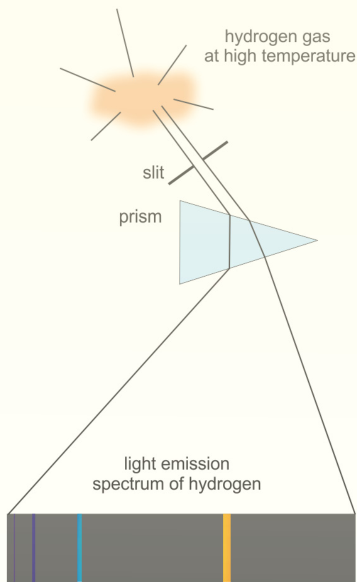
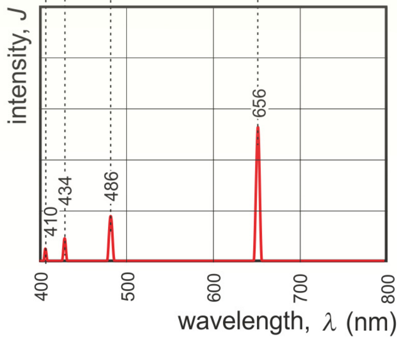
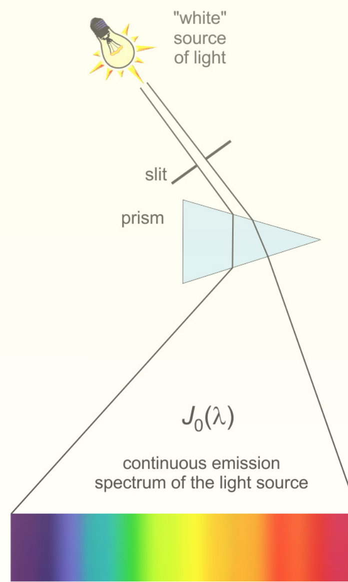
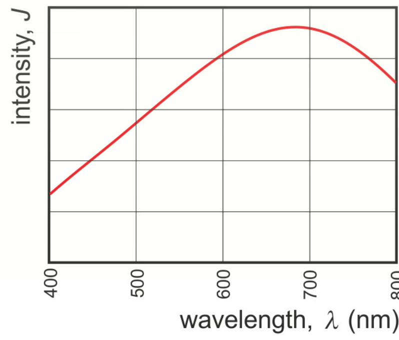
LIGHT ABSORPTION

