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RAPID AND SENSITIVE METHODS FOR THE DETERMINATION OF PROTEINS AND PEPTIDES

SUMMARY OF THE PHD THESIS

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INTRODUCTION

Proteins and peptides are particularly important biochemical components of living matter. The biochemical functions of proteins include catalysis, transport through membranes or bodily fluids, muscle contraction, protection, structure, and metabolic regulation. Proteins play a key role in the proper functioning and survival of organisms, and their homeostasis is associated with the well-being of the body. In the body, peptides can be obtained through enzymatic hydrolysis of proteins, and the sequence of amino acids in a protein is dictated by the nucleotide sequence of a segment of DNA.

This doctoral thesis explores the complexity of protein analysis and proposes contributions to improving the methodology for determining these bioheteropolymers. The complexity and diversity of peptides and proteins arise both from the large number of possible linkages between α-amino acids for their formation, and from the specific conformational and configurational characteristics of their molecules. The thesis focuses on the methods, principles, procedures, advantages, disadvantages, and applications of various techniques used to determine proteins, which are based on the unique characteristics imparted by their specific amino acid sequences. Methods such as Kjeldahl and Dumas target the determination of nitrogen content. Infrared spectroscopy relies on the absorption of specific infrared wavelengths by the peptide bond. Copper–peptide bond interactions allow for the colorimetric quantification of proteins using the biuret, Lowry, and bicinchoninic acid (BCA) methods. Specific amino acids can be involved in detection mechanisms underlying the Lowry, BCA, dye-binding methods, and even UV quantification at a wavelength of 280 nm. The BCA method also utilises the reducing power of proteins in an alkaline solution.

There are various methods for determining proteins and peptides. Peptide biochemistry plays an important role in their study, as well as in the study of proteins and their functions. Determining the protein and peptide content in biological materials is essential in scientific research. Spectrophotometric, fluorimetric, chromatographic, and electrochemical methods are analytical techniques used for the quantification of these components in biological samples. Protein quantification methods are also examined, including techniques such as ultraviolet absorption, the Lowry method, the bicinchoninic acid (BCA) method, the Bradford method, and the Kjeldahl method (Pulikkottil, 2024). The determination of proteins in food and crop seeds is also important for practical reasons (Chang & Zhang, 2017). Therefore, this doctoral thesis addresses the instruments, principles,

procedures, advantages, disadvantages, and applications of various protein analysis methods that rely on the unique characteristics of these macromolecules and their constituent amino acids. Certain methods are implemented as official procedures for establishing nutritional information, while rapid methods may be suitable for quality control, and highly sensitive methods are required for the detection of trace amounts of protein.

The topic of the doctoral thesis, "Rapid and Sensitive Methods for the Determination of Proteins and Peptides", was chosen for several reasons: (i) there is extensive experience in the field of protein research within the Faculty of Chemistry, as evidenced by previous studies on this subject cited in the bibliography of this work; (ii) the practical applicability of these determinations in various fields, from medicine to agriculture and even in school laboratory experiments; (iii) the relatively low complexity of spectrophotometric and fluorimetric measurements, which allows the acquisition of a wide range of experimental data within a short time frame; and (iv) the lack of well-founded studies on improving the rapid and sensitive determination methods of peptide compounds. Consequently, the research undertaken in this thesis continues the ongoing work within the university on protein determination methodology and has become of interest to the global scientific community, as evidenced by its international impact.

It is also important to highlight the significance of protein determination, and for this purpose, it is essential to choose the appropriate technique from the available methods (Janairo et al., 2011). The complexity of research in this field is also linked to the necessity of knowing the specific concentration range in which a method is sensitive. The choice of method is a key factor in reducing errors and ensuring more reliable results (Miranda, 2024). In addition, several factors are taken into account, such as the nature of the protein, the presence of other components in the sample, and the desired speed, precision, and sensitivity of the analysis (Zheng et al., 2017). These and other protein analysis methods differ in terms of their speed and sensitivity. Due to the complex nature of different proteins, challenges may arise in analysing them using existing methods. Certain methods are required as official procedures for establishing nutritional information, rapid methods are suitable for quality control, and highly sensitive methods are necessary for determining trace amounts of proteins (Dee et al., 2024). Therefore, the selection of the method to be used in protein measurements must be made carefully to avoid generating analytical errors (Miranda, 2024). All these aspects were taken into consideration in the research described in this doctoral thesis.

The doctoral thesis *"Rapid and Sensitive Methods for the Determination of Proteins and Peptides"* comprises 229 pages, a total of 92 figures, 7 tables, and 340 bibliographic references. The results presented in the section dedicated to original contributions are the subject of three scientific articles published in Web of Science-indexed journals, with a cumulative impact factor of 5.3 (Mihalcea, E., Enache, A.C., Grădinaru R.V., Drochioiu, G., 2023; Drochioiu, G., Mihalcea, E. et al., 2024; Mihalcea, E. & Drochioiu, G., 2025), as well as two scientific articles published in the proceedings of international conferences (Darie-Ion, L., Drochioiu, G., Pui, A., Mihalcea, E., Grădinaru, V. R., 2021; Mihalcea, E., Drochioiu, G., Jitaru, S. C., Mangalagiu, V., Grădinaru, R.V., 2022).

The author of this doctoral thesis is the first author of three scientific publications. The results obtained from the doctoral research were disseminated at both national (2) and international (3) scientific conferences. Professional development was further supported by involvement in the research project *"Human Capital Operational Programme 2014–2020 – Educational and Training Support for Doctoral Students and Young Researchers in Preparing for Labour Market Integration",* Project Code SMIS 2014+: 153322, during the period 2022–2023.

The doctoral thesis entitled "Rapid and Sensitive Methods for the Determination of Proteins and Peptides" is structured into two main parts: a theoretical part comprising the literature study related to the chosen topic, and a section of personal contributions in which the original results of the doctoral research, as well as the author's own observations, are presented.

The first part contains information regarding the current state of knowledge on various methods used in the determination of proteins and peptides, being divided into three chapters. This section includes information related to the structure of peptide-bonded compounds, the importance of neuroprotective peptides, methods of peptide synthesis, and the main physico-chemical methods used for the structural and conformational characterisation of peptide compounds.

The conclusion of this section is directly related to the main objectives proposed in the development of the doctoral thesis.

The second part is original and presents in detail the personal contributions made to the field of protein and peptide determination methodology, with reference to the applications of new methods and variants of chemical analysis. This part of the doctoral thesis is structured into seven chapters. The first chapter provides information on the biological materials, chemicals, and instruments used in the experimental research, as well

as the methods employed for obtaining and investigating the compounds of interest in this study. The second chapter describes the results obtained through the application of a new variant of the biuret method, as well as its use in the determination of real samples.

The third chapter outlines the potential use of spectrophotometric methods in the characterisation of peptides, with the advantages of spectrophotometric measurements in the UV range being discussed in chapter four. The investigation of the possibility of protein determination based on the reaction of sulfanilic acid with sodium nitrite in an acidic medium is presented in chapter five. Chapter six includes a fluorimetric study on the determination of peptides, amino acids, and proteins. The final chapter of the thesis summarises the scientific activity carried out during the doctoral studies, along with a list of published works.

In this thesis, the following objectives were pursued:

O1 To study the determination of protein and peptide content in biological materials using rapid and sensitive methods, including spectrophotometric, colorimetric, fluorimetric, and chromatographic techniques;

O2 To refine the methodology for protein determination using the biuret method;

O3 To investigate the interference of amino acids and other chemical compounds present in biological environments in the analysis of proteins in living organisms;

O4 To experimentally study the interaction between copper ions and amino acids, as well as other interfering compounds (e.g. Krebs cycle acids);

O5 To disseminate the results obtained through publication in impact factor journals and presentation at various national and international scientific events.

During the research, new objectives were added to enable a deeper understanding of the methodology for determining proteins and peptides, such as:

O6 Determination of proteins using diazotised sulfanilic acid;

O7 Determination of copper ions extracted by proteins and peptides in alkaline medium using a resorcinol-based reaction;

O8 Fluorometric Methods Used in the Study of Proteins and Peptides

The thesis concludes with general conclusions highlighting the novelties and contributions of this doctoral work, followed by the bibliography, a list of abbreviations, and an annex containing the scientific publications presented or published in impact factor journals.

PART II. PERSONAL CONTRIBUTIONS

II.1. REAGENTS, INSTRUMENTS, AND RESEARCH METHODS

Bovine serum albumin was used as the standard protein for spectrophotometric measurements and for testing the newly proposed methods. Amino acids were purchased from Fluka and Merck, while sulfanilic acid and sodium nitrite were acquired from Merck (Darmstadt, Germany). Ethyl alcohol was obtained from Chemical Company S.A. in Iași, Romania.

The Pauly reagent (Ma & Liu, 2023) was prepared by mixing equal volumes of Solution A and Solution B as follows: Solution A was prepared by dissolving 0.1 g of sulfanilic acid (SA) in 2 mL of 2.5 M NaOH, followed immediately by the addition of 20 Ml of 1M HCl. Solution B consisted of 0.1M sodium nitrite. A calibration curve was created using a tyrosine solution in the concentration range of 0–100 µg/mL.

The classical Biuret reagent consists of various concentrations of chemical compounds such as copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) at 3.25 g/L, potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) at 9.02 g/L, and NaOH at 24 g/L. Other authors recommend a Biuret reagent consisting of 9.00 g/L sodium potassium tartrate, 0.024 g/L sodium hydroxide, 1.90 g/L anhydrous copper (II) sulfate, and 5.00 g/L potassium iodide (Laurenciano et al., 2021). To prepare the reagent, 300 mL of 10% (w/v) NaOH is gradually added with stirring to 500 mL of a solution containing 0.3% copper (II) sulfate pentahydrate and 1.2% sodium and potassium tartrate and then diluted to 1 litre. The reagent is stable for several months. When 1 g of potassium iodide per litre is added and the solution is stored in the dark, its stability extends indefinitely. A sample volume is mixed with two to five volumes of reagent; the optimal ratio depends on the maximum protein concentration being analysed. The presence of proteins yields a violet colour with maximum absorbance around 550–555 nm; absorbances are typically read at 540 nm, but the absorbance band maximum can be determined within the 520–570 nm range.

