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Spectrophotometric Analysis of Amino Acids and Proteins

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Abstract- In this work, the spectrophotometric properties of amino acids and proteins are scientifically studied. First, general concepts regarding the biological significance of amino acids and proteins, as well as the main methods used in their analysis, are provided. The theoretical foundations of spectrophotometry—the interaction between electromagnetic radiation and molecules, the Bouguer-Lambert-Beer law, the molar absorption coefficient, and the UV (ultraviolet) absorption properties of amino acids—are highlighted. Furthermore, the spectral characteristics of aromatic amino acids (tryptophan, tyrosine, phenylalanine), the spectrophotometric analysis of proteins, and methods for determining their concentrations are analyzed. In the practical section, Olympiad problems, along with the capabilities, challenges, and limitations of the spectrophotometric method, are presented. At the end of the work, general conclusions and recommendations for future research are provided.

Keywords— Amino acids, proteins, spectrophotometry, Bouguer-Lambert-Beer law, aromatic amino acids, protein concentration.

I. INTRODUCTION

Amino acids and proteins are the fundamental biochemical molecules of all living organisms, playing a crucial role in vital processes. Amino acids are the basic structural units of proteins, and their analysis is of great importance in biological, medical, and biotechnological research. The determination of the quality and quantity of proteins is widely used in many fields, including pharmaceuticals, the food industry, genetic engineering, and medicine[1,2].

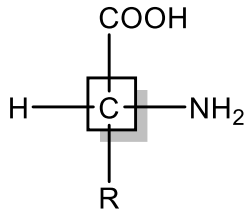
Nitrogen-containing organic compounds that simultaneously contain an amino group – NH2 and a carboxyl – COOH group in their molecule are called amino acids.

Amino acids are derivatives of organic carboxylic acids and are formed as a result of the replacement of one or more H atoms in an acid radical with an amino group. Their general formula corresponds to CnH2n (NH2) (COOH) or H2N–R–COOH.

The following types of isomerism are found in amino acids:

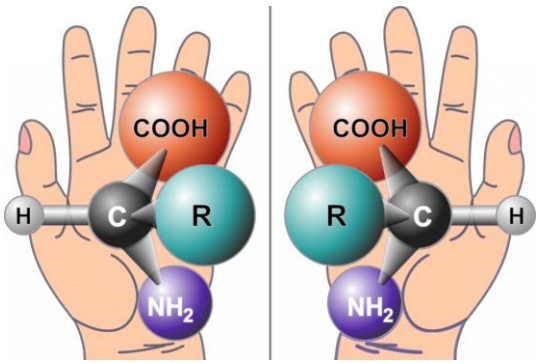
- Chain isomerism, a type of structural isomerism.
- Functional group position isomerism, a type of structural isomerism.
- Interclass (nitro compounds) isomerism, a type of structural isomerism.
- Optical isomerism, a type of spatial isomerism, is encountered.

Optical isomerism (stereoisomerism) is a type of isomerism between compounds that have the same molecular formula and arrangement of atoms, but rotate the plane of polarized light in different directions due to their spatial arrangement. This is the most important type of isomerism for amino acids. Optical isomerism occurs because in most α-amino acids, the α-carbon atom is bonded to four different groups, meaning it has a chiral center (an asymmetric atom).



The following types of optical isomerism are known:

1. Enantiomers are isomers that are mirror images of each other. The molecules have identical chemical and physical properties, but their optical activity is opposite; one rotates the plane of polarized light to the right (dextrorotatory (D,R), +), and the other to the left (levorotatory (L,S), –). This can be imagined just like our two hands: they are mirror images, but the gloves that fit them cannot be worn on the opposite hand (right on the left, or left on the right).



Natural proteins consist only of L-form amino acids. D-forms are typically found in bacteria or synthetic drugs (such as some antibiotics).

