel with Coomassie or silver**



Calculation of protein extinction coefficient

ε_{Total} = number of(Tyr).ε_{Tyr} + number of(Trp).ε_{Trp} + number of(Cys).ε_{Cys}

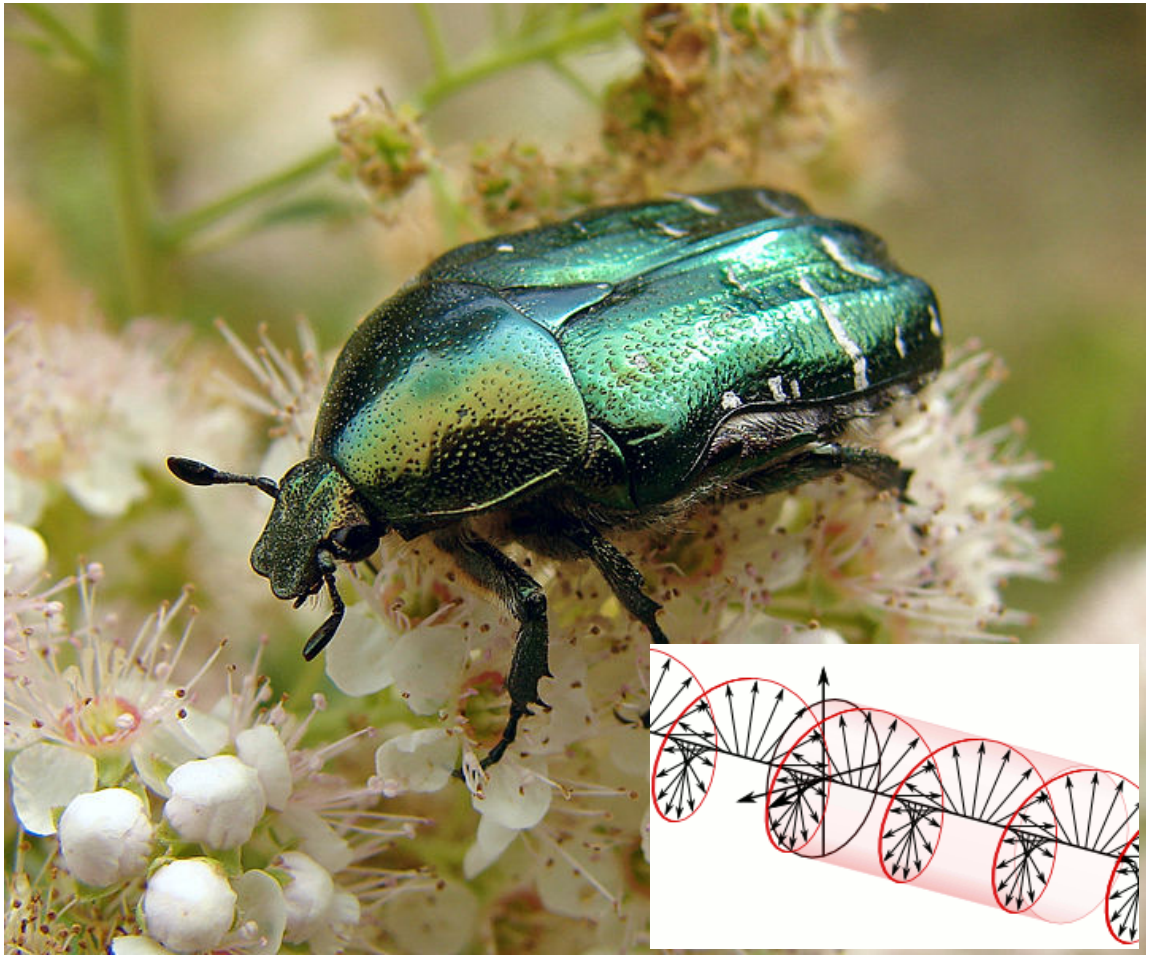
- The extinction coefficients for protein measured in water at 280 nm:

$\varepsilon_{Tyr} = 1490$, $\varepsilon_{Trp} = 5500$, $\varepsilon_{Cys} = 125$

http://www.expasy.org/tools/protparam.html

Gill, S.C. and von Hippel, P.H. (1989). Anal. Biochem. 182:319-326(1989).

