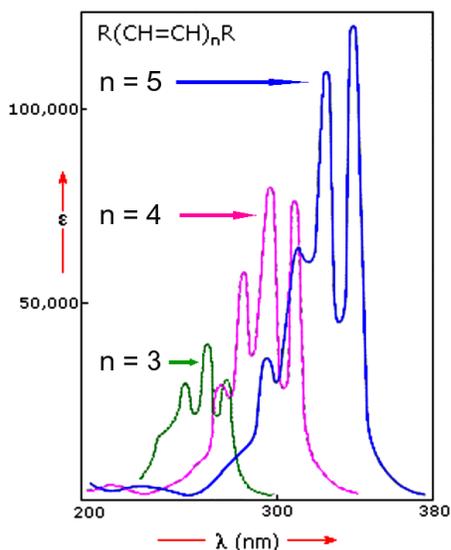


## Absorbance and its application (Varian Cary 50 spectrophotometer)



Absorbance (also called optical density) is a quantitative expression of absorption of light by different materials [1]. In biochemistry, absorptive properties of different solutions are typically used to characterize concentrations of a dissolved analyte or to follow progress of a chemical / enzymatic reaction. When the sample molecules are exposed to light matching a possible electronic transition within

the molecule, some of the light energy is absorbed and the electrons are promoted to a higher energy orbital. An optical spectrometer records the wavelengths at which absorption occurs, together with the degree of absorption at each wavelength. The resulting spectrum is presented as a graph of absorbance ( $A$ ) versus wavelength, as in the isoprene spectrum shown here. Since isoprene is colourless, it does not absorb in the visible part of the spectrum and this region is not displayed on the graph. One can see, that the increasing number of conjugated double-bonds causes



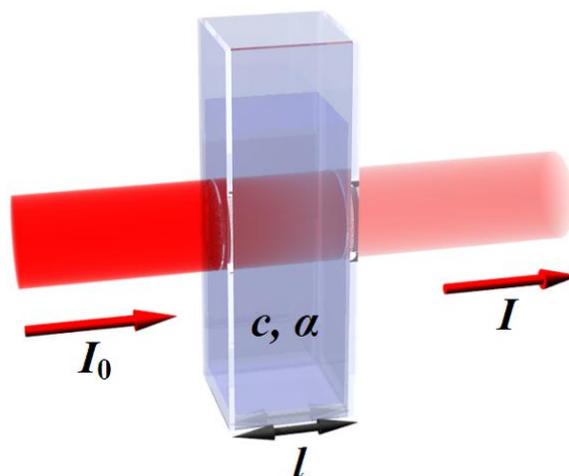
shift of the recorded absorbance spectrum to a longer wavelength (called also bathochromic or red shift), as well as increase in its intensity (hyperchromic shift). From the polyene spectra displayed in the diagram, it is clear that each additional double bond in the conjugated  $\pi$ -electron system shifts the absorption maximum about 30 nm to the right. Also, the molar absorptivity ( $\epsilon$ ) roughly doubles with each new conjugated double bond.

Absorbance usually ranges from 0 (no absorption)

$$A_{\lambda} = \log_{10} \left( \frac{I_0}{I} \right)$$

to 3 (99.9% absorption), and is precisely defined by Beer – Lambert law:

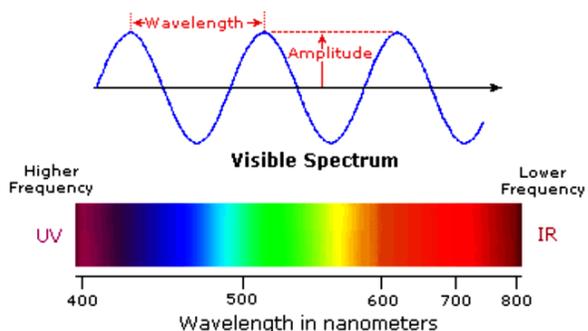
where  $I$  is the intensity of light at a specified wavelength  $\lambda$  that has passed through a sample (transmitted light



intensity) and  $I_0$  is the intensity of the light before it enters the sample (incident light intensity).

Absorbance measurements are carried out in analytical chemistry very often, since the absorbance of a sample is proportional to the concentration of the absorbing species in the sample. Absorbance is also proportional to the thickness of cuvette, but under standard conditions it is invariably equal to 1 cm. Because the absorbance of a sample is proportional to the number of absorbing molecules in the light beam (e.g. their molar concentration in the cuvette), the value of  $A$  can be recalculated to the concentration with help of the coefficient of "molar absorptivity" (molar absorbance,  $\epsilon$ ). Molar absorptivity is defined as optical density of a 1 M solution placed in a cuvette of 1 cm optical length. In this way, the expression for molar concentration of a chemical compound runs as follows:  $c = A / \epsilon$ . Most compounds have the tabulated values of their molar absorptivity, which

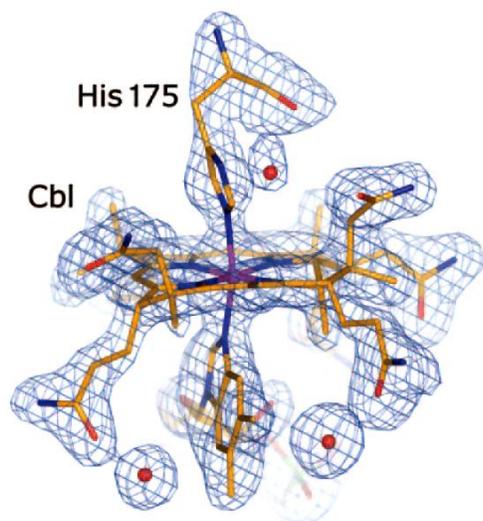
facilitates precise determination of their concentrations.