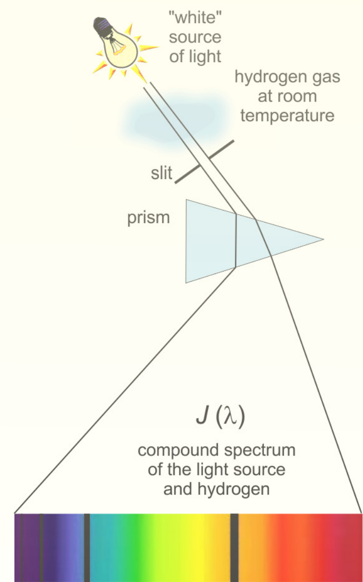
PRINCIPLES OF SPECTROPHOTOMETRY

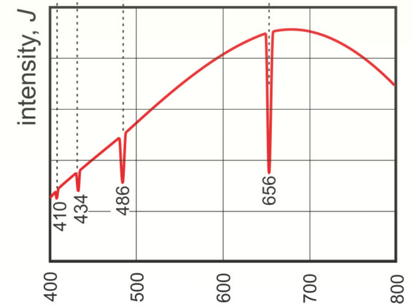


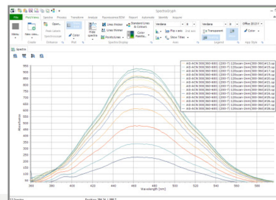


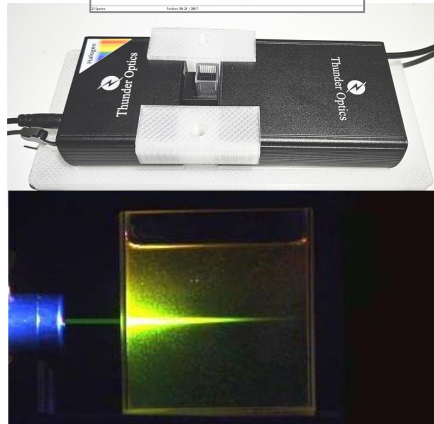


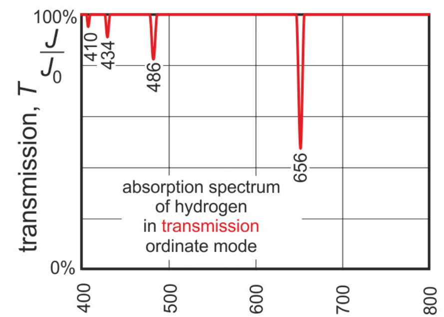


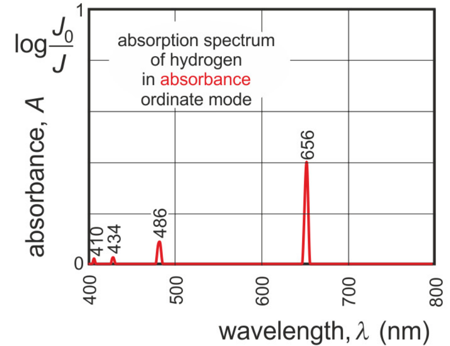


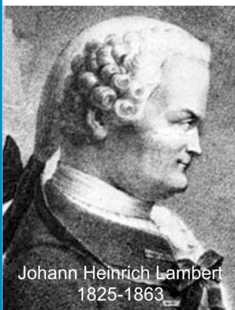


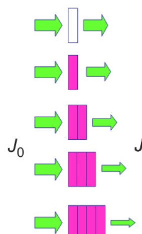












SUMMARY:

LIGHT ABSORPTION: absorption of light photons in matter. On the atomic scale, the energy of the absorbed photon is used for the excitation of the outer-shell electrons.

EXTINCTION (E), OPTICAL DENSITY (OD): The base-10 logarithm of the ratio between the perpendicularly incident light intensity (J_0) and the perpendicularly transmitted light intensity (J), $\lg (J_0 / J)$.

ABSORBANCE (A): The base-10 logarithm of the ratio between the perpendicularly incident light intensity (J_0) and the perpendicularly transmitted light intensity (J), $\lg (J_0 / J)$, assuming that reflection and scattering are negligible and that absorption is the sole process responsible for the intensity decrease.

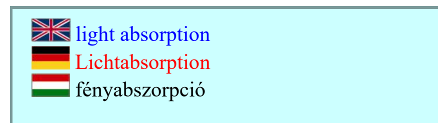
MOLAR EXTINCTION COEFFICIENT ($\varepsilon(\lambda)$): a quantity characteristic of the absorption by the material. It depends on the wavelength and on the characteristics of the material. It gives the optical density of the solution of unit concentration and unit layer thickness. (It does not depend on the concentration and thickness of the material).

BEER-LAMBERT LAW: an expression, valid for dilute solutions, stating that the absorbance of the solution is proportional to the concentration (c) and to the thickness of the material (x): $\log (J_0 / J) = \varepsilon(\lambda) \cdot c \cdot x$, where the proportionality factor is the molar extinction coefficient ($\varepsilon(\lambda)$).

COMPLEMENTARY COLORS: a pair of colors that gives white light as a result of additive color mixing. Such pairs are for example: yellow-blue, green-red, etc. The color of a material apparent from reflected or transmitted light will be the complementary color of the absorbed one.

