Optical Analytical Methods: Focus on Spectrophotometry

Chapter Overview

This chapter introduces optical analytical methods, which represent essential tools in clinical biochemistry. These methods are based on the interaction of electromagnetic radiation with a sample and the subsequent measurement of phenomena such as absorption, emission, fluorescence, or light scattering. Particular emphasis is placed on spectrophotometry, as it forms the cornerstone of routine laboratory diagnostics.

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1 Optical Methods: General Overview

Optical analytical methods use phenomena arising from the interaction of matter and electromagnetic radiation. Electromagnetic radiation is characterized by a wavelength, denoted by the Greek letter λ (lambda) and given in nanometers. The relationship between photon energy and wavelength (or frequency) is as follows:

$$E = h \times f = h \times \frac{c}{\lambda}$$

h ….Planck´s constant
c…..speed of light
f…..frequency

This equation shows that the energy (*E*) of a photon increases with the frequency (*f*) of electromagnetic radiation—that is, with the number of oscillations per second. Since higher frequency corresponds to shorter wavelength (λ), it also follows that the shorter the wavelength, the higher the photon energy.

Light is the visible part of electromagnetic radiation. The wavelengths of visible light (VIS) lie between the wavelengths of ultraviolet radiation (UV) and infrared radiation in the interval 390-760 nm.

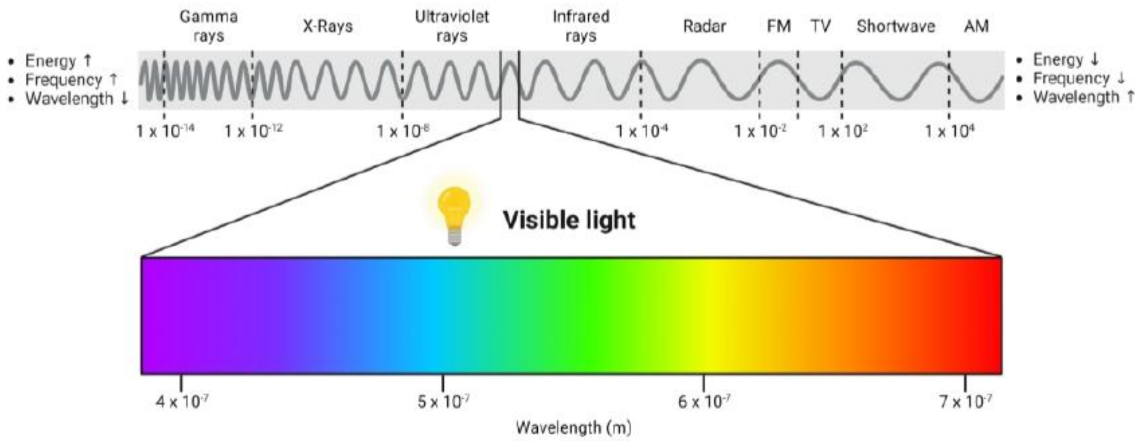
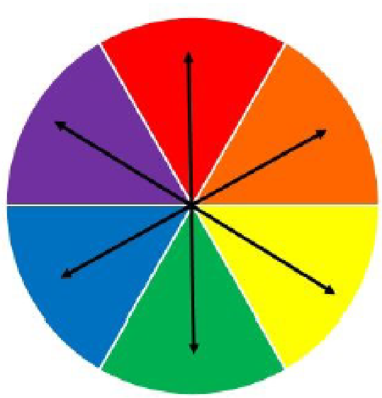


Fig. 1: Electromagnetic radiation

A substance can emit (radiate) or absorb light. If a substance absorbs radiation of a certain wavelength, the radiation of the unabsorbed wavelength will pass through to our eyes. If a substance absorbs all incident/passing radiation, we perceive it as black. If all VIS light passes through, it is perceived as colourless. However, if the substance only absorbs radiation of certain wavelengths in the VIS region, it is coloured. If a substance absorbs, for example, a red colour, we see it as green (the complementary colour to red is green, see the image below).



Colour	Wavelength
red	~ 625 - 750 nm
orange	~ 590 - 625 nm
yellow	~ 565 - 590 nm
green	~ 520 - 565 nm
azure	~ 500 - 520 nm
blue	~ 430 - 500 nm
violet	~ 380 - 430 nm

Fig2: Complementary colours.