The new Biuret reagent (BR) is an alkaline-alcoholic solution prepared by dissolving 20 g of KOH in 100–200 mL of milliQ-grade water, then adding 450 mL of absolute ethanol or 475 mL of 95% (v/v) ethanol and topping up with water to a final volume of 1 litre.

The classical ninhydrin reagent (NR), used for determining lysine in the Biuret supernatant as well as free amino acids in various media, was obtained by dissolving 400 mg of ninhydrin in 25 mL of acetate buffer (pH 5.5), and then adjusting to a final volume of 100 mL with ethylene glycol.

The modified ninhydrin reagent was prepared by dissolving 400 mg of ninhydrin and 400 mg of $CdCl_2$ in 25 mL of acetic acid–sodium acetate buffer (pH 5.5) and making up the volume to 100 mL with glycerol. The solution was freshly prepared before use.

The diazotisation reaction of sulfanilic acid with sodium nitrite in acidic medium at room temperature is well known (see Figure II.1.2).

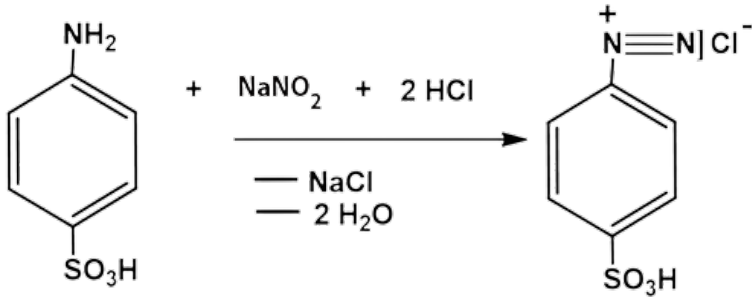

Figure II.1.2. *The Chemical Diazotisation Reaction of Sulfanilic Acid*

The Pauly reaction involves the interaction of histidine and tyrosine with diazotised sulfanilic acid. Tyrosine, histidine, and proteins containing these amino acids form a pink azo dye through a coupling reaction. Upon alkalinisation, the colour intensifies and shifts toward red. The reagents typically used include 1% sulfanilic acid in 10% HCl, 5% $NaNO_2$, and 30% Na_2CO_3. In the present research, the following solutions were prepared:

(i) a 35 mg% (w/v) solution of sulfanilic acid (SA), acidified with 1 mL of 37% HCl and made up to 100 mL in a volumetric flask;

(ii) an aqueous solution of $NaNO_2$ at 30 mg%;

(iii) a tyrosine (Tyr) solution at 30 mg% in 8% Na_2CO_3.

These solutions were more diluted than those commonly reported in the literature, which are typically used for amino acid, peptide, and protein determinations, rather than for the identification of tyrosine and histidine residues in proteins. The chemical coupling reaction of the diazonium salt of sulfanilic acid with tyrosine is shown in Figure II.1.3.

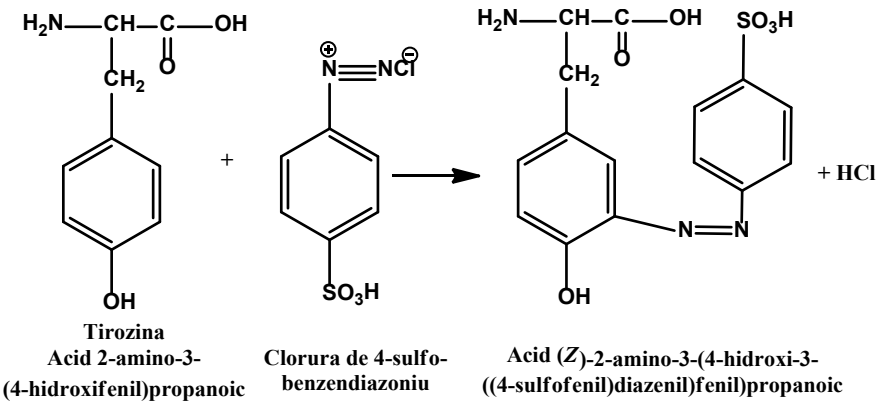

Figure II.1.3. *Coupling Reaction of Diazotised Sulfanilic Acid with Tyrosine*

Infrared Spectroscopic Analysis

Structural modifications of peptides and their complexes with copper ions were evidenced using a classic transmission spectrometer, following pelletisation of approximately 1–2 mg of peptide at 200 atm. FTIR spectra of molecular absorption were obtained using a Fourier-transform infrared spectrophotometer (FT-IR), model Shimadzu 8400S (Duisburg, Germany). The IR spectra were recorded in the range of 4000–400 cm^{-1}, with a spectral resolution of 2 cm^{-1}. Samples were ground with potassium bromide (KBr) powder and pressed into transparent pellets. The resulting FTIR spectra were processed using the software package Origin.

Spectrofluorimetric Studies

2D and 3D fluorescence spectra were recorded using an FP-8350 spectrofluorometer (JASCO, Tokyo, Japan). The peptide or protein solutions analysed were excited at various wavelengths to obtain emission spectra. All measurements were performed at 27 °C using a transparent SUPRASIL® quartz cuvette (with an optical path length of 0.5 cm and a volume of 1.7 mL, Hellma, Mulheim, Germany), mounted in an FMH-802 cell holder.

II.1.5.1. The Micro-Kjeldahl Method

This method involves the digestion of the sample material (plant-based, milk, meat, etc.) with sulphuric acid, in the presence of copper (II) sulphate or mercury (II) sulphate, at approximately 330 °C. Sodium or potassium sulphate is added to raise the boiling point of the mixture. The digestion time is typically 4–6 hours when using $CuSO_4$ as a catalyst, and about 1–2 hours with yellow mercury oxide; in the micro-variant using small quantities of plant material (30–100 mg biological sample), digestion takes only one hour. The resulting

ammonia is distilled in the presence of NaOH or KOH and $Na_2S_2O_3$ and is titrated with HCl or H_2SO_4 of known concentration, using an indicator such as a methyl red–bromocresol green mixture.

II.1.6.1. Protein Determination Using the Proposed Biuret Method

The biuret method is selective, in that the biuret reaction is specific only to peptide compounds. However, substances such as gelatine, zein, peptone, and albumin contain peptide bonds in their molecules, but exhibit differing absorbances, and the maxima of their absorption bands may also vary.

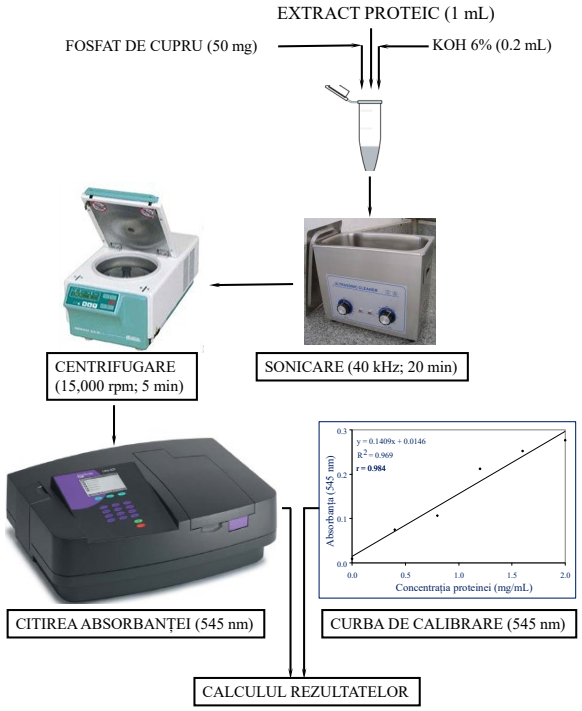

Figure II.1.6. *Methodology for the Determination of Proteins in Solutions by the Biuret Reaction*

II.1.7. The Proposed Biuret Variant

The analysis of the conducted experiments led to a procedure that offers optimal advantages in protein determination. Thus, to 1 mL of protein solution placed in a plastic Eppendorf tube, 0.5 mL of an alkaline–alcoholic solution and 20–30 mg of copper phosphate powder was added. The resulting mixture was sonicated for 30 minutes and then centrifuged at 15,000 rpm for 5 minutes. If the proteins are already in solution, the sonication time can be reduced to just 10 minutes.

The spectrum of the clear supernatant was recorded in the 190–840 nm range, against a blank prepared with the reagents only. Spectra and absorbance values were analysed within wavelength ranges of interest, such as 300–350 nm, 400–700 nm, or specifically at 545 nm and 560 nm. Calibration curves were plotted at the wavelength where the Biuret complex exhibited maximum absorbance, for example around 545 nm or 326 nm. For measurements performed with a microplate spectrophotometer, readings were taken at 560 nm.

In the case of cereal proteins and those from other plant seeds, 50–100 mg of finely ground seed flour was used per analysis. Each sample was treated with 1 mL of alkaline–alcoholic solution and approximately 50 mg of copper phosphate powder. The Eppendorf vials containing this mixture were manually shaken and then sonicated for 30 minutes. After centrifugation at 15,000 rpm for 5 minutes, the clear supernatant was measured using a spectrophotometer in 1 cm cuvettes (plastic cuvettes were used in the visible range), compared to a control prepared with reagents only and treated in the same manner.

