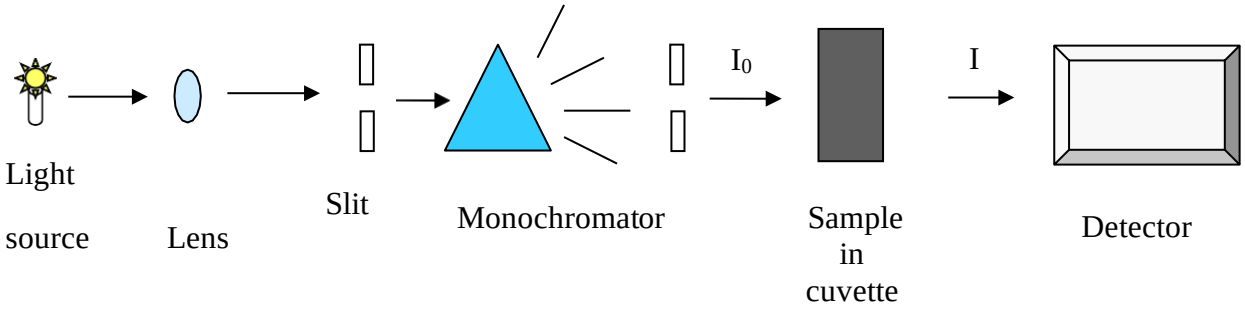
FUNDAMENTALS OF SPECTROPHOTOMETRY

Analytes in clinical biochemistry are most commonly determined spectrophotometrically, using devices called spectrophotometers. Spectrophotometry is an analytical method based on the interaction of electromagnetic radiation with the analyzed solution. A portion of the radiation is absorbed by the analyzed substance, while the remaining radiation that passes through the solution is detected by a detector. The intensity of the radiation after passing through the sample (I) is less than the original intensity of the incoming radiation (I0): I<I0I < I_0I<I0. The amount of absorbed radiation depends on the concentration of the substance in the sample: the higher the concentration of the substance, the more radiation is absorbed. Many substances present in biological fluids (mainly blood and urine) are analyzed spectrophotometrically in clinical laboratories.

Diagram of a spectrophotometer:



For spectrophotometric analysis of colored solutions, electromagnetic radiation in the visible light range (VIS, λ=400−800 nm) is used, with a light bulb as the source. Ultraviolet (UV) radiation (λ=190−400 nm) is also frequently used, allowing analysis of colorless solutions, typically using a deuterium lamp as the light source. Each substance absorbs radiation at a specific wavelength (containing chromophores capable of absorbing this radiation). Radiation of a specific wavelength is referred to as **monochromatic**. It is obtained by splitting polychromatic radiation using an optical grating or prism - for VIS, it is the decomposition of the white light emitted by a light bulb. Proper adjustment of the slit after the monochromator allows only radiation of a specific wavelength to enter the cuvette, while other wavelengths are reflected at different angles. The choice of cuvette depends on the radiation used: glass cuvettes for VIS and quartz cuvettes for UV (as UV radiation is absorbed by glass); special plastic cuvettes are also used.

Principle of analysis used in this practical class: The method is based on forming a colored compound, which can be determined spectrophotometrically in the visible spectrum range.

Complementary colors of visible light (refer to Table 1 and Fig.1):

If a solution **absorbs a specific wavelength** of visible light (middle column), it appears colored to the human eye. The **observed color** of the solution is **complementary** to the absorbed color (right column). Example

- If the solution absorbs radiation between 400 and 480 nm (blue-violet), it will transmit all other colors that will be perceived by the eye as yellow-orange; this means that the yellow-orange color is complementary to the blue-violet color
- If white light is passed through a solution that absorbs radiation between 505 and 555 nm (green), the transmitted radiation and thus the color of the solution will be perceived as red. The original white light contained all VIS wavelengths in a certain ratio, this ratio was disturbed by the passage of light through the solution, and the light, therefore, changed the color
- If red light is passed through the red solution, this radiation will be transmitted, because the red solution does not absorb the red radiation; on the contrary, the green light from the combustion (green is complementary to the red) will be absorbed as it passes through the red solution

wavelength *(nm)*	**absorbed part of the VIS spectrum**	**complementary - permeable colour** *(= determines the colour of the solution)*
350 - 430	Purple	yellow
430 - 475	blue	yellow-orange
475 - 495	Teal	orange
495 - 505	blue-green	red-orange
505 - 555	green	red
555 - 575	yellow-green	magenta
575 - 600	yellow	Purple
600 - 650	orange	blue
650 - 700	red	green

Table 1.:

Fig.1 *Complementary colours are located in the circle opposite each other*

It follows from the above that for spectrophotometric determination we have to choose the wavelength that will be absorbed by the analyzed substance the most. It is optimal if this wavelength is absorbed as little as possible by the other substances present in the solution. The appropriate wavelength is determined before the actual spectrophotometric analysis. We determine the so-called **absorption spectrum** of the sample - as a dependence on the ability to absorb radiation of different, continuously changing wavelengths (see Fig.2). For the analysis itself, the wavelength that is **most absorbed** by the analyzed substance **is selected.**

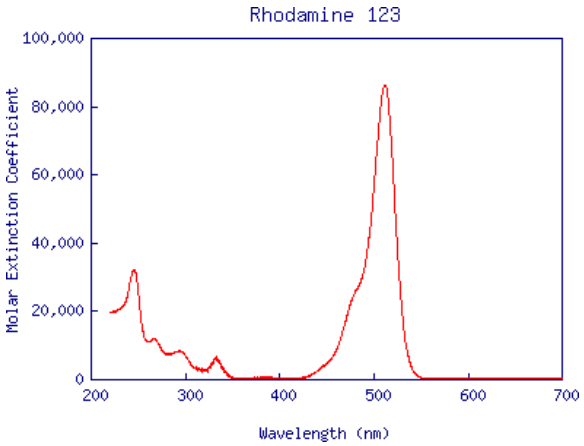


Fig. 2 *Example of the absorption spectrum of the analyzed substance:*
The maximum absorption was found to be around ***500 nm***

Principle of spectrophotometry:

Spectrophotometry is governed by **the Lambert-Beer law**, which relates **the concentration of a substance** in a solution to its **absorbance**, i.e. the ability of molecules of a substance to absorb electromagnetic radiation of a given wavelength. As the light flux passes through the solution, it is weakened - because the particles of substances present in the solution absorb part of the electromagnetic radiation. The radiation that passes through the cuvette hits a detector that measures its intensity. For this reason, a quantity called **transmittance** is introduced, which is defined by:

T = I / I0

where I_0 = intensity of radiation entering the cuvette; I = radiation intensity after passing through the cuvette; Transmittance
(T) takes values from 0 to 1, zero value if all radiation is absorbed, value 1 if all radiation passes through the cuvette

Sometimes, the transmittance is expressed as a percentage: **T = (I / I0) x 100**, i.e., it takes values from 0 to 100%. The amount of absorbed radiation is possible to calculate from the transmittance value:

A = - log T **=log(1/T)** ⇒ **T=10-A**

The quantity A is called **absorbance** and is defined as the negative decimal logarithm of transmittance. It takes values from zero upwards, we usually measure in the range of 0 to 1.5 (or less). Higher absorbance values tend to be less accurate,the measurement always depends on the sensitivity of the detector (or e.g. A = 2 corresponds to T = 0.01, i.e. only one percent of the original radiation intensity fell on the detector; for A = 3 T = 0.001, i.e. only 0.1 % of the original radiation intensity falls on the detector).

Lambert-Beer Law: **A = c . l . e** or **$T = 10^{-c.l.e}$**

A = absorbance; c = molar concentration; l = length of the cuvette, or thickness of the solution layer through which the radiation passes; e = molar absorption coefficient (tabulated value); T = transmittance; The Lambert-Beer law applies to monochromatic radiation and the range of low concentrations, of the order of 10^{-2} $^{mol.}$ l^{-1}.

It follows from the Lambert-Beer law that the higher the concentration of a substance in a solution, the higher the value of the measured absorbance (i.e., the absorbance is directly proportional to the concentration and vice versa). **This relationship is linear**, enabling the creation of calibration curves to determine unknown concentrations. **The absorbance of colored solutions increases with the intensity of their color (darker, more concentrated solutions).**

absorbance A

The Lambert-Beer law resembles the equation of a straight line (**A = c x l x e** ;i.e **y = kx + q**, where x corresponds to the value of c, the value "l x e" corresponds to k, i.e. the slope of the straight line; q = 0, i.e. the dependence of absorbance on concentration passes through the origin), see *The Fig.3*

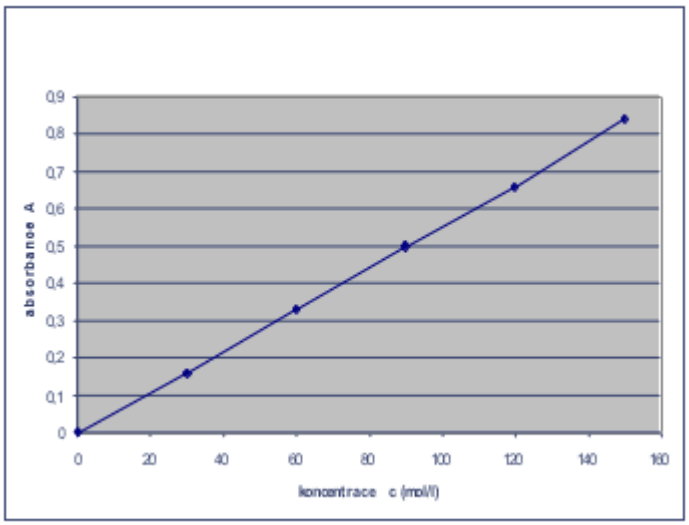


Fig.3 *Dependence of absorbance on concentration*

Transmittance is indirectly proportional to concentration: the higher the concentration of a substance, the less radiation the sample transmits, i.e. less radiation passes through the cuvette → less radiation reaches the detector. There is an exponential dependence between concentration and transmittance (T = 10-c.l.e).