The aim of this practice *is to learn the principles of absorption spectrophotometry and to use this method for the determination of copper ion concentration of an unknown solution.*

Examination of the interaction of light with matter is of great importance in the ***study of biological structures****. The intensity change of the incident light after interaction with matter gives valuable information about the electronic and 3D structure of the participating molecules, and their concentration. The method of spectrophotometry records the wavelength dependence of absorption. It can be used for the quantitative and qualitative analysis of solutions. Because of these advantages, it is widely applied in biology and medical practice.*



The most important applications of spectrophotometry are in the field of ***laboratory diagnostics****. Concentration of various substances (e.g. glucose, cholesterol, etc.) in blood, urine or other body fluids is typically determined by absorption measurement.*

THEORETICAL REVIEW

Light that passes through a layer of matter is attenuated as a result of light absorption and scattering. In dilute solutions, such as the ones examined in this practice, scattering is negligible. Thus, attenuation is essentially solely due to absorption (Fig. 1). The most frequently used quantity that characterizes the extent of absorption is called **absorbance (*A*)** defined as:

$$A = \log\frac{J_0}{J}, \tag{1}$$

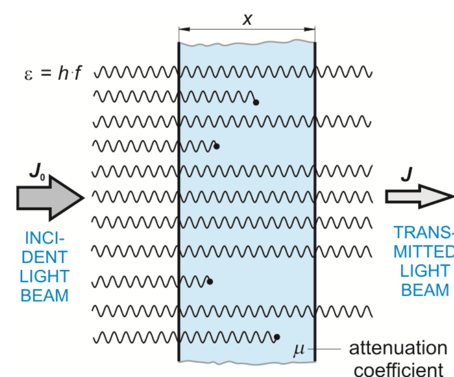


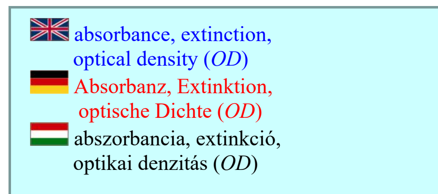
Fig. 1. *Absorption of light in matter.*

where J_0 is the intensity of the perpendicularly incident and J is that of the perpendicularly transmitted light. When the sole process in the material is absorption and scattering or reflection is negligable absorbance equals extinction (E) or optical density (OD).

Alternative to absorption, materials may also be characterized by their light transmission. **Transmittance** (T) or transmission coefficient is defined as the following ratio of intensities:

$$T = \frac{J}{J_0}\ (\cdot 100\ \%), \tag{2}$$

most frequently given in percentage. The relationship between these new quantities is summarized for some situations in the following table.



absorp-tion	J_0 (rel. unit)	J (rel. unit)	J_0/J	$A = \log(J_0/J)$	$T = J/J_0$ (%)
none	100	100	1	0	100%
small	100	50	2	0,301	50%
medium	100	~10	~10	~1	~10%
large	100	~1	~100	~2	~1%
infinite	100	0	∞	∞	0%

The absorption ability of the material is typically different for different wavelengths. This feature is revealed by the graph called **absorption spectrum**, in which absorbance is plotted as a function of wavelength.

The absorption spectrum of atoms and molecules is closely related to their electronic structure. According to the laws of quantum chemistry, electrons of atoms and molecules exist only in well-defined (quantized) energy states. If we provide a ground-state electron with energy corresponding exactly to the difference between the excited- (E_2) and ground-state (E_1) energies, then the electron jumps into the excited state. Thus, if a system of molecules is illuminated with light of matching photon energy (wavelength, color), the system absorbs part of these photons:

$$E_2 - E_1 = \varepsilon = h \cdot f = h\frac{c}{\lambda}, \tag{3}$$

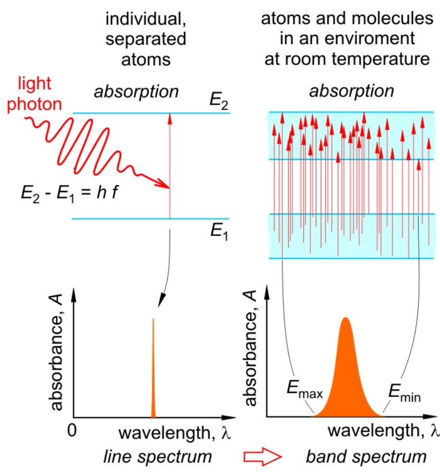


Fig. 2. *Light absorption process on the atomic scale (left). Broadening of the line spectrum into band spectrum (right).*