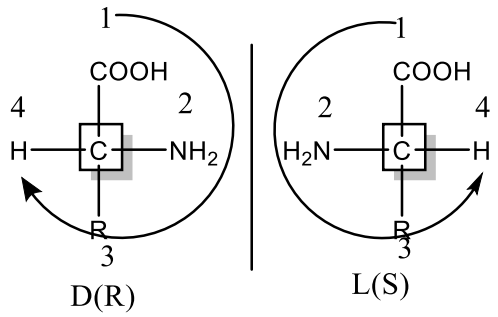
2. Diastereomers are a type of isomerism found in molecules with multiple chiral centers that are not mirror images of each other, where not every center has the same configuration. These molecules have different physical and chemical properties.

Since typical α-amino acids have only one chiral center, diastereomers are usually absent. However, threonine or isoleucine, for example, can have two chiral centers, and thus they have diastereomers.

3. Racemic mixtures (racemates) - A state where optical isomers are mixed in a 1:1 ratio. They neutralize each other's effects and have no optical activity. D-amino acid + L-amino acid = racemic mixture. This condition is most commonly found in synthetically obtained compounds [3,4].

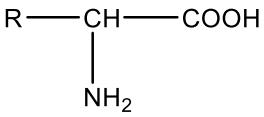
When determining enantiomers, we rank the 4 different groups located around the chiral atom for amino acids according to their chemical priority.

1st priority: -COOH (carboxyl group)
2nd priority: -NH2 (amino group)
3rd priority: -R (radical)
4th priority: -H (hydrogen)
If the sequence of numbers is counterclockwise, it is a levorotatory -L (S) isomer.
If the sequence of numbers is clockwise, it is a dextrorotatory -D (R) isomer.



The number of optical isomers is determined by the formula 2^n. Here, n represents the number of chiral centers.

We classify α-amino acids into types according to the following characteristics.



Based on the nature of R, they are divided into the following types:

- Nonpolar (aliphatic)
- Polar (uncharged)
- Aromatic
- Positive
- Negative

Nonpolar amino acids – amino acids whose radicals are nonpolar and do not bind to water via hydrogen bonds. Examples include glycine, alanine, valine, leucine, and isoleucine.

Polar (uncharged) amino acids – amino acids whose radicals are polar groups that form hydrogen bonds with water and are water-soluble. Examples include aspartic acid amide, glutamic acid amide, serine, and cysteine.

Aromatic amino acids - amino acids with an aromatic ring in their radical. Examples include phenylalanine, tyrosine, and tryptophan. The absorption property of the aromatic ring in these acids is utilized in spectrophotometry to determine the concentration of amino acids and proteins [5,6].

Spectrophotometry method – a method for determining the concentration of a substance based on its light absorption properties according to the Beer-Lambert law. Numerous analytical methods have been developed to identify and measure amino acids and proteins. The most widely used among them is the spectrophotometry method. This method is based on the absorption of ultraviolet (UV) and visible light by molecules, making it a fast and relatively inexpensive method of detection. Spectrophotometry can be used to determine the composition of amino acids, as well as the concentration of proteins.

This article examines the theoretical foundations of the spectrophotometry method, its role in the analysis of amino acids and proteins, practical examples, and solutions to Olympiad problems. It also provides information on the advantages, limitations, and practical applications of the method.

II. SPECTROPHOTOMETRY

Spectrophotometry is a physicochemical method used for the qualitative and quantitative analysis of substances, based on measuring the absorption (or transmittance) of light passing through a sample. Spectrophotometry is based on energy transitions associated with the absorption or emission of light by substances. Changes occurring as a result of the interaction of substances with light are observed through spectra (or spectral absorption curves). The analysis of these spectra provides information about the composition and quantity of the substance.

Spectrophotometry is primarily used in the following ranges:

- Ultraviolet (UV): In the 200-380 nm range.
- Visible light (Vis): In the 380-780 nm range.
- Near infrared (NIR): In the 0.78-30000 μm range.