***Interesting fact**: Why is blood red? Oxyhemoglobin absorbs green and blue light, which is why it appears red to us. When hemoglobin breaks down into bilirubin and biliverdin, the color changes—explaining the gradual "greening" and "yellowing" of bruises.*

The fundamental measurement of interactions between electromagnetic radiation and matter is based on the fact that part of the light is absorbed, part is transmitted, and part may be re-emitted or scattered. These processes can be described either by directly defined quantities (absorbance, transmittance) or by measuring the intensity of the resulting light (emission, fluorescence, scattering).

- **Absorbance** – expresses how much of the incident light is absorbed by the sample.
- **Transmittance** – expresses how much of the incident light passes through the sample.
- **Emission** – measures the intensity of light that the sample itself emits.
- **Fluorescence** – measures the intensity of light emitted by the sample after it has first absorbed radiation.
- **Scattering** – measures the intensity of light that the sample has redirected from its original path.

2 Absorption of radiation

When monochromatic light passes through a coloured solution, it is absorbed: this means that the radiation that leaves the sample (I) is smaller than the radiation that entered the sample (I0). The ratio of these quantities expresses permeability – transmittance (T), sometimes it is expressed as a percentage (T x 100). The more radiation the sample absorbs, the lower the **transmittance.**

$$T = \frac{I}{I_0}$$

I_0…. incident radiation

I…. transmitted radiation

Absorbance (A) is the logarithm of the reciprocal of transmittance. The more radiation the sample absorbs, the higher the absorbance.

$$A = -logT = log\frac{1}{T} = log\frac{I_0}{I}$$

Transmittance indicates the relative amount of radiation passed. The more radiation the sample absorbs, the lower the transmittance. Transmittance values range from 0 to 100 %. For absorbance values, the more radiation the sample absorbs, the higher the absorbance.

Absorbance has the advantage of being directly proportional to the concentration of the analyte, whereas transmittance changes in a non-linear manner. For this reason, absorbance—rather than transmittance—is used in laboratory practice for the quantitative determination of analytes.

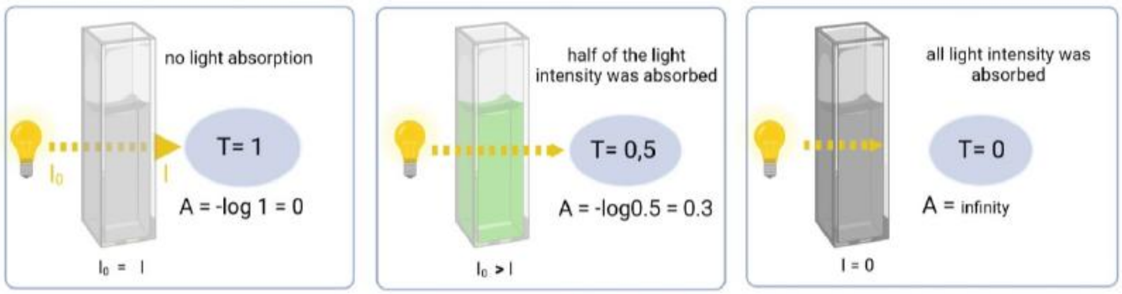


Fig 5: Relationship between transmitance and absorption .

2.1 Absorption spectrum

Each absorbing substance has its characteristic absorption maximum (one or more). This can be determined by measuring the absorbance at individual wavelengths. We then obtain an absorption spectrum. The absorption spectrum is the dependence of the absorbance on the wavelength A = f (λ). If more absorbing substances are present in the solution, the determined absorbance value is the sum of the absorbance values of the individual components in the mixture.

We do not find too many coloured substances in the human body, except for haemoglobin and its metabolites and the skin pigment melanin. Haemoglobin is formed from heme (tetrapyrrole with bound Fe2+) and the protein globin. Oxygenated haemoglobin is called oxyhaemoglobin. Bilirubin is formed from released haemoglobin during the breakdown of erythrocytes. In clinical practice, for example, bilirubin is determined by direct spectrophotometry in new-borns when neonatal jaundice is suspected.