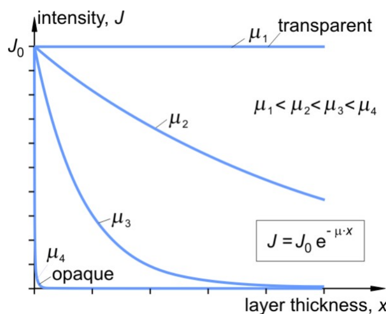


Fig. 3. *Attenuation law plotted on the linear scale as the function of thickness for different attenuation coefficients.*

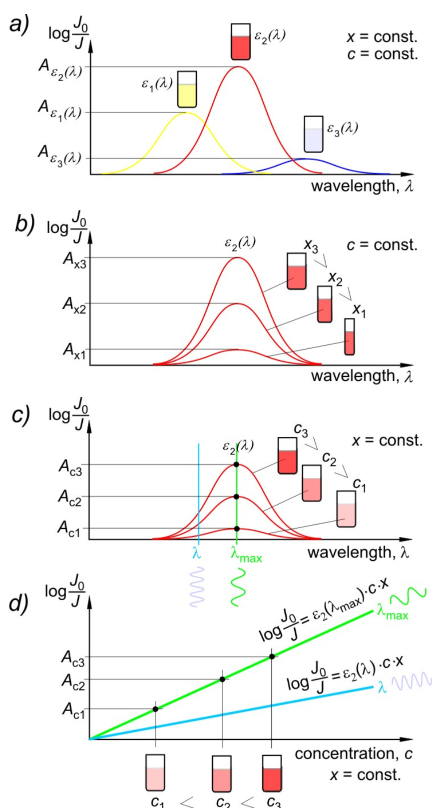


Fig. 4. *Absorption spectra: absorbance vs. wavelength as*
a) material,
b) thickness,
c) concentration varies.
d) Absorbance vs. concentration (concentration calibration curve).

where ($E_2 - E_1$) is the energy difference required for the excitation, ε is the photon energy, h is Planck's constant, f is the frequency of light, λ is the wavelength and c is the speed of light.

From the excited state the system will return to the ground state, loosing its excess energy. In most cases this process involves heat exchange with the environment in many small steps (relaxation).

The above simple model does not explain why solutions of identical molecules never display linear spectra containing discrete peaks at distinct photon energies (wavelengths) but spectra with wide, broadened bands. In case of molecules, the electronic states are slightly altered by molecule's vibrational and rotational states, but this effect is not expected to cause such a broadening by itself.

Broadening of spectral lines in solution has two main reasons. First, solute molecules are within an environment of the solvent molecules so that they can interact with each other. Second, the measurements are usually performed at room temperature (290 - 300 K) where thermal motion is extensive. The large number of thermally-driven interactions among the solvent and solute molecules causes alteration of the molecular energy levels, leading to spectral broadening. Thus, every molecule requires somewhat different excitation energy (Fig. 2).

The amount of absorption can be calculated from the general form of the attenuation law, because, as we mentioned before, the light scattering can be neglected:

$$J = J_0 \, e^{-\mu \cdot x}, \qquad (4)$$

where J_0 is the perpendicularly incident and J is the perpendicularly transmitted light intensity, μ is the attenuation coefficient characteristic for the type of the material and for the wavelength of light, called **absorption coefficient** in this case, and x is the layer thickness (Fig. 3).