The calibration curve was generated either using an alkaline–alcoholic BSA solution (1 mL) or with protein values previously determined using the micro-Kjeldahl method.

II.2. DETERMINATION OF PROTEINS AND PEPTIDES VIA THE BIURET REACTION

This doctoral thesis aims to study and improve the method for protein and peptide determination based on the well-known Biuret reaction, which involves the formation of coloured complexes between compounds containing peptide bonds and copper ions in a basic medium. Accordingly, the classical reaction was first studied, involving the formation of Biuret complexes between peptide compounds and copper ions complexed with tartrates (the original method used Rochelle salt—i.e., the double salt of sodium and potassium tartrate) in an alkaline environment. Since copper ions precipitate in alkaline solutions, they are first complexed with Rochelle salt before reacting with proteins. Upon heating urea to over 170 °C, it condenses with the release of one molecule of ammonia, forming an organic compound called biuret. This is the simplest compound that, in the presence of copper ions, forms a violet-coloured complex—hence the name “Biuret reaction.”

The investigation of the Biuret reaction included the following: (i) the study of the classical method; (ii) the characterisation of the new variant proposed in this work; (iii) the measurement of Biuret absorbances across a wide wavelength range using a standard spectrophotometer and comparison with values measured using a microplate reader; the

study of the Biuret reaction in the ultraviolet region; the application of the improved Biuret method to real sample analysis.

II.2.1. The Biuret Reaction

The classical Biuret method is fast, easy to apply, and independent of the amino acid composition of the protein (Liu & Pan, 2017). A variant of the Biuret method has been proposed for corn-based products using an alkaline reagent containing sodium dodecyl sulphate and thermal treatment. The Biuret method is based on the reaction of copper (II) ions with the peptide bonds of proteins, resulting in a violet-coloured protein–copper chelate. A minimum of two peptide bonds is required per Cu^{2+} ion. The Biuret method is frequently used as a reference method for total protein determination (Zhang et al., 2017). In addition, the protein content of artificial solutions was determined using the Biuret method with BSA as the standard (Zhang et al., 2017).

II.2.2. Comparative Study

This chapter examines two key aspects: Firstly, the use of the classical Biuret reagent versus the alkaline–alcoholic solution with insoluble copper phosphate, and secondly, the application of the new variant of the Biuret method for protein determination across various environments—from artificial solutions to real samples—in comparison with established methods. To assess the efficiency of the classical Biuret reagent, 4 mL of Biuret reagent (containing copper sulphate and sodium–potassium tartrate) was added to 1 mL of BSA solution, with concentrations in the range of 0–10 mg/mL. A calibration curve using BSA was plotted, and the standard curve revealed the **low sensitivity** of the classical method (see **Figure II.2.1**).

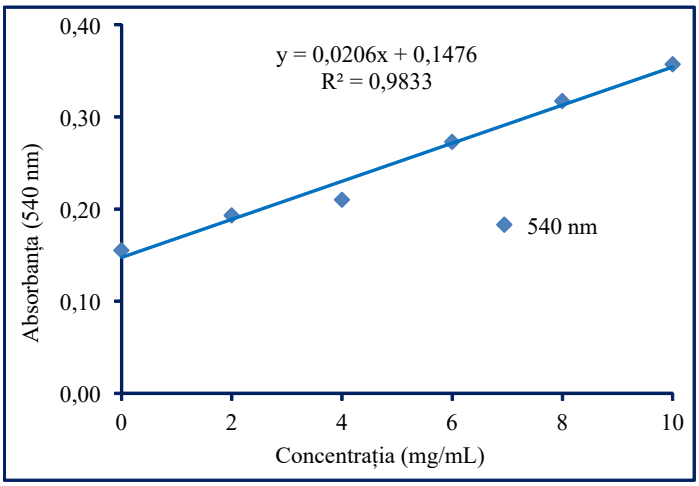

Figure II.2.1. *Calibration Curve Prepared with BSA (0–10 mg/mL) and Classical Biuret Reagent*

This result is also because the reagent volume used was four times greater, which led to a dilution of the Biuret solution. The reaction occurred over a very short time span of just one minute, at which point the maximum absorbance of the formed Biuret complex was recorded. Its stability was also high, allowing the samples to be read using a colorimeter or spectrophotometer over a longer time window.

These values were compared with those obtained using the proposed Biuret variant. The new method applied in this experiment consisted of treating six 1 mL volumes of BSA at concentrations of 0, 2, 4, 6, 8, and 10 mg/mL, respectively, with 1 mL of alkaline–alcoholic solution (prepared from 45 mL absolute ethanol, 2 g KOH, and water to a final volume of 100 mL), along with approximately 50 mg of insoluble copper phosphate powder. The mixture was sonicated for 30 minutes, centrifuged at 15,000 rpm, and measured at 540 nm. This variant yielded higher Biuret absorbance values than those obtained using the classical method (see **Figure II.2.2**).

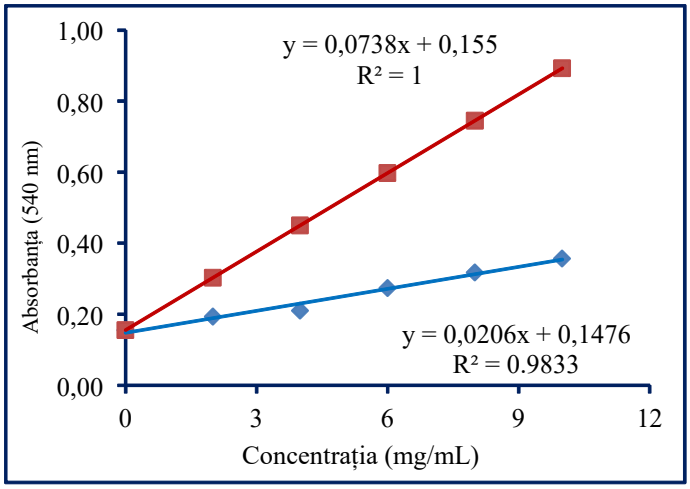

Figure II.2.2. *Comparison Between Calibration Curves Prepared with BSA (0–10 mg/mL) for the Copper Phosphate Reagent (red) and the Classical Biuret Reagent (blue)*

The calculated regression equations and correlation coefficients between absorbance values and BSA concentrations confirmed the significantly better results obtained with the improved method ($A_{540} = 0.0738 \cdot c + 0.155$, $r = 1$ and, respectively, $A_{540} = 0.0206 \cdot c + 0.1476$, $r = 0.991$, where A_{540} represents the absorbance at 540 nm and c the BSA concentration in mg/mL).

These experiments involving copper phosphate substitutes led, in all cases, to opalescent supernatant solutions. It was concluded that copper phosphate can be used, from which proteins extract copper ions to form the Biuret complex; however, it is essential to check the clarity of the resulting Biuret solutions. Filtering the samples after performing the Biuret reaction also yielded satisfactory results, though the duration of the procedures increased accordingly. Unfortunately, this method for obtaining clear Biuret solutions is time-consuming, and some of the analyte solution remains on the filter paper. As a result, larger volumes of reagents and biological materials are required for analysis.

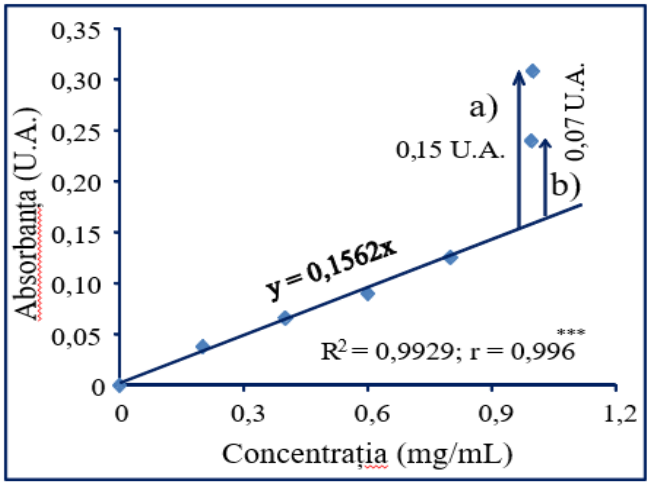

Figure II.2.7. *Calibration Curves in the Range 0–1 mg/mL BSA: (a) Before Biuret Value Correction and (b) After Adjustment for Opalescence Contribution*

II.2.3. UV-Vis Spectra of the Biuret Complex

The absorption spectra in the wavelength range of 300–700 nm for BSA solutions of various concentrations are shown in Figure II.2.8. Two absorption maxima are visible in this figure: one at 326 nm and another at 545 nm. Since copper ions were extracted from the insoluble copper phosphate in proportion to the protein concentration, the peak at 326 nm may be particularly useful for detecting low concentrations of proteins (below 2 mg/mL). At this wavelength, the absorbance values were four times higher than those recorded at 545 nm.