- **Violet:** 400 - 420 nm
- **Indigo:** 420 - 440 nm
- **Blue:** 440 - 490 nm
- **Green:** 490 - 570 nm
- **Yellow:** 570 - 585 nm
- **Orange:** 585 - 620 nm
- **Red:** 620 - 780 nm

Spectrophotometers measure absorbance in the regions of UV (200 – 400 nm) and visible light (400 – 800 nm). Any real instrument has a limited range

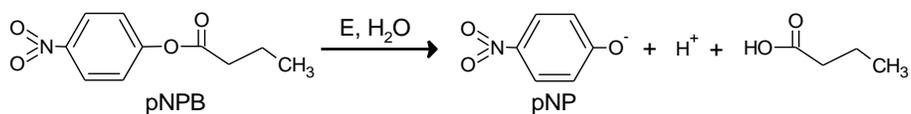
of accurately measured absorbance. A good instrument (including Varian Cary 50) can detect  $A$  in the region of 0.001 – 3 (99.9% – 0.1% Transmittance). The major parameters, that determine accuracy are signal to noise ratio and isolation from stray light. The first characteristic is important for measurement of a very low absorbance, because noise (i.e. spontaneous fluctuations in the intensity of the source light) hinders evaluation of a very small difference in absorbance. Likewise, stray light (which is not intended to be in the optical system) will mask a very high absorbance where almost no light passes through the sample inside the cuvette compartment.



Quite often the absorbance spectrum provides an important information about the structure of a molecule and its interactions with other compounds. This particularly concerns the corrin containing metallo-organic ligands, where stretching of coordination bonds and oxidation – reduction of metal ions causes a remarkable response in the system of conjugated  $\pi$ -bonds in the surrounding corrin ring. Among interesting examples, we can mention prediction of histidine –

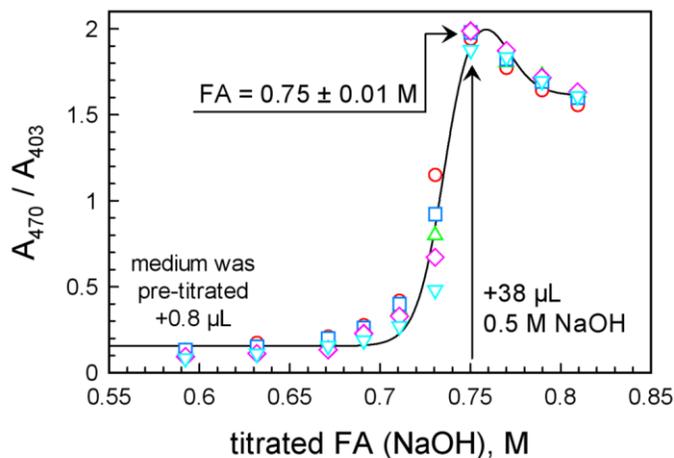
cobalt coordination in the complex between aquo-cobalamin and transcobalamin, deduced from the obtained absorbance spectrum [2]. This conjecture was later confirmed by crystallographic studies of this complex [3].

Absorbance measurements are often used to record reactions accompanied by cleavage of substrates. This can be illustrated by a lipase-catalysed hydrolysis of a synthetic substrate. Lipases catalyse cleavage of ester bonds between organic alcohols and fatty acids  $R-O-CO-(CH_2)_i-CH_3$  [4]. Most of them have also an esterase activity. The below example describes hydrolysis of a synthetic substrate p-nitrophenyl butyrate (pNPB). The reaction is accompanied by liberation of a product p-nitrophenol (pNP), which is easily detectable because of its yellow colour at slightly alkaline pH ( $\epsilon_{400} = 11\ 000$ , pH 7.5,  $I = 0.1\ M$ ).



Velocity of enzymatic reaction ( $v = dp/dt = dA_{400} / \epsilon_{400} / dt$ ) can be therefore measured on a spectrophotometer. Formation of a micella by pNPB at high substrate concentration causes acceleration of hydrolysis by lipases with surface activation [4]. No effect is observed for the enzymes without this activation mechanism. Other enzymes, whose activity can be measured in a similar way, are proteases. In many other reactions, production of an “optically neutral” compound is followed by a sequential

process, which rapidly consumes this product in a reaction accompanied by optical response.



Some chromophores exhibit sensitivity to pH by binding and dissociating a proton, which is accompanied by redistribution of conjugated double-bonds and appearance or disappearance of the colour. Spectral or fluorescent response from such chromophores can be used under titration of acids (including fatty acids in oils). A micro-titration test with pyranine is currently under development.

## Literature

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