Absorbance that was introduced earlier is derived from the equation (4) as:

$$A = \log \frac{J_0}{J} = \log e \cdot \mu \cdot x \, . \qquad (5)$$

In case of **dilute solutions**, if the solvent does not absorb in the applied wavelength range (i.e., it is transparent, just like water or alcohol in the visible range), then the absorption coefficient μ is proportional to the concentration of the solute, and the right side of the previous equation will become:

$$\log e \cdot \mu \cdot x = \varepsilon(\lambda) \cdot c \cdot x \, , \qquad (6)$$

where $\varepsilon(\lambda)$ is called (decadic) **molar extinction coefficient** (extinction coefficient for short), which depends characteristically on the wavelength for the given material. It gives the optical density of the solution of unit concentration and unit layer thickness. (It does not depend on the concentration and thickness of the material. The attribute "molar" indicates that concentration is measured in M, that is, mol/l units.)

Beer-Lambert law that is applicable for dilute solutions is expressed as:

$$\log \frac{J_0}{J} = \varepsilon(\lambda) \cdot c \cdot x \, . \qquad (7)$$

Most often the absorbance (log (J_0 / J)) *versus* wavelength (λ) plot is given as the **absorption spectrum** (Fig. 4a, for three chemically different solutions). According to equation (7) the absorbance of a certain solution is proportional to the layer thickness x (Fig. 4b) and to the concentration c at every wavelength of the absorption spectrum (Fig. 4c). Notice that the proportionality constant $\varepsilon(\lambda)$ is different for every wavelength (Fig. 4d). When one measures the absorbance of a solution at a certain wavelength λ, provided that x and $\varepsilon(\lambda)$ are known, the concentration c can be calculated. This is the **principle of the spectrophotometric determination of concentration**, a technique widely used in medical laboratories. Concentration can be determined most accurately at the wavelength corresponding to the maximum (λ_{max}) of the absorption spectrum (Fig. 4), where the slope of the straight line of Fig. 4d is the largest. This curve is frequently used for calibration.

Absorption spectra of solutions of three different materials, but of the same concentration, placed in the cuvettes of the same thickness are plotted in Fig. 5 (C, L and M curves). Notice that the different materials absorb at different wavelength ranges, and their extinction coefficients ($\varepsilon(\lambda)$) in the absorption bands differ significantly (the height of the curves is different).

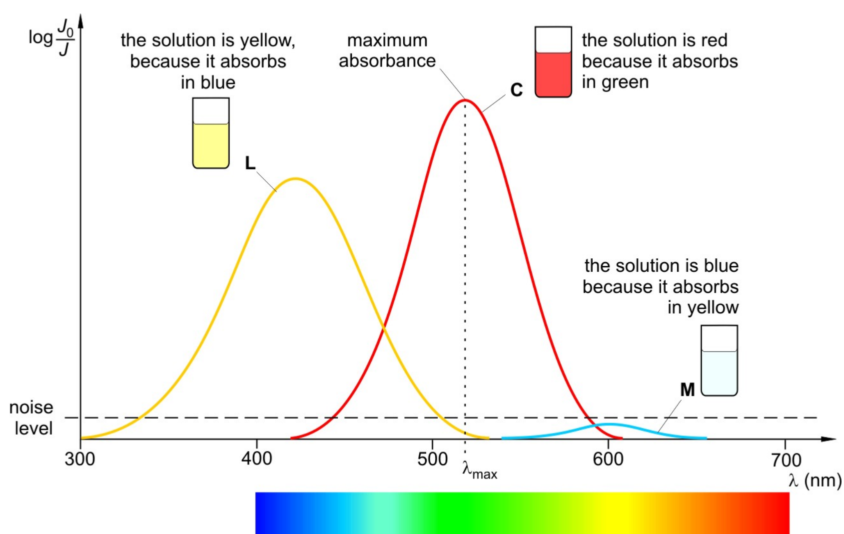


Fig. 5. Absorption spectra of solutions of three different materials, but of the same concentration, placed in cuvettes of identical thickness. Solution marked M is so transparent that it is not measurable with the spectrophotometer of the given noise level.