Spectrophotometers consist of the following main parts:

- Light source: Generates a light beam.
- Monochromator: Isolates light of the desired wavelength.
- Cuvette compartment: A space for placing the sample.
- Detector-recorder: Records changes in light.

Spectrophotometry is widely used in various fields, including:

- Chemistry: In the qualitative and quantitative analysis of substances.
- Biology: In the analysis of biological samples (blood, urine, etc.).
- Food industry: In controlling the quality and composition of food products.
- Pharmaceutical manufacturing: In controlling the quality and quantity of medicines.
- In environmental protection: Water and air quality control [7,8].

Spectrophotometric analysis of amino acids and proteins.

Properties of amino acids in spectrophotometry.
Among amino acids, those with aromatic residues (tryptophan, tyrosine, phenylalanine) absorb UV rays in the 260-280 nm range, which allows them to be determined by the spectrophotometric method.

- Trp (Tryptophan) — strong absorption at 280 nm
- Tyr (Tyrosine) — moderate absorption at 280 nm
- Phe (Phenylalanine) — very weak absorption in the region below 260 nm.

Table 1: Molar absorption coefficients of amino acids

Amino acid	Wavelength (nm)	ε (L·mol−1·cm−1)
Trp	280	~5500
Tyr	280	~1490
Phe	260	~200

Determination of protein concentration by the spectrophotometric method.
Because proteins contain many aromatic amino acids, their concentration is measured at 280 nm. The total ε value of a protein is considered equal to the

sum of the ε values of the aromatic amino acids in its composition, and the Beer-Lambert law is used in spectrophotometric measurements.

To calculate the concentration of proteins and amino acids in spectrophotometry, the following steps must be performed.

- Passing UV light with a wavelength of 280 nm through the protein solution.
- Calculating ε (molar absorption coefficient) or obtaining it from literature sources
- Cuvette (usually 1 cm)
- Determining the concentration using the Beer-Lambert law:

Separation of mixed spectra and components. Spectrophotometric methods help determine the concentration of each component in a mixture; if each substance has a unique spectrum, each component in the mixture is determined using spectral measurements and mathematical analysis.

Analytical methods based on spectrophotometry include:

- Biuret method — measuring protein through a color reaction at 540 nm,
- Bradford method — protein concentration is determined through a blue complex (595 nm).
- Lowry method — protein is determined through a color reaction (750 nm) with phenol and Cu2+.

However, although these methods are spectrophotometric, their mechanism differs from the specific absorption properties of amino acids.

III. ASPECTS TO CONSIDER IN SPECTROPHOTOMETRY

- Solution preparation: Protein and amino acid solutions must be prepared correctly (pH, ionic strength, impurities).
- Calibration: The instrument is calibrated using standard solutions.
- Concentration range: The Beer-Lambert law only works within a certain concentration range.
- Additions: Interfering substances and contaminants in the spectrum must be taken into account[9-15].

Problems related to spectrophotometry

The results obtained in spectrophotometry are calculated based on the Beer-Lambert law. The mathematical expression of this law is as follows:

$$A = lg\frac{I_0}{I} = \varepsilon \cdot c \cdot l$$

Where:

A – the absorbance property of the solution, i.e., optical density

I_0 – the amount of incident light

I – the amount of transmitted light

ε – molar absorption coefficient (epsilon)

c – concentration (mol/L)

l – cuvette path length (cm)

Problem 1. Determining the molar absorption coefficient of amino acids.

Tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) have the following molar absorption coefficients at 280 nm:

- ε (Trp) = 5500 $L \cdot mol^{-1} \cdot cm^{-1}$
- ε (Tyr) = 1490 $L \cdot mol^{-1} \cdot cm^{-1}$
- ε (Phe) = 0 $L \cdot mol^{-1} \cdot cm^{-1}$ (i.e., almost no absorption)

The glucagon peptide consists of 2 Trp, 2 Tyr, and 1 Phe residues. Calculate the total molar absorption coefficient of the peptide at 280 nm.