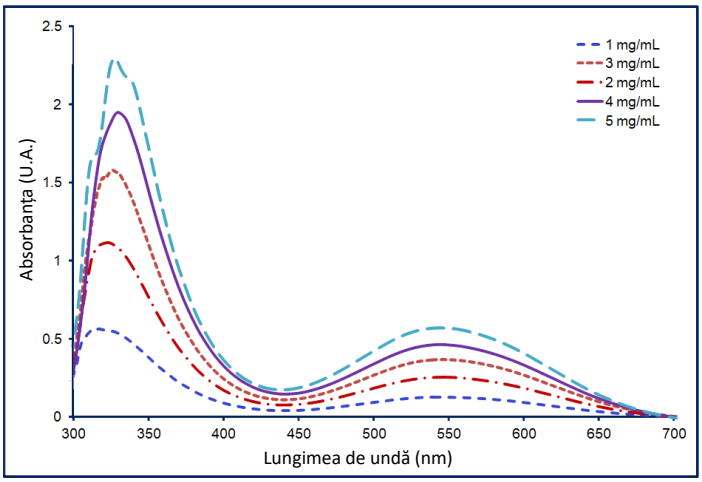

Figure II.2.8. *UV-Vis Absorption Spectra of the Biuret Complex for BSA Solutions of Various Concentrations*

The calibration curves obtained using BSA at the two wavelengths are presented in Figure II.2.9. It was found that, with modern spectrophotometers, absorbance values greater

than 1.0 AU can be accurately measured. Thus, the calibration curve plotted at 326 nm was linear up to approximately 2.5 AU. In both cases, the correlation coefficient r was highly significant according to the student's t-test. The R^2 values were 0.99 and 0.9959, respectively, and the regression equations calculated from the absorbance data and corresponding concentrations were: $A_{326} = 0.4328 \cdot c + 0.1886$, $A_{545} = 0.1164 \cdot c$

The calibration curves were linear within the concentration ranges of 1–5 mg/mL and 0–5 mg/mL, respectively, with the second line intersecting the ordinate at the origin.

Since older spectrophotometers were scaled for an absorbance range of 0.0–1.0 AU, and readings beyond 1.0 AU were not feasible, it was previously recommended that measurements be performed in the 0.2–0.8 AU range, where a proportional relationship between absorbance values and analyte concentration could be reliably maintained. In contrast, modern spectrophotometers—including the one used in this doctoral research—offer significantly higher sensitivity, making it possible to perform determinations well beyond the unit absorbance value.

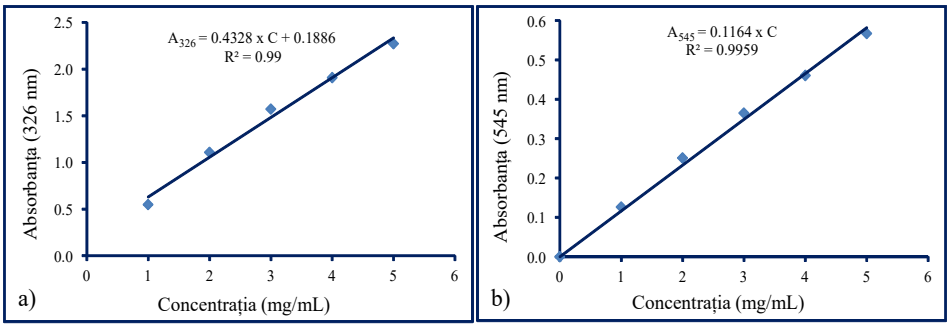

Figure II.2.9. *Calibration Curves for Protein Determination at 326 nm (a) and 545 nm (b)*

Therefore, the possibility of determining proteins using the proposed Biuret method at absorbance values exceeding 1.0 AU was investigated. The calibration curves proved to be linear within the range of 0–2.5 AU, as shown in Figure II.2.9. In conclusion, this study demonstrates that protein determinations can be successfully conducted over a wider absorbance range using modern spectrophotometers.

II.2.5. Real Samples

A modified Biuret method was applied to corn-based products, using an alkaline reagent containing sodium dodecyl sulphate and thermal treatment (Liu & Pan, 2017). However, the classical method, which relies on copper sulphate and sodium–potassium tartrate, presents several disadvantages. It was found that starch, fibres, oils, and other non-protein substances in the turbid solution interfere with colorimetric determinations.

In a separate experiment, 20 additional corn samples were tested to evaluate the correlation between Biuret values and those obtained using the micro-Kjeldahl method (see Figure II.2.12). Crude protein content was determined based on a calibration curve that correlated Biuret absorbance values with the protein content of the corn samples as measured by the micro-Kjeldahl method. The crude protein content determined using the Biuret method was comparable to that obtained by the micro-Kjeldahl method.

The use of an ultrasound bath enhanced complex formation in the presence of alkaline–alcoholic solutions and insoluble copper phosphate. Ethanol prevented the opalescence caused by starch in the corn samples, while the phosphate powder served as the source of copper ions that reacted with the peptide bonds of the proteins to form the Biuret complex. The regression equation was as follows: $B = 1.0664 \cdot K - 0.7313$ ($R^2 = 0.9755$; $r = 0.987$) where B is the percentage of crude protein determined by the Biuret method and K represents the values obtained by the micro-Kjeldahl method.

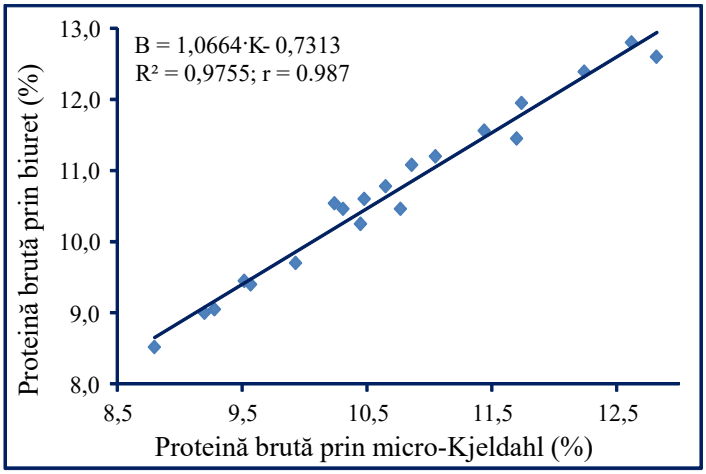

Figure II.2.12. *Strong Correlation Between Crude Protein Values Determined Using the Proposed Biuret Method and Those Obtained by the Classical Micro-Kjeldahl Method*

Regarding the determination of proteins using the Biuret method, as reflected in Figure II.2.12, pure protein was previously determined using the Barnstein method, yielding relatively lower values compared to crude protein determined by the micro-Kjeldahl method, in which 50 mg samples were analysed. Since the determination of pure protein using this method is particularly laborious, it was proposed to measure Biuret absorbance in many samples—for example, 50 samples per day—from which samples with low, medium, and high absorbance values across the full range are selected to construct the calibration curve.

II.2.6. Protein Determination in Meat

A volume of 0.3 mL of the sample under investigation (meat suspension) was vigorously mixed with 1 mL of alkaline–alcoholic reagent in a 2 mL Eppendorf tube, followed by the addition of approximately 50 mg of insoluble copper phosphate. The vials containing the mixtures were vigorously shaken and then subjected to ultrasonic bath sonication for 30 minutes. After sonication, the tubes were centrifuged at 18,000 rpm for 7 minutes. The clear supernatant was measured using a spectrophotometer in the wavelength range 200–700 nm, against a reference sample prepared with reagents only.

A strong absorbance was observed in the ultraviolet region, while the Biuret absorbance at 545 nm showed a value of 0.485 for the first sample in the series (see Figure II.2.13).

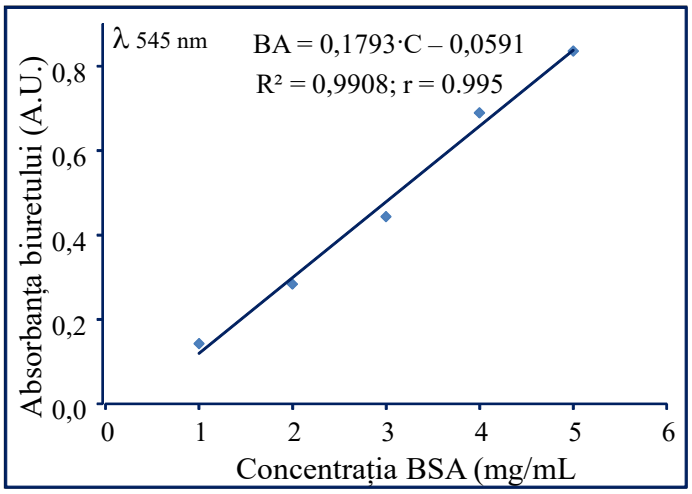

Figure II.2.14. *Protein Calibration Curve Using BSA*

Initially, the Biuret reaction was tested using various volumes of meat suspension to determine the optimal sample volume that would fall within the absorbance range of 0–1 absorbance unit (AU) on the spectrophotometer at 545 nm—the wavelength at which the Biuret complex of proteins exhibits maximum absorbance. To calculate the protein concentration in the meat sample, a calibration curve prepared with BSA in the concentration range 1.0–5.0 mg/mL was used (Figure II.2.14). Since it is much easier to perform these calculations using an Excel spreadsheet, the regression equation used was BA = 0.1793·C – 0.0591 from which the protein concentration was calculated using the formula: C = (BA + 0.0591) / 0.1793 where BA is the absorbance value of the Biuret complex formed with BSA, and C is the protein concentration expressed in mg/mL BSA. By applying this formula, a value of 4.215 mg of protein was obtained in a 0.3 mL solution volume. Since 10 g of beef

yielded 200 mL of suspension, from which 0.3 mL was used in the assay, the calculated protein content was: C = (4.215 mg / 0.3 mL × 200 mL) / 10 g = 281 mg/g protein
This corresponds to 28.1% protein, a value which falls within the normal protein content range for beef (26.0–30.3%).

II.2.7.3. Duration of Extraction Under Ultrasonic Treatment

The classical procedure, which relies on standard mechanical agitation between corn flour and alcohol, failed in extracting the full zein content from corn flour during the 270-minute experimental period. Without sonication of the alcoholic solutions mixed with corn flours, only approximately 40% of the maximum extractable zein content was obtained, as compared to the extraction achieved after 30 minutes of ultrasound treatment.

