PHYSICO-CHEMICAL ANALYTICAL METHODS

Physico-chemical methods are divided into:

a) *optical* - based on the interaction of electromagnetic radiation with the analyte,
b) *chromatographic* - separation methods in which substances are separated between the stationary and the mobile phase on the basis of different affinities for these phases,
c) *electrophoretic separation methods* in which substances carrying an electric charge are separated by a direct electric field;
d) *electrochemical* - based on measurement of electrical quantities (current, voltage, electrical resistance, conductivity, etc.).

OPTICAL METHODS

Optical analytical methods represent a wide range of physico-chemical methods and are based on the interaction of electromagnetic radiation with the analyte. As a result of this interaction, atoms, molecules, ions or radicals are excited, which is associated with absorption and spontaneous emission of radiation. Depending on whether there is emission or absorption of radiation during the measurement, this is an emission or absorption spectral analysis. Optical methods include:

- spectrophotometry
- luminescence spectral analysis
- atomic emission spectrometry (AES) - flame photometry
- atomic absorption spectrometry (AAS)
- refractometry
- nephelometry and turbidimetry
- polarimetry

SPECTROPHOTOMETRY

Spectrophotometry is an optical analytical method. It is an absorption spectral analysis in the visible and UV range (200-800 nm), which is based on observation of changes in radiation absorption by the examined solution.

If continuous radiation (i.e., radiation of different wavelengths) is passed through the solution, its intensity decreases at certain wavelength intervals. Reduction of light intensity can be expressed quantitatively by:

a) **absorption B** (Io - I/Io) - indicates how much of the incoming radiation was absorbed

a) **transparency T** (I/Io) - transmittance, which in turn indicates how much radiation has passed through the solution.
From that follows: B = 1 - T resp. B = 100-T (%).

A negative decimal logarithm of permeability is called **absorbance A**, logarithm of the ratio of the intensity of light entering (Io) to the intensity of light leaving the color layer (I)

$$\mathbf{A = - \log T = \log \frac{1}{T} = \log \frac{Io}{I}}$$

The relationship between radiation absorption, solution layer thickness and solution concentration is expressed by the **Lambert-Beer law**, which states that absorbance (A) is proportional to layer thickness (d) and solution concentration in mol.l^{-1} (c)

$$\mathbf{A = \varepsilon . d . c}$$

ε is the **molar absorption coefficient** (1. mol^{-1}.cm^{-1}) and expresses the absorbance of a 1 mol.l^{-1} solution in a 1 cm thick cuvette. Its value depends on the quality of the substance and the wavelength. Values of this coefficient usually lie in the range 10^3 to 10^4, maximum 10^5. The magnitude ε indicates the sensitivity of the method concerned.

USE OF SPECTROPHOTOMETRY

1. **Measurement of absorption spectra (curves)**- monitoring absorption dependence on light wavelength. If we measure the absorbance of a solution at different wavelengths and express the dependence graphically, we obtain the absorption spectrum of the substance. The **absorption spectrum** thus expresses the dependence of the absorbance on the wavelength at a constant concentration and the same layer thickness of the test solution, **A = f (λ)**.
In the wavelength range at which light absorption occurs (absorption band), the absorption maximum appears on the absorption curve. Measurement of absorption spectra is important in studying the **structure of substances**. Certain characteristic groups in the substance molecule form absorption bands with maxima at a certain wavelength, e.g. the oxo group has an absorption maximum at 280 nm, the nitro group at 366 nm etc.

2. The relation of the structure of substances and their spectral properties is also used for **monitoring of the course of chemical reactions**. In biochemistry, the most common difference

in absorption spectra between oxidized and reduced form of the same substance is used to determine enzyme activities (the so-called kinetic method). E.g. reduced coenzyme NADH (its hydrogenated pyridine ring) has a broad absorption maximum at 340 nm, while its oxidized form of NAD^+ (an aromatic pyridine ring) does not absorb light in this region (Fig. 1).

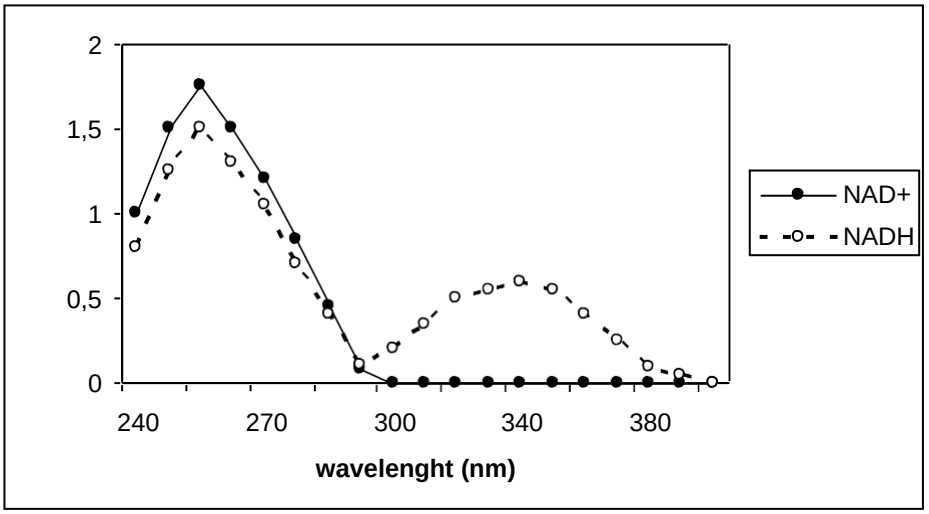

Fig.1. Absorption spectra of the oxidized and reduced forms of pyridine nucleotides, NAD^+ and NADH (c = 0.1 $mmol.l^{-1}$).