Circular polarization



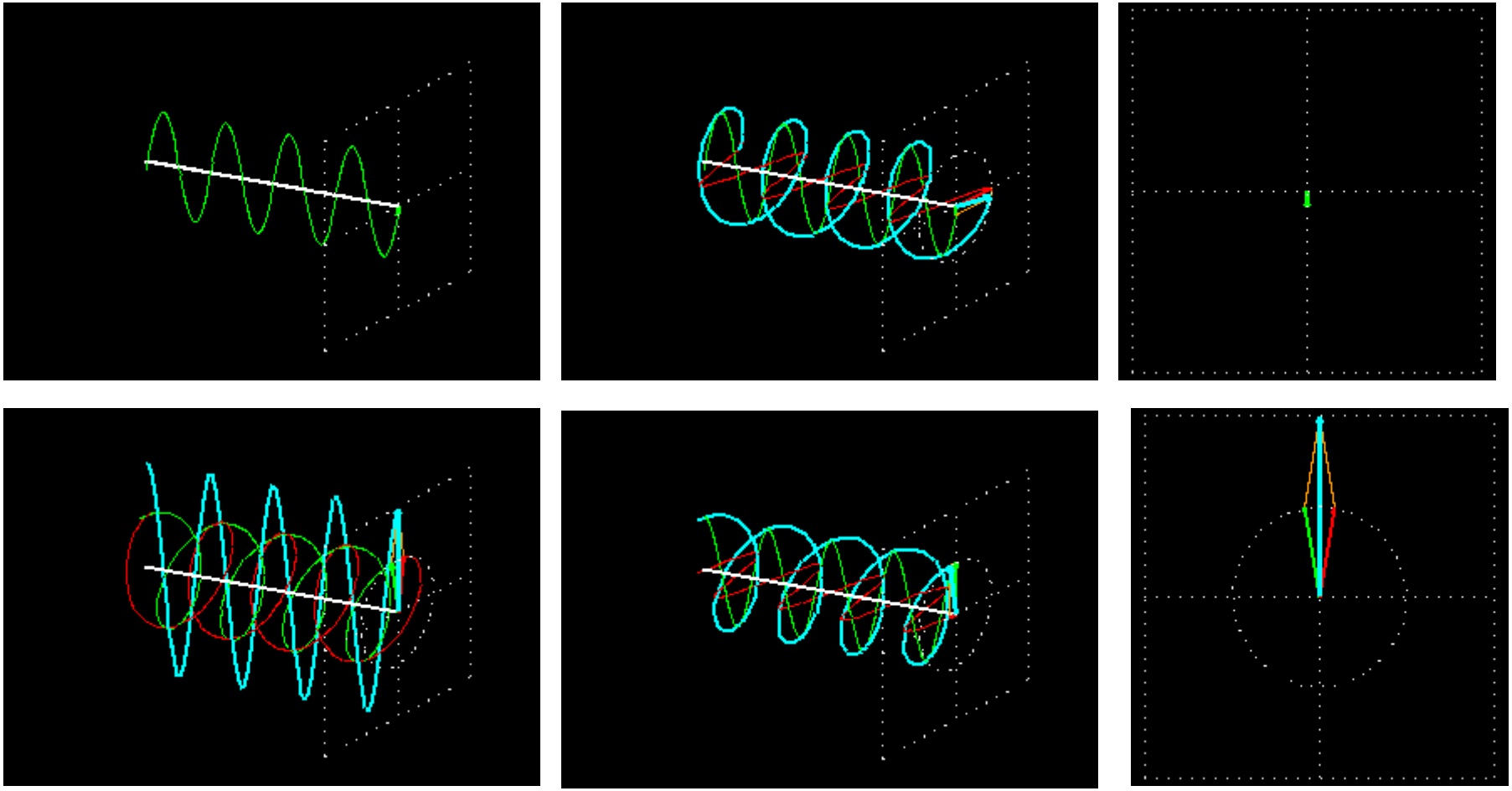
Circular dichroism in structural analysis of molecules