Figure II.2.15 shows the relationship between Biuret absorbance at 545 nm of finely ground seed samples (particle size: 100 µm) and the duration of sonication of the plastic Eppendorf tubes containing the reaction mixtures. All samples consisted of 50 mg of corn flour from the same corn variety. One of the samples was not sonicated but instead manually agitated for one minute, yielding an absorbance value of only 0.639 AU. The maximum absorbance was recorded after 30 minutes of sonication (A_{545} = 1.537 AU), while 1 mL of 5 mg/mL BSA dissolved in an alkaline–alcoholic solution showed 1.57 AU. In fact, the regression equation obtained was: $A_{545} = 0.2964 \cdot c$ ($R^2 = 0.998$) where c is the BSA concentration in mg/mL. Based on this, the protein concentration of the sample was calculated to be 9.80%, which is within the normal range for corn seeds.

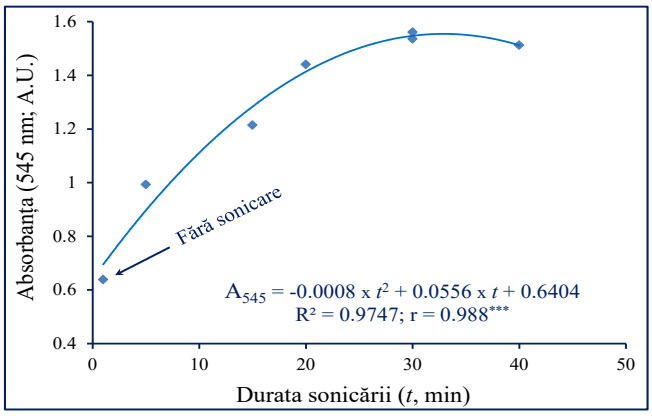

Figure II.2.15. *Determination of Protein Extraction Time from Corn Samples Under Ultrasonic Treatment (Mihalcea et al., 2025)*

The results obtained indicated that complete extraction of α-zein from the analyzed corn flour was achieved using the alcoholic extraction solution, with the remaining residue

containing only trace amounts of zein solution. Moreover, MALDI-ToF MS mass spectrometry confirmed that the alcoholic solutions contained almost exclusively α-zein (Figure II.2.16).

II.2.8. Advantages of the New Biuret Variant

The method proposed in this study is relatively simple and does not require complex equipment, making it accessible to less well-equipped laboratories, particularly those specialising in the analysis of biological materials. The reagents are inexpensive and easy to obtain or prepare, and the method itself does not entail high costs. Results can be obtained in a short time and from a relatively large number of samples, which is particularly advantageous for routine analyses. The sensitivity of the proposed method is moderate (1–20 mg), but it was significantly improved during this research. Notably, the reagent-to-sample ratio was reduced, and in addition, absorbance readings could also be taken at 326 nm, contributing to a significant increase in sensitivity. The method is generally capable of detecting moderate protein concentrations, making it suitable for many biological and biochemical applications. An improvement was proposed for enhancing the sensitivity of Biuret-based determinations in diluted protein solutions, though the method was applied to biological materials such as meat and cereal flours. The method proved to be robust and yielded consistent results. This improved variant can be used to analyse a wide range of biological samples.

The improved Biuret method, which uses insoluble copper phosphate powder, offers numerous advantages, particularly when applied to protein determination in plant-based materials. The Biuret supernatant can be read with a standard spectrophotometer or colorimeter at 545 nm, or at 560 nm when using microplates. Additionally, a portion of the supernatant can be recovered and used for other determinations, without the need to re-weigh the analysed material. Moreover, using the same supernatant is beneficial for reducing reporting errors related to the amino acid content or other components relative to the total protein content.

Specific volumes from the same supernatant (obtained after extraction and centrifugation) were pipetted and treated with reagents specific to determine two essential amino acids, tryptophan and lysine (Drochioiu, G., Mihalcea, E., et al., 2024).

Tryptophan reacts with 0.1 M glyoxylic acid in 7 N sulphuric acid and 1.8 mM ferric chloride, with a reaction time of 30 minutes (Nurit, E. et al., 2009), while lysine was treated with a ninhydrin-based reagent. Calibration curves were plotted at the wavelengths at which

the coloured compounds resulting from the amino acid reactions showed maximum absorbance. The absorbance values were compared with concentrations determined using standardised methods. In this case, only 50 mg of ground seed flour per sample was required to quantify three different components (total protein and two amino acids).

Another advantage of the proposed method is the low interference of copper ions in the ultraviolet region, where the Biuret complex exhibits significant absorbance of electromagnetic radiation. Both the test sample and the blank can be appropriately diluted with Biuret reagent or alkaline solutions and read at 220–230 nm. In this way, the reaction can be carried out with microlitre-scale volumes, and the method's sensitivity allows readings of 0.7–1.0 mL volumes in quartz cuvettes in the UV range.

II.2.9. Interference from Proteinogenic Amino Acids

All determinations were performed at a concentration of 2 mg/mL of either protein or amino acid (Figure II.2.17). During this research, it was observed that gelatine does not exhibit UV absorbance, yet the Biuret reaction yields a relatively high absorbance value in the visible region. It was also noted that proteins exhibit Biuret absorption maxima at slightly different wavelengths, highlighting the necessity of constructing individual calibration curves for each protein. This phenomenon may be explained by the amino acid composition of each protein. For example, BSA contains amino acid residues in its molecule that are capable of binding copper ions more strongly, potentially shifting the absorbance maximum to longer wavelengths.

Furthermore, while tryptophan showed weak absorbance at the wavelength characteristic of the Biuret complex, serine suggested strong interference when present at the same concentration as proteins. This behaviour is particularly important in protein determinations from immature plant seeds or hydrolysed materials, where free amino acid content is high. These findings led to a focused investigation of the role of proteinogenic amino acids in protein determination using the new Biuret method, which is based on the mobilisation of copper ions from insoluble copper phosphate powders.

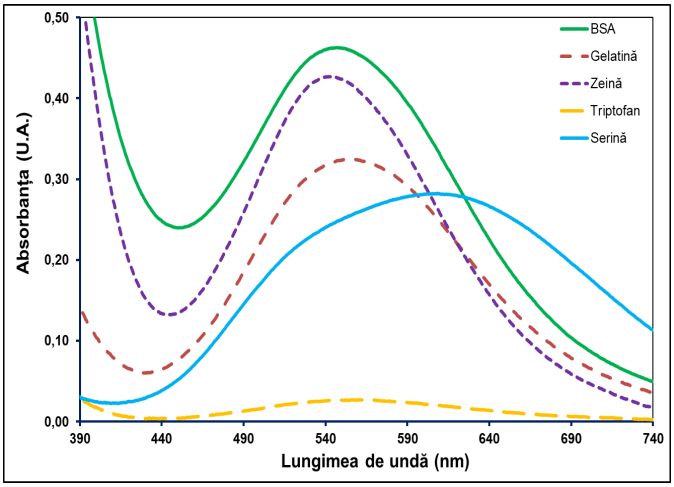

Figure II.2.17. *Absorption Spectra of Selected Proteins and Amino Acids Treated with Alkaline–Alcoholic Solution in the Presence of Insoluble Copper Phosphate. Concentrations: 2 mg/mL in Aqueous Solution, Except for Zein (2 mg/mL in 70% Alcohol) (Mihalcea et al., 2022).*

The interference of two amino acids was investigated: tryptophan, which showed a maximum absorbance at 556.5 nm, and serine, at 605.5 nm. The peak absorbance values of these complexes were also significantly different (tryptophan: 0.027 AU; serine: 0.282 AU). As such, the Biuret absorbance in the determination of BSA would increase from 0.463 AU to 0.490 AU (+5.83%) in the presence of tryptophan but would rise to 0.711 AU (+53.56%) due to the presence of serine.

In this doctoral thesis, the Biuret absorbance of proteinogenic amino acids was investigated both at their respective absorbance maxima and at 545 nm, the wavelength at which the protein Biuret complex absorbs (Figure II.2.18). For this purpose, both BSA protein and the individual amino acids were measured at a concentration of 2 mg/mL.

The results revealed that some amino acids cause major interference, while others show minimal interference in protein analysis using the Biuret method. These findings have already been submitted for publication in *Revue Roumaine de Chimie* (Mihalcea, E. & Drochioiu, G., 2025).

Individual amino acids extract copper ions from insoluble copper phosphate to form complexes with different absorbance maxima. As shown in **Figure II.2.18**, the highest absorbance was observed for histidine, followed by serine and glycine, despite their differing absorbance maxima (Mihalcea et al., 2025). At the opposite end of the scale was arginine, which, despite containing four nitrogen atoms capable of binding copper ions, showed weak interaction—along with tryptophan and leucine, which exhibited the lowest interference.

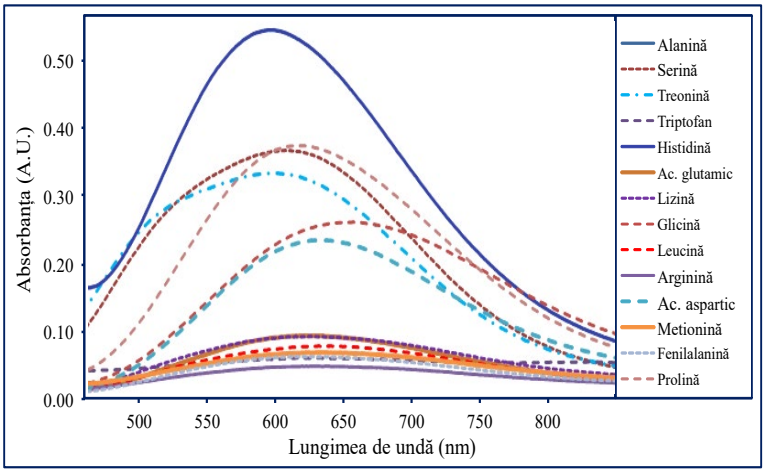

Figure II.2.18. *Absorption Spectra of Proteinogenic Amino Acids Subjected to the Biuret Reaction (2 mg/mL Amino Acids)*

From Figure II.2.18, it can be observed that only a few amino acids exhibit weak interference, while most show significant interference if present at high concentrations. Therefore, it is advisable to purify proteins before performing the Biuret reaction, particularly if they are suspected to be accompanied by free amino acids. It is also evident that the binding of copper ions is the source of the intense absorbance displayed by amino acids in alkaline medium.