Solution: To determine the molar absorption coefficient of the peptide, the molar absorption coefficients of each amino acid are added together.
ε (glucagon) = 2×5500 + 2×1490 + 1×0 = 11000 + 2980 + 0 = 13980 $L \cdot mol^{-1} \cdot cm^{-1}$

Problem 2. Calculating the concentration of the glucagon solution.

The optical density of a glucagon solution at 280 nm in a 1 cm cuvette is 0.95. If the total molar absorption coefficient of glucagon is 13980 $L \cdot mol^{-1} \cdot cm^{-1}$, find the concentration of glucagon in the solution in mol/L.

Solution: According to the Beer-Lambert law:

$$A = \varepsilon \cdot c \cdot l$$
$$0{,}95 = 13980\ L \cdot mol^{-1} \cdot sm^{-1} \cdot c(mol \cdot l^{-1}) \cdot 1sm$$
$$c = 6{,}8 \cdot 10^{-5}\ M$$

Additional question: If the molecular mass of glucagon is 3482 g/mol, determine the mass concentration (g/L) of glucagon in the solution.

Solution:
Mass concentration = c×Mr = 6.8×10^{-5} mol/L × 3482 g/mol = 0.237 g/L

Problem 3. International Chemistry Olympiad (IChO) 2007.
To determine the amino acid composition spectrophotometrically using Ellman's reagent, the absorbance was measured at 412 nm. In the experiment

- Solution volume (V) = 0.5 L,
- Optical density (A) = 0.75,
- Molar absorption coefficient (ε) = 14150 $L\cdot mol^{-1}\cdot cm^{-1}$,
- Cuvette thickness (l) = 1 cm,
- If the dilution factor (n) is equal to 10,

Question: Determine the amount of amino acid (mol) in the solution.

Solution: From the Beer-Lambert law:

$$A = \varepsilon \cdot c \cdot l$$
$$0{,}75 = 14150\ L\cdot mol^{-1}\cdot sm^{-1}\cdot c(mol\cdot l^{-1})\cdot 1sm$$
$$c = 5{,}3\cdot 10^{-5}\ M$$

$5{,}3\cdot 10^{-5}\ M\ \cdot 10 = 5{,}3\cdot 10^{-4}\ M$ Initial concentration of the solution
To find the moles:
n (mol) = c×V = 5.3×10^{-4} M×0.5 L = 2.65×10^{-4} mol.

Problem 4. Local olympiad question.
When the optical density of an unknown protein placed in a 1 cm cuvette was measured at 280 nm, it was found to be 0.539. Before determining the optical density of the unknown solution on the spectrophotometer, the following calibration data were collected. Determine the concentration of the protein in the unknown solution.

Table 2: Concentration and optical density of protein solutions

No.	Concentration (mol/L)	Optical density (OD)
1	0.03 M	0.162
2	0.06 M	0.33
3	0.09 M	0.499
4	0.12 M	0.67
5	0.15 M	0.84

Solution
Method 1. Using the calibration data, we determine the extinction coefficient of the solution.
Molar absorption coefficient for experiment 1:

$$A = \varepsilon \cdot c \cdot l$$
$$0{,}162 = \varepsilon \cdot 0{,}03M \cdot 1sm$$
$$\varepsilon = 5{,}4$$

Molar absorption coefficient for experiment 2:

$$A = \varepsilon \cdot c \cdot l$$
$$0{,}33 = \varepsilon \cdot 0{,}06M \cdot 1sm$$
$$\varepsilon = 5{,}5$$

Molar absorption coefficient for experiment 3:

$$A = \varepsilon \cdot c \cdot l$$
$$0{,}499 = \varepsilon \cdot 0{,}09M \cdot 1sm$$
$$\varepsilon = 5{,}5$$

Molar absorption coefficient for experiment 4:

$$A = \varepsilon \cdot c \cdot l$$
$$0{,}67 = \varepsilon \cdot 0{,}12M \cdot 1sm$$
$$\varepsilon = 5{,}58$$

Molar absorption coefficient for experiment 5:

$$A = \varepsilon \cdot c \cdot l$$
$$0{,}84 = \varepsilon \cdot 0{,}15M \cdot 1sm$$
$$\varepsilon = 5{,}6$$

Table 3: Concentration, optical density, and molar absorption coefficient of protein solutions.