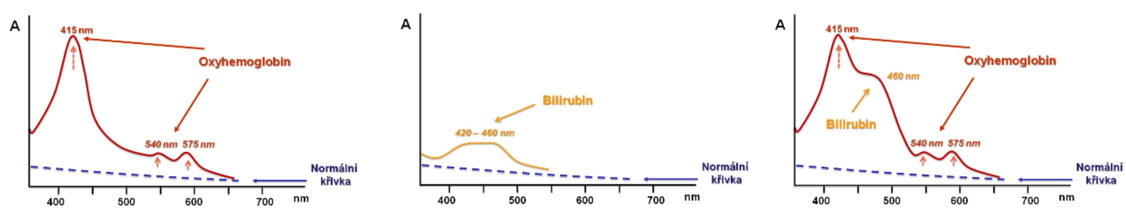


Fig. 4: Absorption spectra of oxyhemoglobin (left) and bilirubin (center); on the right, their mixture shows a visible overlap in the range of 415–460 nm. This principle is applied in cerebrospinal fluid spectrophotometry to distinguish fresh bleeding (hemoglobin) from older bleeding (bilirubin) in the diagnosis of subarachnoid hemorrhage.

2.2 Absorption Methods – Spectrophotometry

Absorption methods are based on the principle that molecules in a sample absorb light of a specific wavelength. A light beam passes through a cuvette, and the detector records the difference in intensity before and after transmission. The result is the absorbance value, which is directly proportional to the concentration of the substance according to the Lambert–Beer law.

Practically all biological fluids can be measured, including serum, plasma, urine, or cerebrospinal fluid. The applications in medicine are extensive—from determining the concentration of glucose, lipids, and bilirubin to measuring enzyme activities—making spectrophotometry a cornerstone of routine clinical biochemistry.

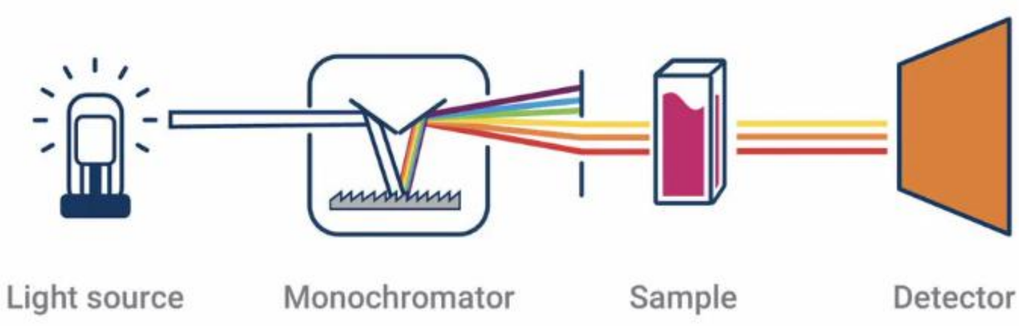
The measuring devices are called spectrophotometers, and in modern laboratories they are often integrated into automated biochemical analyzers.

Principle of Measurement:

A spectrophotometer consists of a light source, a monochromator, and a detector. The monochromator is responsible for isolating light of a specific wavelength. From its exit slit, monochromatic light (of a defined wavelength) emerges, which can be selected on the instrument.

The light then passes through the sample contained in a cuvette. The detector measures the intensity of the transmitted light. First, the intensity is recorded using a blank (reference sample), which contains all components of the solution except for the colored analyte. When light passes through the actual sample, its intensity decreases due to absorption by particles in the solution.

The spectrophotometer thus measures the difference between the intensity of the incident light (I_0) and the intensity of the transmitted light (I).



(Source: https://www.agilent.com/en/product/molecular-spectroscopy/uv-vis-uv-vis-nir-spectroscopy/double-beam-spectrophotometers)

Lambert-Beer law

There is a linear relationship between the concentration of a substance in a solution and its absorbance of the solution, i.e., a more concentrated solution absorbs more radiation. This allows us to calculate the concentration of a substance in a solution by measuring its absorbance. Absorbance is also directly proportional to the optical path (l) and the properties of the absorbing substance (ελ). The validity of the law is limited to dilute solutions (in highly concentrated solutions the relationship will not be linear).

The relationship between absorbance and concentration is given by the equation:

$$\boldsymbol{A_\lambda = \varepsilon_\lambda \cdot c \cdot l}$$

- **A** … absorbance (dimensionless quantity)
- **ε** … *molar absorption coefficient (dm3 /mol.cm) corresponds to the absorption of a substance at a certain wavelength in a solution with a concentration of 1 mol/l in a 1 cm cuvette.*
- **c** … *solute concentration (mol/l)*
- **l** … the thickness of the absorbing layer (cm), e.g., cuvettes

The validity of the law is limited to diluted solutions. At higher concentrations, deviations occur (non-linearity).