Colored transparent solutions absorb in the visible spectral range only at characteristic wavelengths. Our eye percepts only this transmitted light, which is the **complementary color** of the absorbed light (e.g., a solution that absorbs in green will appear red, and the one absorbing in blue will appear yellow and *vice versa*). Colorless materials (e.g. water, alcohol) transmit light in the whole visible spectral range. Chlorophyll absorbs red and blue, thus for us it appears to be green (Fig. 6).

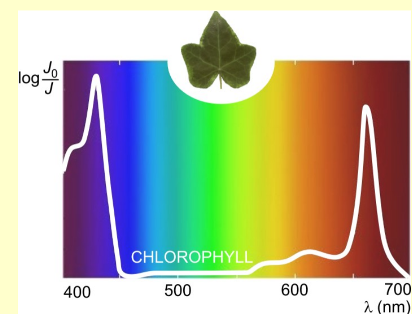


Fig. 6. Absorption spectrum of chlorophyll. Chlorophyll in the green leaves of plants absorbs in red and blue, thus it appears green for the human eye.

The location of absorption maxima (there may be more than one) is related to the **electron excitation energies characteristic for the molecular structure** as described by equation (3).

The height of the absorption maximum changes proportionally with the concentration, provided that the thickness stays constant (see formula (7) and Fig. 4c). This proportionality is used for **concentration determination**.

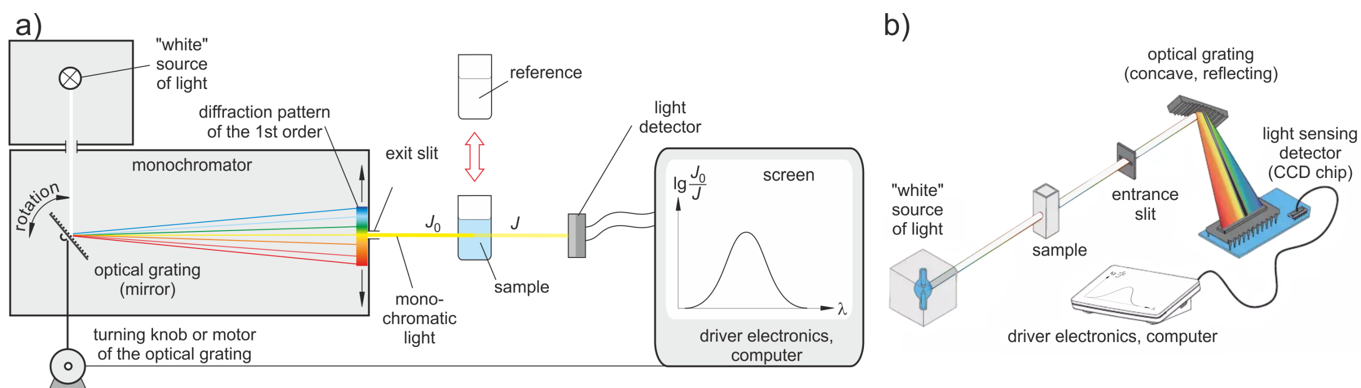


Fig. 7. Classical (a) and modern (b) spectrophotometer.

Absorption spectra are measured using an **absorption spectrometer** (spectrophotometer) (Figure 7). In spectrometers with a simpler design, the wavelength-dependent absorbance of the **sample** and the **reference** (solvent) is measured separately at each wavelength. More modern instruments operate automatically over a wide wavelength range (e.g., 190–900 nm), which includes the ultraviolet (UV, $\lambda < 400$ nm) and infrared (IR, $\lambda > 800$ nm) regions outside the visible spectrum. The so-called double-beam spectrometers record the data of the sample and the reference simultaneously, so the absorbance as a function of wavelength can be displayed immediately with the help of the built-in computer.

The **reference** is measured to take into account the spectral sensitivity of the detector and the possible wavelength dependent absorbance of the solvent.