- Circular dichroism is caused by asymmetry of molecular structures
- Optically active molecules of sugars and amino acids are part of biological molecules
- In the case of arrangement of monomeric units into a helix, it leads to a significant enhancement of optical activity of the whole macromolecule
- Optical activity of solutions of biological molecules is used to describe their structure and especially their structural changes

Principle of CD

- The material is **an optically active** if it rotates the plane of polarized light
- Plane-polarized light can be decomposed into a left-handed and right-handed component of circularly polarized light.
- If left-handed component of circularly polarized light goes through the environment at different speeds (has a different refractive index n) than the right-handed component of circularly polarized light => it causes **twisting of plane polarized light**
- Left-handed component of circularly polarized light is absorbed differently than right-handed component of circularly polarized light => it causes a change from plane-polarized light to **elliptically polarized**
- **Circular dichroism** is defined as the difference between the extinction coefficient of the left- and right-handed component of circularly polarized light **$CD = \Delta\varepsilon = \varepsilon_L - \varepsilon_P$**
- **ellipticity** θ – in degrees is often measured
- **Twisting of plane polarized light and characterization of elliptically polarized light gives information about structure of molecules in solution**

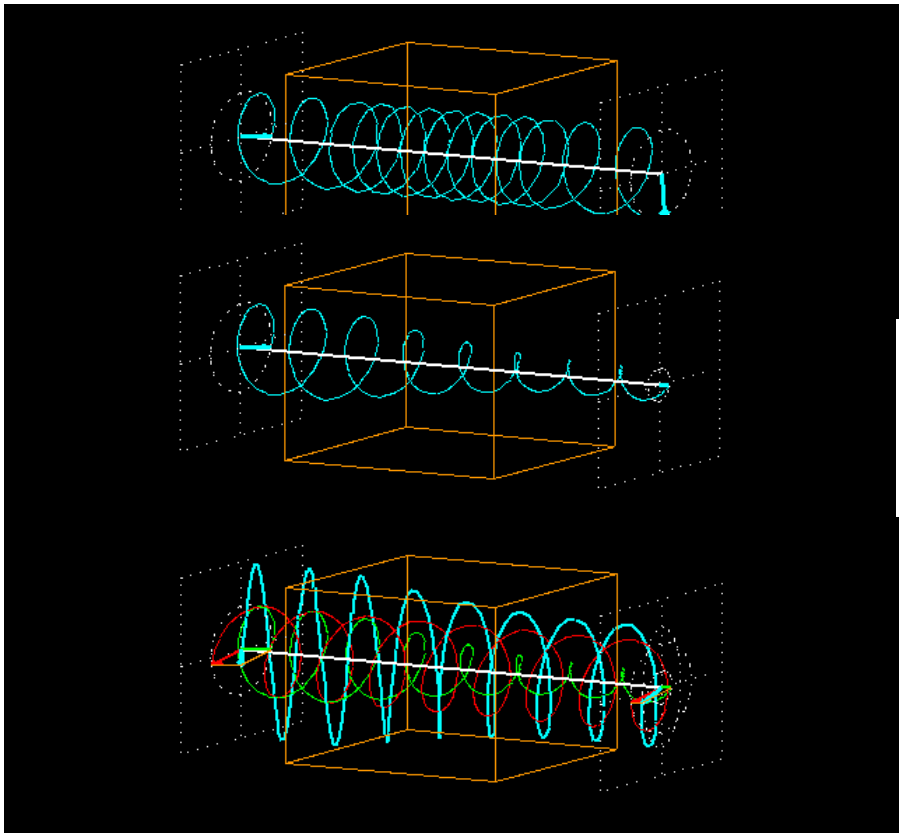
Decomposition of plane-polarized light to circularly polarized component