Concentration (mol/L)	Optical density (OD)	ε – molar absorption coefficient
0.03 M	0.162	5.4
0.06 M	0.33	5.5
0.09 M	0.499	5.5
0.12 M	0.67	5.58
0.15 M	0.84	5.6
		ε (average) ≈ 5.5

We will determine the concentration of the solution using the optical density and sedimentation constant of the solution with an unknown concentration given in the problem statement.

$$A = \varepsilon \cdot c \cdot l$$
$$0{,}539 = 5{,}5 \cdot c \cdot 1sm$$
$$c \approx 0{,}1\ M$$

Method 2.

We determine the unknown concentration using graph paper. To do this, using the calibration data, the optical density of the solution is plotted on the x-axis and the concentration of the solution on the y-axis, a corresponding straight line is drawn, and the concentration corresponding to the optical density in the problem statement can be determined.

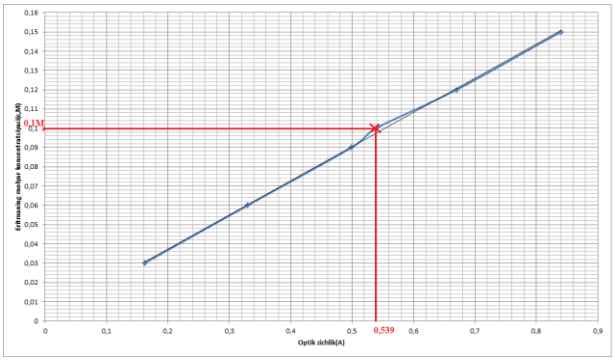


Figure 1. The relationship between the optical density of the protein solution and its concentration. As seen in the graph, at an optical density of 0.539, the corresponding solution concentration is approximately 0.1M.

IV. PROBLEMS AND LIMITATIONS

Although the spectrophotometry method has many advantages, there are certain problems and limitations in its application. Scientifically, they can be divided into the following groups:

Main problems

- Spectral deviation: at high concentrations, a deviation from the Beer-Lambert law is observed.
- Instrumental errors: instrument malfunctions or decreased detector sensitivity may lead to incorrect results.

Effect of impurities

- Additional absorbance is observed as a result of other components in the solution.
- The solvent can also have absorbance within a certain range.

Calibration difficulties

- Calibration lines do not always form an ideal straight line.
- Errors in preparing standard solutions affect the results.

Limitations of spectrophotometry

- Sensitivity: it is difficult to detect substances at very low concentrations.
- Selectivity: it is difficult to distinguish substances that absorb in the same range.
- Measurement range: limited to the UV-visible spectrum.

V. CONCLUSION AND RECOMMENDATIONS

Spectrophotometry is one of the most effective and widely used methods in biological and chemical analyses, particularly for determining the amount of amino acids and proteins. The main advantages of this method are its speed, high sensitivity, and the ability to use simple equipment.

The absorption spectra of aromatic amino acids (tryptophan, tyrosine, phenylalanine) in the ultraviolet range are of great importance for the qualitative and quantitative assessment of proteins. Colorimetric reactions (Biuret, Lowry, and Bradford methods) further expand the capabilities of spectrophotometry.

Recommendations for the future

- Using modern high-precision spectrophotometers and automated systems.
- Expanding practical laboratory sessions for students.
- Combining spectrophotometry with other analytical methods for researchers.
- Solving problems related to this topic is necessary to more fully understand spectrophotometry.

Thus, spectrophotometry is a relevant and promising direction in the analysis of proteins and amino acids in the fields of biology and chemistry.

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