The spectrophotometric method is also suitable for studying complexes. (A complex is a compound in which the d-orbitals of a metal ion participate in the formation of a chemical bond.) One group of biologically important proteins, the chromoproteins, contains prosthetic groups (coenzymes), several of which (e.g., hemin, cytochromes) are complexes of organic compounds (ligands) and metal ions. The metal ions alter the electronic structure of the organic compound, which in turn changes the compound's light absorption (color) and biological function. Determining both the amount (concentration) and the stability of these complexes can be important. These questions can also be addressed using spectrophotometric methods.

In the practical session, we introduce the Ames MiniLab PC **mini-photometer**, which is suitable for determining hemoglobin concentration in whole blood, as well as urea, glucose, bilirubin (in adults and newborns), cholesterol, HDL-cholesterol, and triglyceride concentrations in serum or plasma. In each case, the concentration determination is based on absorbance measurement at 546 nm, following reaction with an appropriately chosen reagent.

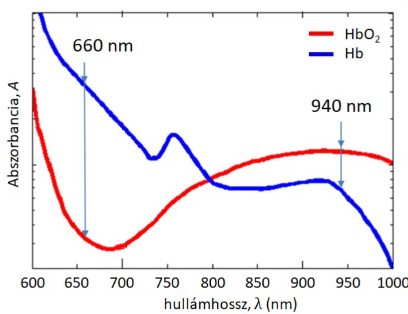


Fig. 8. *Absorption spectrum of hemoglobin and oxigen-saturated hemoglobin.*

Hemoglobin absorption also plays an important role in the rapidly developing field of optical tomography, which produces images based on changes in the intensity of infrared light scattered while passing through tissues. Since hemoglobin absorption in the infrared region is much more pronounced than that of most tissue-forming cells, the intensity of the scattered light decreases when the light passes through blood-rich tissue. This principle enables, for example, the investigation of cerebral oxygen supply or the determination of the size and location of hematomas formed within the skull.

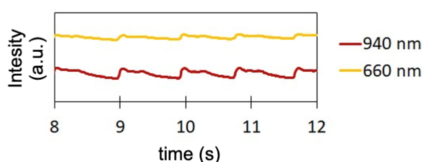


Fig. 9. *Typical recording of a pulse oximeter.*

According to the considerations above, concentration determination of dilute solutions can be simplified into the measurement of absorbance, which is a rather straight-forward technical task that can be automated. The sensitivity of the method can be further enhanced by using color reagents with large extinction coefficients specific for the molecule to be detected. With a pre-established calibration curve at hand the unknown concentration can be obtained in a single measurement.

MEASUREMENT

1. DEMONSTRATION MEASUREMENT — PULSE OXIMETRY

The examination of the hemoglobin molecule, which is important for oxygen uptake and transport, by pulse oximetry is based on the fact that hemoglobin molecules in different states exhibit distinct optical spectra (Fig. 8). The absorbance of oxygen-saturated hemoglobin (HbO_2) is low in the 650–700 nm range, while above 900 nm, in the infrared region, it is typically higher. The spectrum of unsaturated hemoglobin (Hb) shows the opposite trend. In pulse oximetry, light of given wavelengths is passed through a relatively thin body part (e.g., fingertip or earlobe), and the intensity of the emerging light is measured on the opposite side. In such thin tissues, the absorption by other tissue components is considered constant, so changes in the transmitted light intensity will depend on changes in the arterial blood volume. Since arterial blood volume varies in a pulsatile manner according to heart rate (pulse), this pulsation will also appear in the measured signal (Fig. 9). This gives the method its name ("pulse oximetry"). (Some devices use obliquely incident light beams that are partly absorbed and partly scattered; in such cases, full transmission through the body part is not required.) The measured absorbance, according to the Lambert–Beer law, also depends on the thickness of the tissue layer under examination, which is not precisely known and varies between patients. To determine the concentration of oxygen-saturated hemoglobin, two light beams of different wavelengths are applied (e.g., red light at 660 nm and infrared light at 940 nm). From the ratio of the absorbances measured at these two wavelengths, oxygen saturation can be calculated using an empirical formula.