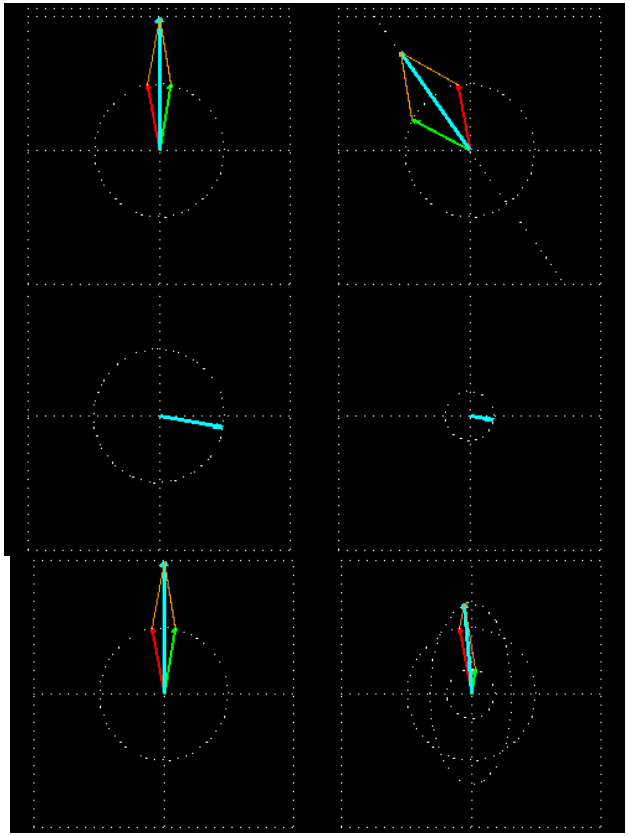


http://www.enzim.hu/~szia/cddemo/edemo0.htm

Change of polarization in asymmetrical environment

- In the environment where the left-handed circularly pol. light moves differently than the right-handed, there is a change in mutual shift of circularly polarized components, which causes the **twist of the polarization plane**.
- In the case of different absorption of left- and right- circul. pol. light also, the **elliptically polarized light** was formed



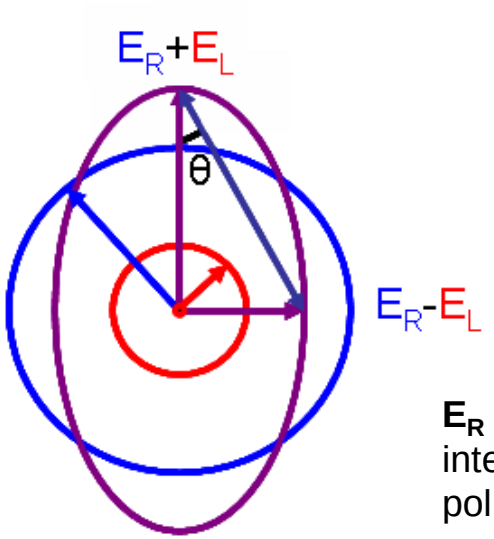


Ellipticity

$$\tan\theta = \frac{E_R - E_L}{E_R + E_L}$$

$$\theta = \frac{2.303}{4}(\varepsilon_L - \varepsilon_P)\cdot\frac{180}{\pi}[°]$$

$$\theta = 3.298\cdot(\varepsilon_L - \varepsilon_P)$$



E_R vector of electric. intensity of right-handed pol. component

E_L vector of electric. intensity of left-handed pol. component

Ellipticity is angle that characterizes the rate of change of plane-polarized light to elliptically polarized.