The micro-Kjeldahl method is accurate, and its precision depends on the fineness of the sample grinding and the quantity of plant material used. However, the method has low throughput and relatively high reagent and energy consumption, making it more difficult to apply in plant breeding research. Nevertheless, its micro-variants are still used due to their accuracy and low material requirements, which are especially important during the early stages of breeding experiments. Protein concentrations can be determined simply by reading the Biuret absorbance of a sample at 540 nm, or at 750 nm in the Lowry method, and at 595 nm in the Bradford assay (Martina & Vojtech, 2015).

The sensitivity of the classical method for determining total serum protein is indeed low, with a detection limit generally between 0.2–1.7 g/L. However, this is sufficient for estimating total serum protein, which normally ranges between 60–80 g/L. In the classical Biuret method, the reaction involves cupric ions binding to peptide bonds, forming a tartrate-stabilised complex that absorbs at 540–550 nm (Sapan & Lundblad, 2015). The authors of the cited study explained that proteins and other polypeptide compounds extract Cu^{2+} ions from their complexes with tartrates. What this doctoral thesis demonstrates is that, because proteins form highly stable Biuret-type complexes, they are also capable of extracting copper ions from insoluble copper phosphate (CuPi).

Applications of the New Method - Following the determination of proteins using the proposed Biuret variant, tryptophan was also quantified in corn samples to identify high-quality maize varieties for use in plant breeding research (Drochioiu, Mihalcea et al., 2024). Maize quality is defined not only by its nutritional value and biochemical grain composition, but also by the protein content and quality of the kernel. High-quality maize varieties contain a higher percentage of crude protein and a balanced amino acid profile, in which essential amino acids, especially lysine and tryptophan, are present in increased proportions. To improve the biological quality of maize, opaque-2 mutant genes were introduced—these result in the formation of kernels with an opaque appearance. These genes contribute to a higher content of lysine and tryptophan, which are generally deficient in normal cereals, while simultaneously reducing prolamin content, such as zeins. As a result, corn proteins are more efficiently digested by monogastric animals. There is a positive correlation between the quality of maize and the quality of meat from animals fed with it. High-quality, protein- and amino acid-rich maize is also a valuable food source for human consumption, helping to prevent deficiencies such as pellagra and other conditions associated with diets based on low-quality maize.

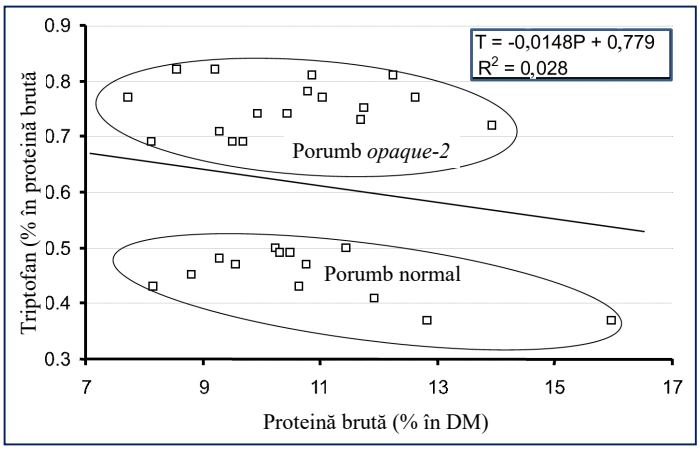

Figure II.2.19. *Correlation Between Tryptophan Content (% in Protein) and Total Protein Content (% in DM, Dry Matter) in Selected Samples of Normal and Opaque-2 Maize (Drochioiu, Mihalcea et al., 2023)*

Since the lysine content in maize seeds is approximately four times higher than that of tryptophan, it is sufficient to use only the tryptophan content in maize seeds to evaluate their nutritional quality (Nurit, 2009). Accordingly, Figure II.2.19 shows a clear separation between the tryptophan content of opaque-2 maize samples (ranging from 0.70–0.85% in crude protein) and that of normal maize (0.30–0.50%). This allows for the differentiation of mutant varieties from normal ones without the need for costly genetic analyses.

Specific experiments were carried out to improve a method that proved to be selective, rapid, and inexpensive, and applicable to a wide range of biological materials. Compared to the methods, the Biuret method offers the advantages of easy operation, excellent accuracy, and lower interference. The principle of this method lies in the ability of peptide bonds, formed at the junction between two adjacent amino acid residues, to bind with two copper ions (Cu^{2+}) under alkaline conditions, forming a violet–purple complex. The intensity of the colour is proportional to the protein content of the sample.

II.3. DETERMINATION OF PEPTIDES

II.3.5. Determination of Tryptophan-Containing Peptides

Spectrophotometric measurements were performed on tryptophan solutions (Figure II.3.8), presenting spectra for tryptophan concentrations in the range of 1–10 µg/mL. These are very low concentrations, enabling sensitive measurements of this amino acid in solution. At the same time, peptides containing tryptophan can also be easily determined in the UV region, as the method is particularly sensitive. During HPLC separation of these peptides, the usefulness of tryptophan is evident in monitoring absorbance at 280 nm.

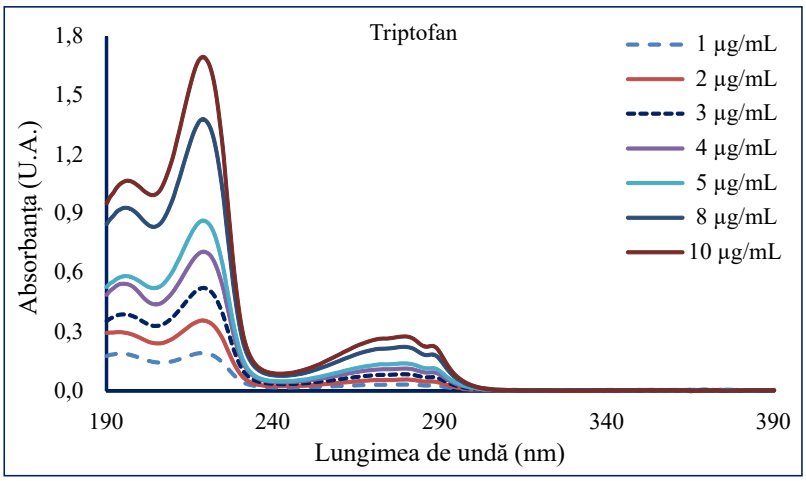

Figure II.3.8. *UV Spectra of Aqueous Tryptophan Solutions*

The calibration curves were plotted using the data presented in **Table II.3.1**. From this table, it is evident that tryptophan exhibits maximum absorbance in the ultraviolet region at 219 nm (1.697 AU). Although tryptophan also absorbs strongly and specifically at 280 nm, it can be observed that the absorbance at this wavelength is six times lower than at λ_{max} = 219 nm (1,697/0,277 = 6,12).

Table II.3.1. *Absorbance Values of Tryptophan at Different Wavelengths and Concentrations in Aqueous Solution*

Wavelength (nm)	**Absorbance (AU)**						
Concentration(µg/mL))	*1,0*	*2,0*	*3,0*	*4,0*	*5,0*	*8,0*	*10,0*
196	0,187	0,295	0,389	0,543	0,583	0,931	1,068
205	0,145	0,242	0,331	0,440	0,524	0,838	1,002
214	0,174	0,318	0,458	0,617	0,754	1,206	1,482
219	0,193	0,358	0,524	0,708	0,865	1,381	1,697
220	0,191	0,354	0,520	0,703	0,858	1,368	1,681
280	0,033	0,058	0,085	0,114	0,140	0,224	0,277

To determine peptides that contain a tryptophan residue in their primary structure, the dipeptide glycyl-tryptophan (denoted Gly-Trp or GW) was selected for study, as it is simple and easy to analyse, contains glycine (which does not absorb at 280 nm), and was readily available or easy to synthesise. This peptide, containing only a single double bond (within the peptide group), is not capable of producing a response in the Biuret reaction. However, it represents an ideal candidate for spectrophotometric and fluorometric studies due to the presence of tryptophan in its structure. Additionally, this peptide has been used in various research projects within the biochemistry laboratory (Ciobanu et al., 2015).