***Interesting fact**: The color of red wine directly depends on the concentration of anthocyanins and other pigments present in grape skins. The more of these compounds are extracted during fermentation and maceration, the more intense the wine's color becomes. This is a textbook example of a "natural" Lambert–Beer law: higher concentration → higher absorbance → deeper color.*

Validity and Limitations of the Law

The Lambert–Beer law does not hold under all conditions—deviations may occur:

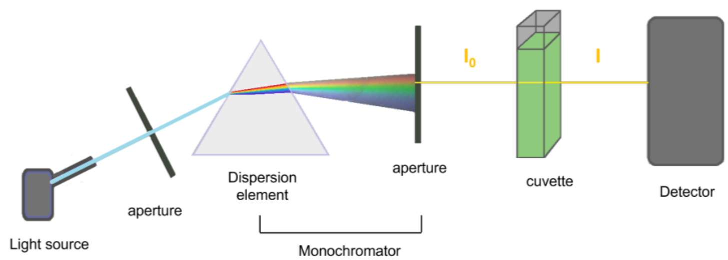
- at excessively high concentrations (molecular interactions, light scattering)
- when using unsuitable cuvettes (scratched or cloudy glass)
- at inappropriate wavelengths (outside the absorption maximum)
- if the analyte undergoes chemical changes during measurement (e.g., oxidation of Fe^{2+} → Fe^{3+})

The linear range of the Lambert–Beer law—the relationship between analyte concentration and absorbance—is valid only within a certain interval:
– At low absorbance values (<0.1), the difference between I_0 and I is very small, making the measurement imprecise and easily affected by instrumental noise.
– At high absorbance values (>1.0), almost no light passes through the sample, so the detector records only minimal intensity, leading to errors and deviations from linearit.

? ***Question:*** *What is the relationship between absorbance and concentration according to the Lambert–Beer law? Why should absorbance values for measurements be kept within the range of 0.1–1.0?*

2.2.1 Spectrophotometer – Components

A spectrophotometer is an instrument that measures the amount of light absorbed by a solution. It consists of several key components, each with a specific function.



(Source: https://lcms.cz/products/1311)

Fig. 5: Diagram of a spectrophotometer.

<u>Main components of a spectrophotometer:</u>

1. Light source:
- For the visible spectrum (VIS, 400–700 nm), a tungsten lamp is used.
- For the ultraviolet spectrum (UV, 200–400 nm), a deuterium lamp is required.
- High-quality spectrophotometers combine both sources for a wider measuring range.

2. Monochromator:
- A device that isolates a single wavelength from white light.
- Filters, prisms, or diffraction gratings are commonly used.
- Enables specific measurement of absorbance at the chosen λ.

3. Cuvette:
- A small glass or plastic container holding the sample.
- The standard cuvette path length is 1 cm.
- Cleanliness and correct alignment in the light path are essential.

4. Detector:
- A photodiode or photomultiplier that measures the intensity of light after passing through the sample.
- The signal is converted into an electrical impulse and processed further.

5. Output unit:
- Display panel, printer, or digital transfer into the laboratory information systém.
- Displays absorbance, transmittance, or the calculated concentration.

?

***Question:** Which part of the spectrophotometer is responsible for selecting a specific wavelength?*

2.2.2 Applications of Spectrophotometry in Medicine

Spectrophotometry has broad applications in clinical laboratories. It enables rapid, reproducible, and relatively simple determination of substances that either naturally absorb light in the UV/VIS range or can be chemically converted into absorbing products.

Measurements are performed at the wavelength where the solution shows maximum absorption (the *absorption maximum*). This maximum is a characteristic property of a colored compound, independent of its concentration. In VIS spectrophotometry, the absorption maximum corresponds to the complementary color of the solution.

Spectrophotometry can thus be used for:

- **Direct determination** of naturally absorbing substances (e.g., analysis of hemoglobin and bilirubin absorption maxima to detect and estimate the age of bleeding).
- **Indirect determination** of colorless substances, which must first be converted into colored products by chemical reactions—so-called *indicator reactions*.