If the light is plane polarized θ =0

If the light is circularly polarized θ =45 °

Principle of CD

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- Plane-polarized light can be decomposed into a left-handed and right-handed component of circularly polarized light.
- If left-handed component of circularly polarized light goes through the environment at different speeds (has a different refractive index n) than the right-handed component of circularly polarized light => it causes **twisting of plane polarized light**
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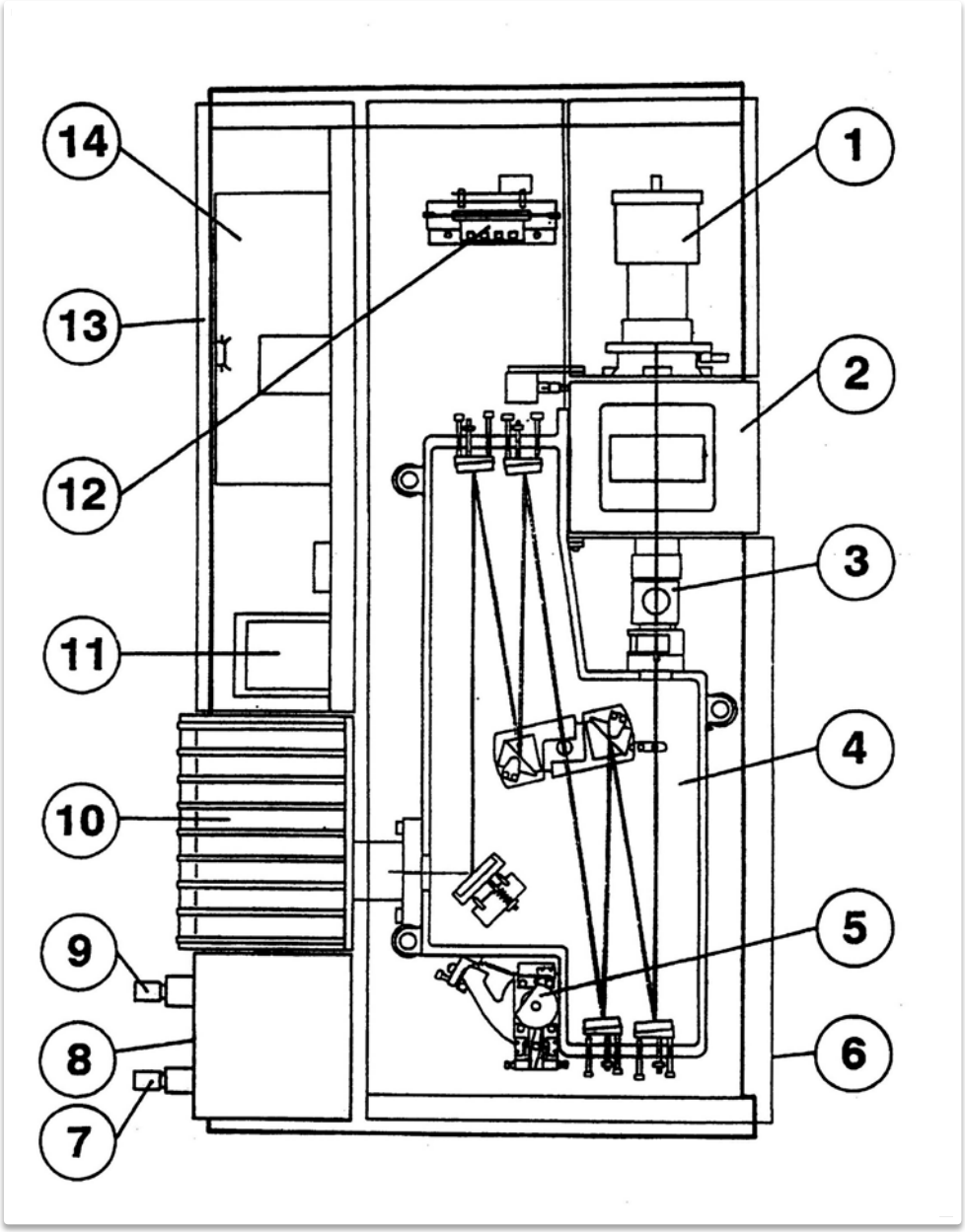
- **Circular dichroism** is defined as the difference between the extinction coefficient of the left- and right-handed component of circularly polarized light **$CD = \Delta\varepsilon = \varepsilon_L - \varepsilon_P$**
- **ellipticity** θ – in degrees is often measured
- **Twisting of plane polarized light and characterization of elliptically polarized light gives information about structure of molecules in solution**