To determine the concentration of the analyzed substance, it is first necessary to measure and construct a calibration curve. **The calibration curve** (Fig. 4) for photometric determinations is a graphical representation of the **relationship between the absorbance** of the solution **and the concentration** of the analyzed substance present in it. The curve can be processed on a computer, for manual processing we will need to use graph paper. So-called **standard solutions** of the analyzed substance (calibration solutions) are used to construct the calibration curve. **A standard solution** is a solution of a substance with a known composition and concentration. In practice, at least three, preferably more, standard solutions of varying concentrations are used. These can be best prepared by diluting a concentrated **stock solution** of the standard into several lower concentrations. The concentration range should be wide enough to ensure that the results of analyzing samples with unknown concentrations fall within the lowest and highest values of the calibration curve.

By plotting the measured absorbance values of the standard solutions (y-axis) against their concentrations (x-axis), we obtain **a linear relationship** between absorbance and concentration (see Lambert-Beer law). When constructing the calibration curve, the individual points are not directly connected; instead, a straight line is fitted as closely as possible to all the points. The calibration line should also pass through the origin (the intersection of the x and y axes). Standard solutions should be processed simultaneously with samples of unknown concentrations to maintain consistent analytical conditions, including potential inaccuracies, such as during pipetting.

After measuring the absorbance of the standard solutions and the unknown samples and constructing the calibration curve, the measured absorbance value of the unknown samples is plotted on the calibration curve. By projecting a perpendicular line from the point where the absorbance value corresponds to a point on the calibration curve, the concentration of the substance in the analyzed sample can be read on the x-axis.

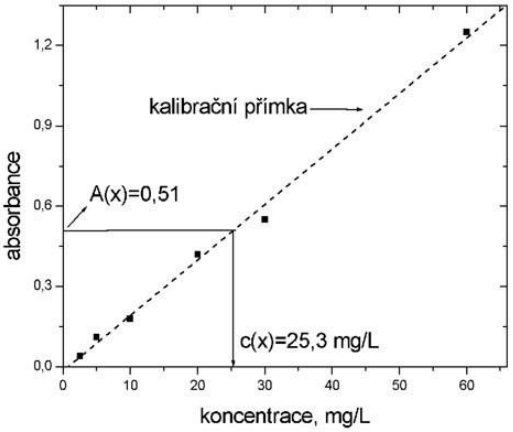


Fig.4 *Calibration curve (x = unknown sample, A = absorbance, c = concentration)*

Absorbance is **measured against a so-called blank sample (blank test).** This solution contains the same amount of all added reagents as the analyzed sample (standard or sample with unknown concentration**), but without the analyzed substance itself.** The pipetted volume of the analyzed substance (standard or urine) is replaced in the blank test with the same volume of distilled water.

The term **'measured against a blank sample'** means that the photometer is zeroed using the blank sample before measuring the absorbance: the blank sample is poured into the cuvette, and the measured absorbance is set to zero. During the measurement of absorbance for standard solutions or samples with unknown concentrations, any 'background absorbance,' not caused by the analyzed substance, is automatically subtracted. In some cases, added reagents may absorb the monochromatic radiation, which, without using a blank test, would lead to falsely higher absorbance readings and, thus, an overestimated concentration.

In practice, the photometer is usually first **zeroed with water** (a colorless liquid, the solvent itself, with an absorbance of 0). Then, the blank test is poured into the cuvette, and the measured absorbance value is recorded (for control purposes; with proper preparation of the reaction mixture, the same absorbance should be obtained in subsequent measurements). The photometer is then zeroed again. If the photometer is only zeroed with water, the absorbance value of the blank test must be subtracted manually from the absorbance of each standard or sample to ensure that the absorbance corresponds solely to the analyzed substance. This ensures that the absorbance and, therefore, the resulting concentration, are not distorted by any other substance present in the solution.

The final value of the quantitative determination (i.e., the concentration being determined) depends on the **accuracy** and diligence of **processing**. Adding only 'approximate' amounts of reagents, inaccurate pipetting, improper handling of the photometer (e.g., incorrect zeroing, dirty cuvettes, residues of more concentrated solutions in the cuvette when measuring less concentrated solutions, diluting samples with water remaining in the cuvette after rinsing)—all of these can lead to inaccurate results."