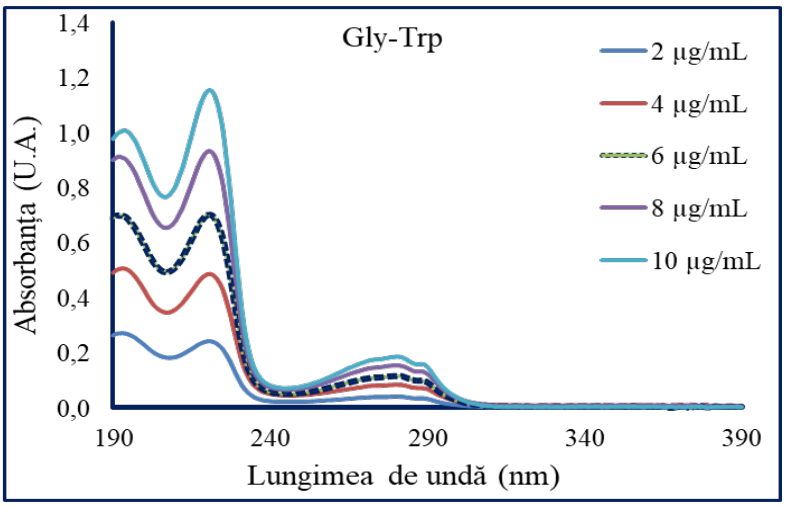

Figure II.3.10. *UV Spectra of Aqueous Solutions of Glycyl-Tryptophan*

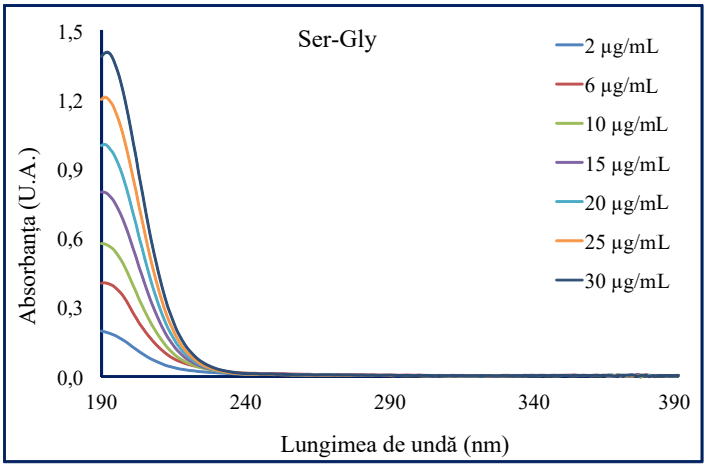

Figure II.3.11. *UV Spectra of Aqueous Solutions of the Peptide Seryl-Glycine (Ser-Gly, SG)*

From the figure above, it is evident that the seryl-glycine peptide exhibits strong absorbance in the far-ultraviolet region, at wavelengths below 205 nm, and much weaker absorbance above 220 nm. It is worth noting that much higher concentrations were used compared to those for the GW peptide (Figure II.3.10). These findings suggest the potential for erroneous measurements if such a peptide is separated using an HPLC instrument and quantified at 220 nm. Table II.3.2 supports this conclusion, as the presented data show that the maximum absorbance occurred at 192 nm.

Table II.3.2. *Absorbance Values of the Seryl-Glycine (SG) Peptide at Different Wavelengths and Concentrations*

Wavelength (nm)	**Absorbance (AU)**						
Concentration (µg/mL)	*2,0*	*6,0*	*10,0*	*15,0*	*20,0*	*25,0*	*30,0*
192	0,191	0,404	0,570	0,792	1,000	1,206	1,406
205	0,093	0,197	0,283	0,402	0,512	0,624	0,739
220	0,027	0,053	0,059	0,073	0,087	0,100	0,120

Using the values from Table II.3.2, calibration curves were plotted, which can be used for quantitative determination of this peptide in its solutions (Figure II.3.12). The very high correlation coefficients confirmed the feasibility of sensitive detection of this peptide, particularly at 192 nm, although it can also be reliably evaluated at 205 nm. The calibration curves were linear across the entire concentration range of 2–30 µg/mL.

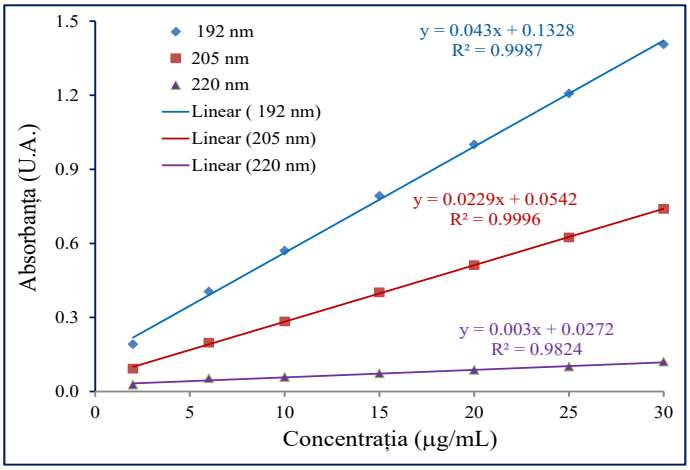

Figure II.3.12. *Calibration Curves for the Seryl-Glycine Peptide in the Concentration Range of 2.0–30.0 µg/mL at Different Ultraviolet Wavelengths*

The significance of this approach lies in the fact that it enables the easy determination of very low concentrations and quantities of peptides that do not absorb at higher wavelengths, such as λ = 280 nm. In this way, their concentration in diluted solutions can be accurately determined before being used in other experiments.

II.4. UV SPECTROPHOTOMETRIC STUDIES

Spectrophotometric methods for analysing total protein content are generally simple, rapid, and sensitive. Such sensitive protein assays may have applications in forensic science, the detection of protein contaminants in pharmaceuticals, and a range of other research-relevant fields.

II.4.1. Far-UV Spectrophotometric Studies

To improve protein determination methodology, spectrophotometric measurements in the UV range were performed to develop extremely sensitive procedures. The results indicated that high-level purification of proteins and peptides is essential prior to measurement to ensure accurate concentration values.

Therefore, the experiment was repeated using higher concentrations of BSA, specifically in the range of 0.005–0.100 mg/mL, to better capture the differences between A280 and A190 values (Figure II.4.2). The high sensitivity of protein determination in the far-UV region resulted in absorbance values above 1.0 AU for BSA. The results clearly demonstrate the exceptional sensitivity of measurements near 190 nm, allowing detection of

BSA concentrations below 0.01 mg/mL. Figure II.4.2 also shows that as protein concentration increases, the absorption maximum shifts toward longer wavelengths. For example: at 0.005 mg/mL BSA, the absorbance maximum occurred at 190 nm (0.369 AU); at 0.02 mg/mL, it shifted to 192 nm (1.383 AU); at 0.1 mg/mL, it further shifted to 200 nm (2.605 AU). Since protein determination is commonly performed at 205 nm, 214 nm, and 220 nm—the latter often used with HPLC instruments—the absorbance of BSA at these wavelengths was also investigated.

First, BSA absorbance at 280 nm was compared with that around 190 nm. While 0.1 mg/mL BSA yielded A280 = 0.069, the absorbance at 200 nm was A200 = 2.605 AU, which is approximately 37.75 times higher (2.605 / 0.069 = 37.75). **Table II.4.1** presents the BSA absorbance values at various wavelengths.

Table II.4.1. *BSA Absorbance Values in the Ultraviolet Range at Different Wavelengths*

Wavelength (nm)	**Absorbance (AU)**			
	0,005 mg/mL	0,01 mg/mL	0,02 mg/mL	0,1 mg/mL
205 nm	0,151	0,296	0,631	2,468
214 nm	0,081	0,153	0,321	1,479
220 nm	0,062	0,111	0,233	1,078
280 nm	0,071	0,011	0,015	0,069

II.4.2. Protein Absorbance at 280 nm

As several proteins were studied to improve the methodology for protein determination using the Biuret method, their UV-Vis absorption spectra were recorded, focusing only on the representative wavelength range of 250–400/450 nm (Figure II.4.3). Significant differences in the UV absorbance values between 250–300 nm for these proteins led to the conclusion that only some of them can be reliably determined using this method.

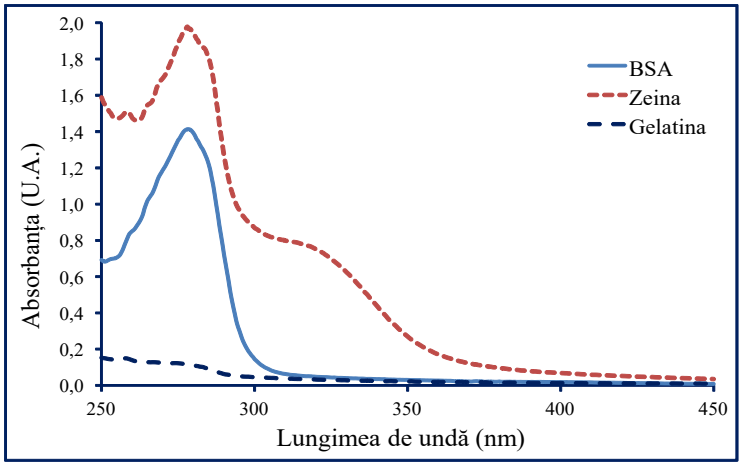

Figure II.4.3. *UV-Vis Spectra of BSA, Zein, and Gelatin Proteins (Concentration: 2 mg/mL)*

From the analysis of the three ultraviolet absorption spectra, it can be observed that zein exhibits a maximum absorbance at 278 nm (1.977 AU), which is significantly higher than that of BSA (278.5 nm: 1.413 AU). This difference can lead to errors in concentration determination. Moreover, the absorbance of accompanying coloured compounds is not known, making it difficult to subtract their contribution from the total absorbance value at 274 nm or 278 nm.

II.4.4. Determination of Proteins in the UV Range Using the Biuret Reaction

In this experiment, aqueous solutions of BSA with concentrations ranging from 0 to 0.1 mg/mL were used. To 1 mL of BSA solution, approximately 50 mg of copper phosphate powder and 0.1 mL of 2% KOH solution were added. The Eppendorf tubes containing the reaction mixture were sonicated for 15 minutes, followed by centrifugation at only 5000 rpm. UV-Vis spectra were recorded in the wavelength range 190–690 nm, using quartz cuvettes with a 1 cm path length (Figure II.4.4). The blank sample (control), prepared using only the reagents, showed intense absorption in the 190–220 nm range, and subtracting this spectrum from that of the BSA solutions produced fringes in this region. Therefore, only the range 220–570 nm was analysed to capture both the ultraviolet absorbance of the biuret complex and its visible absorbance around 540–550 nm. A strong absorbance of the biuret complex at 229 nm was observed — a result not previously reported in the literature. Moreover, the absorption bands were particularly narrow, especially at higher BSA concentrations. The sensitivity of detection at 229 nm was remarkably high: for instance, at a concentration of 0.1 mg/mL, an absorbance of 2.819 AU was measured. Modern spectrophotometers allow for accurate measurement of absorbance values greater than 1.0

AU, as supported by various recent scientific studies (Chen et al., 2009; Babakhanova et al., 2022).