Spectrophotometry is essential for measuring biomolecule concentrations, monitoring enzyme activities, and detecting antibodies in immunodiagnostics. Its advantages are speed, accuracy, and a high degree of automation.

Determination of Biomolecule Concentrations (Selected Analyses):

These compounds are key markers of metabolism and are routinely monitored in laboratories.

- **Glucose (blood sugar):** Measured enzymatically; glucose is converted into a colored product, and the color intensity reflects the glucose level. Essential for diagnosing diabetes mellitus.

- **Cholesterol:** Determined similarly via enzymatic reactions; the resulting color indicates the cholesterol concentration (e.g., in blood). Important for assessing the risk of atherosclerosis and cardiovascular disease**.**
- **Urea:** Measured using urease, which breaks down urea, and the product is transformed into a colored compound. Significant for evaluating kidney function.

Protein determination in serum is performed by colorimetric reactions:

- **Biuret method:** Copper ions react with peptide bonds in proteins, forming a violet-blue color. It is simple, reliable, and widely used in laboratories.

Enzyme Activities:

Clinical laboratories frequently measure enzyme activity in blood—elevated levels indicate tissue damage.

- **ALT (alanine aminotransferase) and AST (aspartate aminotransferase):** Increased in liver cell injury (e.g., hepatitis).
- **GGT (gamma-glutamyltransferase):** Elevated mainly in liver and bile duct diseases, often also in alcohol abuse.
- **ALP (alkaline phosphatase):** Elevated in cholestasis or in bone disorders (e.g., Paget's disease, bone tumors).

Principle of measurement: A suitable substrate is added to the sample, and the enzyme in the blood catalyzes its transformation. During the reaction, either a colored compound is produced or disappears (sometimes a compound absorbing in the UV region). The spectrophotometer continuously monitors the change in absorbance over time, and the reaction rate corresponds to enzyme activity. The result is expressed in U/L (enzyme units, where one unit converts 1 µmol of substrate per minute).

? ***Question:*** *In some cases, it is not the enzyme itself but a coenzyme, such as NADH, that is monitored. Can you explain the principle of this determination and why the wavelength of 340 nm is specific for measuring enzyme reactions involving NADH?*

Immunoanalytical Methods:

A frequently used immunoanalytical method is **ELISA (Enzyme-Linked Immunosorbent Assay)**. It relies on the antigen–antibody interaction. An enzyme is linked to the antibody (or antigen), and after adding a substrate, a color reaction is produced. The spectrophotometer measures the color intensity, which is directly proportional to the amount of the analyte in the sample—for example, a hormone, virus, or antibody.

3 Fluorescence

Fluorescence is a physical phenomenon in which a molecule absorbs light of a shorter wavelength (e.g., ultraviolet) and subsequently emits light of a longer wavelength in the visible spectrum. Molecules capable of this process are called *fluorophores*. The process itself is extremely rapid, occurring within nanoseconds.

The principle of fluorescence methods is based on exciting the sample with a light source (UV lamp, laser) and detecting the emitted radiation, whose intensity is proportional to the concentration of the fluorescent substance. Fluorescence may occur naturally in some biomolecules (e.g., porphyrins, flavins) or be induced in molecules that are specifically labeled with fluorophores.

Fluorescence methods allow the analysis of a wide variety of biological samples—from fluids (e.g., blood serum) and cell suspensions to tissue sections. Instruments used include:

- **Fluorimeters** – determine fluorescence intensity, spectrum, and lifetime.
- **Fluorescence microscopes** – visualize the localization of fluorophores in cells and tissues.
- **Flow cytometers** – measure fluorescence of individual cells passing through a laser beam; widely used in hematology and immunology.

A major application is **immunofluorescence**, in which fluorophore-labeled antibodies bind to a specific antigen, allowing highly sensitive and specific detection. Fluorescent labeling is also applied beyond proteins, for example in the detection of nucleic acids, such as **fluorescence in situ hybridization (FISH)** or **real-time PCR** with fluorescence detection.

Due to its high sensitivity and specificity, fluorescence has broad applications in medicine—from detecting hormones, vitamins, and tumor markers to detailed monitoring of cells and their structures in diagnostics and research.