These pyridine nucleotides are coenzymes of many dehydrogenases, enzymes that catalyze dehydrogenation of substrates. The function of the coenzyme is in reversible hydrogen bonding. If NADH is formed in the enzymatic reaction, the absorbance increases at 340 nm and if NAD^+ is formed, the absorbance at 340 nm decreases. The change in absorbance per unit of time is directly proportional to the rate of enzyme reaction, i.e. enzyme activity. Enzyme assay methods based on measuring the decrease or increase of NADH (or NADPH) absorbance are widely used in biochemical analyzes, namely enzyme activity determination, enzyme determination of concentration of substances (products of metabolism, exogenous substances or their metabolites) in biological samples (blood, urine, cell tissues) and food analyzes.

When measuring the absorption spectra of a solution of a given substance with a known concentration, the molar absorption coefficient ε, which is **the absorbance value at the wavelength of the absorption maximum of the substance and the layer thickness of 1 cm**, can be read from them. From the absorption spectra of the solutions at the one-unit concentration of the solution, the value of the molar absorption coefficient can be read directly from the graph as the absorbance at the absorption maximum (at 340 nm is ε = 6.2 $mmol^{-1}.l.cm^{-1}$). In spectrophotometric determination of the concentration of substances we measure the absorbance at the wavelength that corresponds to the maximum on the absorption curve.

3. **Determination of low concentrations of** colored **substances**, substances giving a color reaction, but also colorless substances that absorb light in the invisible area. In terms of determining of the concentration of substances, the dependence of absorbance on the concentration of the solution at a constant wavelength (wavelength of the absorption maximum of the substance) and a constant layer thickness of the solution is monitored, **A = f (c)**.

Absorbance is a linear function of concentration, so we get a straight line in the graphical representation of the Lambert-Beer law. The deviation from the line dependence is called deviation from the Lambert-Beer law (Fig. 2). Line slope A = ε. c. d is the molar absorption coefficient value.

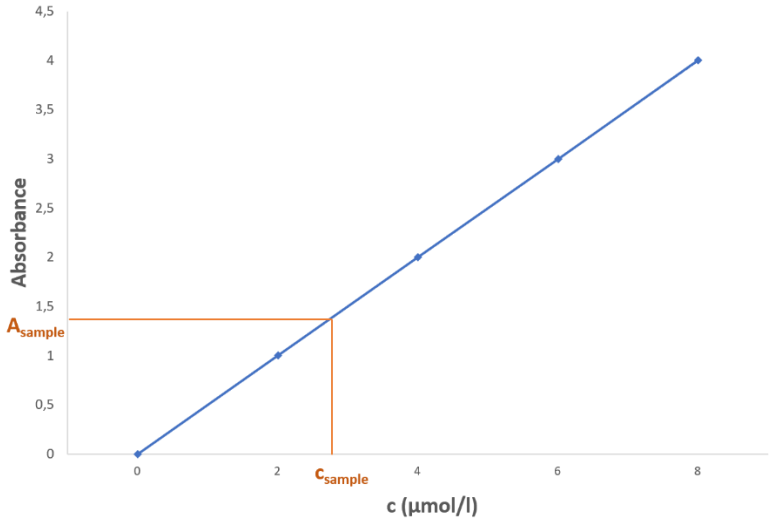

Fig. 2. Analytical curve

The validity of the Lambert-Beer law is experimentally verified by measuring the absorbance of a series of standard solutions (with a known concentration). By constructing of an **analytical curve**, which is a graphical representation of the absorbance (y-axis) versus concentration (x-axis), the Lambert-Beer law concentration interval is defined. It can be seen from the course of the analytical curve (Fig. 2) that the **law applies only to sufficiently diluted solutions** (low concentrations). At higher concentrations of the substance, so-called negative deviation due to association of absorbing molecules occurs. Dependence of absorbance on concentration is not further linear. Therefore, we can only work in the concentration range where the dependence of A on c is linear.

CONCENTRATION OF UNKNOWN SUBSTANCE in the Lambert-Beer Law validity can be determined in three ways:

1. **Subtracting from the analytical curve** (Fig. 2). The analytical curve enables to determine the concentration of samples by interpolation on the basis of measured absorbances. Due

to variations in the Lambert-Beer law, we prefer to determine the concentration using the analytical curve of the substance.