Scheme of instrument for measuring circular dichroism



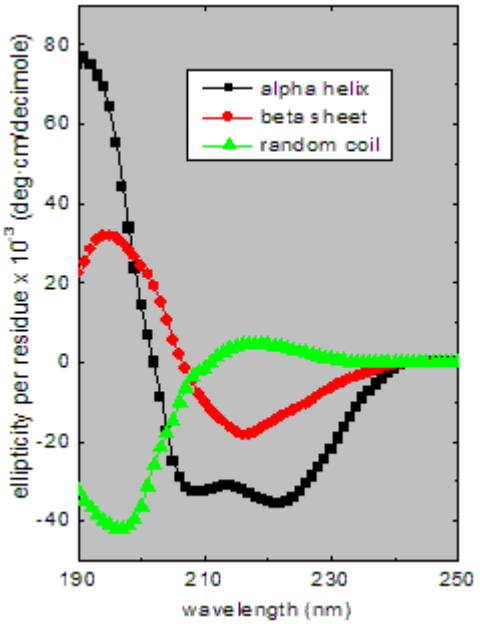
1. photomultiplier
2. space for the cuvette
3. **modulator**

   liquid crystal which is piezoelectrically with alternating current compressed and stretched and thus modulates alternately the circularly polarized beam to the right and to the left
5. monochromator
10. lamp

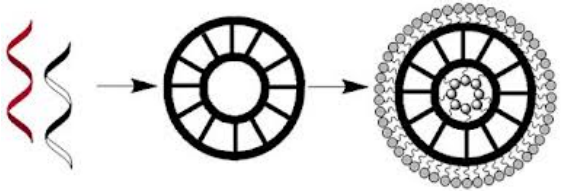
Kindly provided by prof. Vorlíčkovou

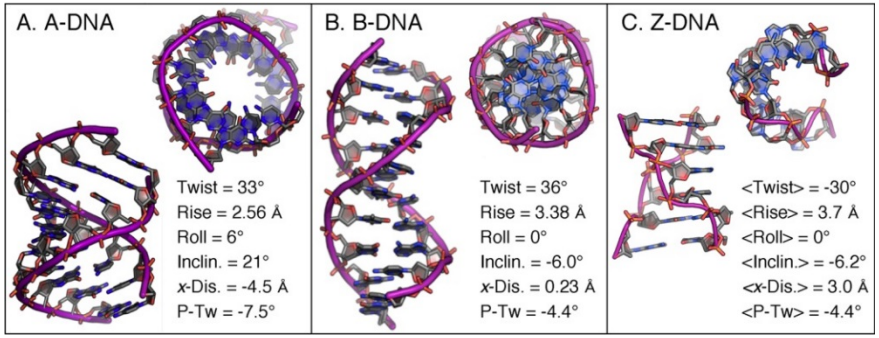
Utilization of CD spectroscopy

- Determination of the secondary structure of biomolecules
- Determination of the relative amounts of individual conformations (α helix, β sheet for proteins)
- Observation of slight structural changes
- Structural transitions of DNA (A,B,Z) and proteins
- Determination of thermal stability
- Observation of the protein-protein and protein – DNA interactions
- Observation of protein tertiary structure



CD spectroscopy of DNA





http://www.ibp.cz/cs/oddeleni/cd-spektroskopie-nukleovych-kyselin/informace-o-oddeleni

Vorlíčková, M., Kypr, J. and Sklenář, V.: NUCLEIC ACIDS: (c) SPECTROSCOPIC METHODS, Encyklopedia of Analytical Science, vol. 6, sec. ed., *Elsevier*, Oxford, (2005) 391-399

Next: transfer of power (energy) at a distance