Note: *Fluorescence should be distinguished from phosphorescence—while fluorescence occurs within nanoseconds, phosphorescence is a slower phenomenon that can last seconds to minutes.*

Interesting fact*: Tonic water contains quinine, which fluoresces blue under UV light—this is why tonic seems to “glow” in nightclubs. Toothpaste or white clothing often contain optical brighteners that fluoresce in the blue region, making them appear whiter.*

4 Emission

Emission occurs when atoms or molecules absorb energy (e.g., from a flame, electrical discharge, or plasma) and are promoted to an excited state. Upon returning to the ground state, they release energy in the form of light radiation. Each element emits light at characteristic wavelengths, producing a unique emission spectrum. This provides both qualitative information (which element is present) and quantitative information (its concentration), since the intensity of emitted light is directly proportional to the amount of the element in the sample.

Measurement techniques include flame photometers or emission spectrometers, and in modern practice often inductively coupled plasma (ICP) sources. Samples are typically liquids such as blood serum or urine.

In clinical biochemistry, emission methods are particularly important for determining electrolyte concentrations—sodium, potassium, calcium, and magnesium. These elements are essential for normal physiological functions, including fluid and ion balance, nerve and muscle activity, and cardiac regulation. Electrolyte analysis is therefore crucial in diagnosing and monitoring kidney diseases, cardiac disorders, and metabolic disturbances.

Interesting fact*: Every element has its own characteristic emission spectrum. This principle is used to determine the composition of stars by analyzing their light. The same principle applies in flame photometry in the lab. It also explains the colors of fireworks: red (Sr), green (Ba), blue (Cu).*

5 Light Scattering

Light scattering occurs when a beam of light encounters particles in a solution and changes direction. Two related methods are based on this principle: **turbidimetry** and **nephelometry**.

- **Turbidimetry** measures the decrease in intensity of the light beam after passing through the sample.
- **Nephelometry** measures the intensity of light scattered to the side at a defined angle.

Both methods are used to determine the concentration of suspended particles or macromolecules. Commonly analyzed samples include serum, plasma, and urine, which may contain proteins, immune complexes, or cellular elements. In clinical practice, light scattering methods are applied, for example, in determining protein concentrations (immunoglobulins, fibrinogen, C-reactive protein) or in assessing urine turbidity in urinary tract infections. The instruments used are turbidimeters, nephelometers, or spectrophotometers equipped with side detectors.

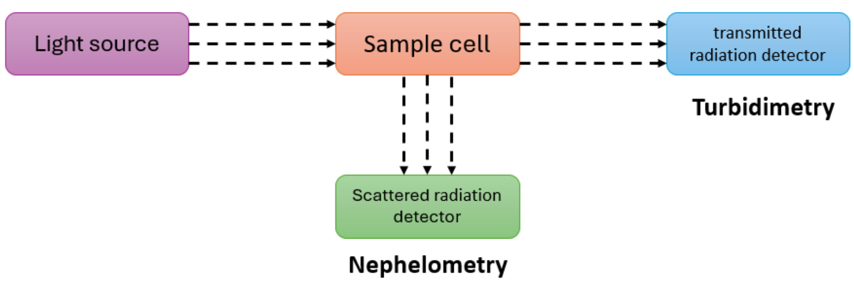


Fig. 5: Detector placement in light scattering methods.

? ***Question:*** *Which type of optical method would be most suitable for measuring urine turbidity in suspected urinary tract infection?*

Summary

Optical methods are fast, sensitive, and indispensable in clinical biochemistry. The main measurable phenomena include absorption, transmittance, fluorescence, emission, and light scattering. The most important method is UV/VIS spectrophotometry, based on the Lambert–Beer law, which enables the measurement of analyte concentrations as well as enzyme activities. In practice, it is routinely used for the determination of glucose, lipids, bilirubin, hemoglobin, enzymes, and nucleic acids. Correct interpretation of results requires awareness of possible interferences—such as hemolysis, icterus, or lipemia. Modern laboratory systems allow full automation of these measurements, ensuring high accuracy and reproducibility.

Control Questions

1. What is the difference between absorbance and transmittance?
2. State the Lambert–Beer law and the variables it includes.
3. How do turbidimetry and nephelometry differ?
4. Give an example of direct and indirect determination of a substance using spectrophotometry.
5. What are the most common interferences in spectrophotometric measurements in clinical biochemistry?
6. What is the role of the blank sample in spectrophotometry?