2. **-Calculation from the relationship** (at the same layer thickness) after measuring the absorbance of a solution with an unknown concentration (A_{sample}) and the absorbance of a standard solution ($A_{standard}$), whose exact concentration is known ($c_{standard}$).

$$\boldsymbol{c_{sample} = \frac{A_{sample}}{A_{standard}} \cdot c_{standard}}$$

3. **Calculation based on the knowledge of the molar absorption coefficient (ε)**, the value of which can be read from the absorption spectra of the substance or from chemical tables. To calculate the unknown concentration (c_{sample}), we must measure the absorbance of the solution of the substance (A_{sample}) at the same wavelength for which the value of ε (wavelength of the absorption maximum) is determined and multiply the absorbance by appropriate dilution of the sample and reaction mixture.

$$\boldsymbol{c_{sample} = \frac{A_{sample}}{\varepsilon . d}}$$

When determining the concentration of an unknown substance, we always measure the absorbance of the solution at the wavelength of the absorption maximum of the substance. The measurement is carried out in the range of absorbances from 0.1-0.8 (this corresponds to a transmittance of 10 - 80%). With an absorbance above 1, less than 10% of the light passes, so we can make a big mistake in determining of A. Self-photometric measurement error is 0.2-1%. Then the total relative measurement error is 0.5-3%.

Spectrophotometry together with other spectral methods such as IR, UV, NMR and mass spectrometry are used extensively in the study of the structure of substances, which are often combined with each other or with other physical methods.

METHODS OF ISOLATION AND SEPARATION OF SUBSTANCES

The methods used for isolation - separation and purification of substances are divided as follows:

• for a **homogeneous system** - crystallization, distillation, extraction, precipitation, dialysis, chromatography and electrophoresis

• for **heterogeneous system** - filtration, sedimentation, centrifugation and sublimation.

In the following textbook we focus only on the basic principles of selected methods.

SEDIMENTATION

Sedimentation, or settling, is a physical process in which solid particles of suspensions are deposited by gravity. The settling rate depends on the size, shape and density of the grains as well as on the viscosity and density of the liquid phase of the suspension.

Estimation of erythrocyte sedimentation is used in clinical practice. It is a simple examination to confirm the inflammation taking place in the body. If proteins present in the body are typical of inflammation, they alter the rate of erythrocyte sedimentation. Although sedimentation does not tell us what the disease is, it reminds us that something is happening in the body and it is necessary to investigate the cause.

SPIN (CETRIFUGATION)

Centrifugation is a physical method of separating solid or liquid particles with different density by centrifugal force, which exceeds the gravitational force many times (9.81 $m.s^{-2}$). It is carried out in centrifuges.

The **centrifugal force** (R) indicates how many times the centrifugal acceleration is higher than the Earth's gravitational acceleration (n.g).

$$\mathbf{R = 1.118.\ N^2.\ r.\ 10^{-5}}$$

r - turning radius, N – rpm

For a reproducible description of the spin, not only the indication of centrifugal acceleration is enough, but we also need to know the turning radius. Then the relative centrifugal force can be read either from the so-called centrifugal force nomogram to convert speed to R, or calculated from the formula.

E.g. In the centrifuge, the bottom of the cuvette is at a distance of 5 cm from the axis of rotation and the rotation speed is 58,000 rpm. What is the relative centrifugal force? $R = 1.118.\ 58\ 000^2.\ 5.\ 10^{-5} = 188{,}000\ g$

Differential centrifugation is a method that enables to fractionate a mixture of particles with a sufficiently different sedimentation coefficient by gradually increasing the multistage relative centrifugal force. The following fractions are obtained by differential centrifugation of the cell homogenate (after cell membranes have been broken):

Nuclei and cell debris: 600 g, 10 min
Mitochondria and lysosomes: 10,000 g, 30 min
Microsomes: 100,000 g, 60 min

Density gradient centrifugation is used to divide substances with close sedimentation coefficient values. Particles whose density is higher than the gradient density will sediment. Sucrose is most often used to prepare gradients.
The fraction that settles on the bottom of the cuvette during centrifugation is called **sediment** and the **supernatant** is liquid over the sediment (Fig. 3). Thick-walled glass tubes or cuvettes, or at a higher rotation speed of a plastic tube, can be used as centrifuge tubes. When centrifuging, load centrifuge tubes (cuvettes) into the centrifuge always balanced and opposite each other. Do not open the centrifuge until the centrifuge has stopped completely.

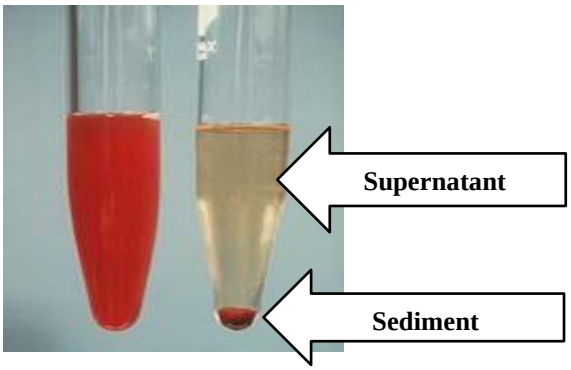

Fig. 3. Separation of the blood by centrifugation into supernatant (plasma / serum) and sediment (blood cells).