However, the degree of oxygen uptake is not determined solely by the concentration of hemoglobin. The effective binding of oxygen by the hemoglobin molecule also strongly depends on the oxidation state of the iron ions located in the heme groups. For example, the Fe^{3+} ion found in methemoglobin is incapable of binding oxygen and therefore cannot fulfill the physiological function. Even Fe^{2+} ions within hemoglobin can only bind oxygen if the appropriate binding site is free (deoxyhemoglobin) or occupied by a loosely bound ligand (CO_2). Strongly bound ligands, such as carbon monoxide (CO) or the cyanide ion (CN^-), paralyze the normal function of hemoglobin. Since the oxidation state and chemical environment of the iron ions modify the interaction of hemoglobin with light and thus its absorption spectrum, the spectral characteristics also provide information about the types of hemoglobin present in the blood and potential pathological alterations.

2. DETERMINATION OF METHYLENE BLUE CONCENTRATION

As a model substance, we use an aqueous solution of **methylene blue**. The solution of methylene blue (Fig. 10) is blue in color (absorbing in the yellow region) and has a very high extinction coefficient. Therefore, in the practical session, the substance will be examined only in highly diluted solutions.

To determine the wavelength corresponding to the absorption maximum (λ_{max}) of methylene blue, we record the absorption spectra of the solutions. The spectra are recorded individually using the Thunder Optics spectrophotometer (Fig. 11).

TASKS:

1. Record the absorption spectra of the solutions with known concentrations.

2. From the spectrum of the most concentrated solution, read and note the absorbance values corresponding to the following wavelengths: λ = 400, 450, 500, 550, 600, 620, 640, 660, 680, 700, 750, and 800 nm. Using these data, plot the absorption spectrum of methylene blue in your lab report.

3. From the obtained absorption spectra, determine the wavelength corresponding to the absorption maximum (λ_{max}). This wavelength will be the same for all solutions. Using λ_{max}, calculate the average energy required for the electronic transition according to equation (3).

4. Plot the absorbance values measured at λ_{max} for the known-concentration solutions as a function of concentration, and construct a calibration curve.

5. Record the absorption spectrum of the solution with unknown concentration, then determine the absorbance at λ_{max}. Using the calibration curve, determine the unknown concentration.

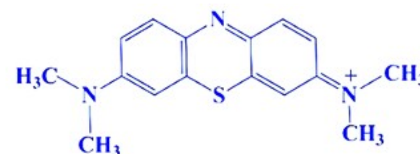


Fig. 10. *Structure of metylene blue*

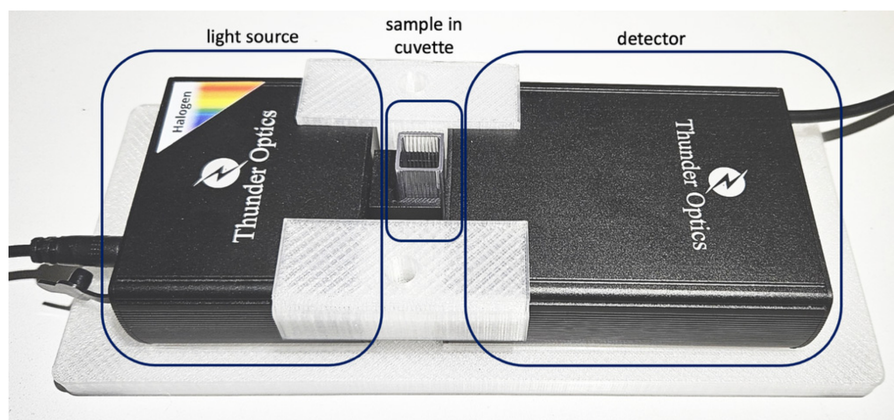


Fig. 11. *Parts of the Thunder Optics spectrophotometer.*