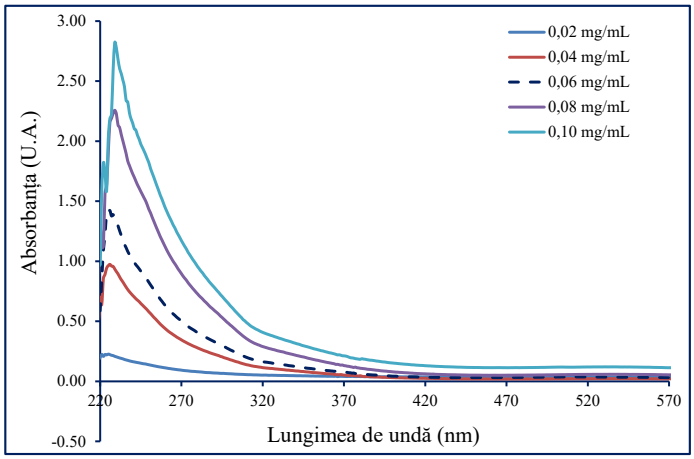

Figure II.4.4. *UV-Vis Spectra of the BSA Biuret Complex at Different Protein Concentrations*

The molecular weight of BSA is 66,463 Da (approximately 66.5 kDa), and its molar extinction coefficient is 43,824 $M^{-1}cm^{-1}$ at 279 nm. Thus, the molar absorption coefficient of BSA in the biuret reaction, with absorbance read at 229 nm, was calculated to be 1,873,591.97 $M^{-1}cm^{-1}$. This value is 42.75 times higher than the native extinction coefficient of BSA at 279 nm. The proof of the existence of the biuret complex lies in its absorption at 540 nm. The same complex also shows strong absorption at 229 nm (Figure II.4.5). Although the absorbance values of the biuret complex at 545 nm were low (C = 0.04 mg/mL, A_{545} = 0.023 AU; C = 0.08 mg/mL, A_{545} = 0.058 AU), the shape of the spectrum and the presence of a distinct absorption band at 545 nm confirmed the formation of the biuret complex, even at low concentrations.

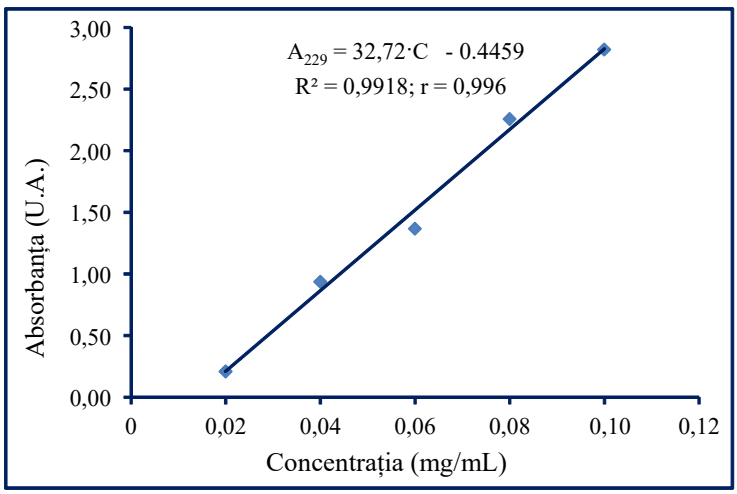

Figure II.4.5. *Calibration Curve in the Ultraviolet at 229 nm, Obtained with BSA at Concentrations of 0.02–0.10 mg/mL*

The increase in the sensitivity of the biuret method was studied by measuring absorbance values at 229 nm instead of 545 nm, and a significant increase in biuret absorbance at 229 nm was observed. The ratio between the absorbance values of the biuret complex at the two BSA concentrations (0.04 mg/mL and 0.08 mg/mL, respectively) showed the following results: 0.935/0.023 = 40.65 and 2.257/0.058 = 38.9 (as shown in the spectra in Figure II.4.6). Therefore, measuring absorbance values at 229 nm is approximately 40 times more sensitive than absorbance measurement at 545 nm.

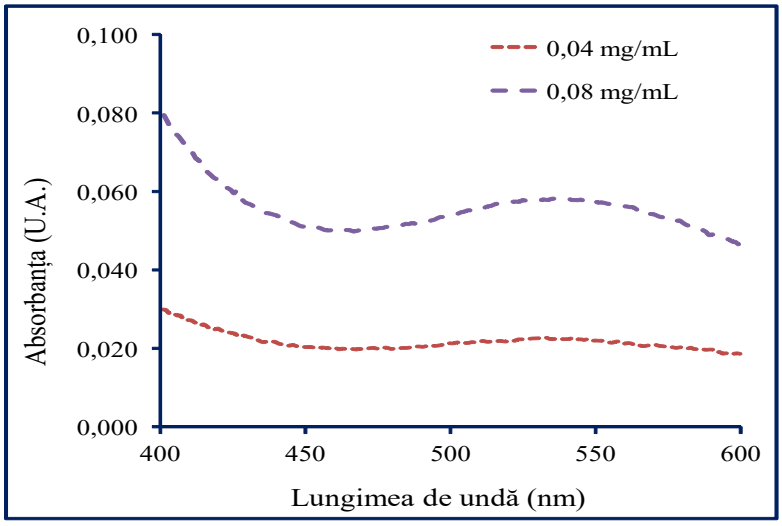

Figure II.4.6. *Visible spectra of BSA biuret solutions at concentrations of 0.04 mg/mL and 0.08 mg/mL BSA, respectively.*

II.5. RESULTS OBTAINED. DETERMINATION OF PROTEINS WITH DIAZOTIZED SULFANILIC ACID

Sulfanilic acid contains an amino functional group that can be diazotized not only at 0–5 °C but even at room temperature. The coupling of the diazonium salt of sulfanilic acid with two amino acids—histidine and tyrosine—present in protein and peptide molecules, leads to the formation of yellow-red azo derivatives, which can be quantified calorimetrically.

II.5.1. Reaction of Tyrosine with Diazotized Sulfanilic Acid

The phenolic ring of tyrosine reacts with the diazonium salt, generating a coloured azo compound. The absorption of this compound was studied spectrophotometrically in the UV-Vis domain. Because tyrosine undergoes a coupling reaction with diazotized sulfanilic acid, it is possible to quantify tyrosine in its solutions. This reaction results in the formation

of a coloured azo compound, and the absorbance values of this compound are directly proportional to the tyrosine concentration.

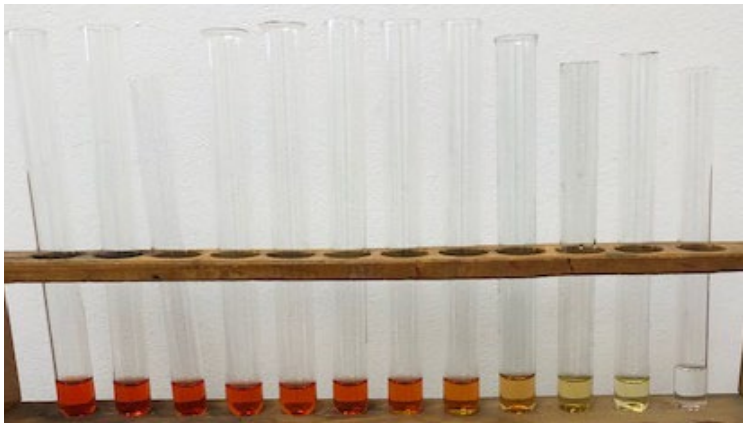

Figure II.5.2. *The Pauly Reaction of Tyrosine (Concentrations 0–300 µg/mL) with Diazotized Sulfanilic Acid.*

To become a reliable method, for example, one for determining tyrosine and peptides containing this amino acid, it is necessary to study the interference from other substances such as phenols and other compounds that may interfere in the Pauly reaction. Therefore, in this study, only the possibility of spectrophotometrically determining compounds containing tyrosine in their molecular structure—such as zeins, the alcohol-soluble proteins of corn—was investigated.

II.5.2. Reaction of Histidine with Diazotized Sulfanilic Acid

Histidine is an amino acid containing an imidazole ring, with the molecular formula $C_4H_9N_3O_2$ and a molar mass of M = 155.15 g/mol. The aromatic ring has a basic character that allows it to couple with a diazonium salt. To carry out the reaction of histidine with the diazonium salt of sulfanilic acid, three solutions were prepared as follows: solution 1: 350 µg/mL sulfanilic acid acidified with 1 mL of 37% HCl, made up to 100 mL; solution **2**: 300 µg/mL sodium nitrite ($NaNO_2$); solution 3: 300 µg/mL histidine in 8% sodium carbonate (Na_2CO_3). The sulfanilic acid dissolved easily in the presence of hydrochloric acid. To obtain the diazonium salt at room temperature, 1 mL of Solution 2 was added dropwise to 1 mL of Solution 1, followed by mixing. In a series of test tubes, 1 mL of Solution 3 (with histidine concentrations ranging from 0 to 300 µg/mL) was pipetted, followed by 1 mL of the freshly prepared diazonium salt. During the addition of the diazonium salt to the histidine solution, a light red solution was observed, indicating the formation of the azo derivative.

Table II.5.1. *Procedure for preparing the mixtures used to determine the colourimetric (spectral) response at different concentrations of tyrosine*

Test tube	1	2	3	4	5	6	7	8	9	10	11
Na_2CO_3 8%, mL	1	0,9	0,8	0,7	0,6	0,5	0,4	0,3	0,2	0,1	0,0
Tyrosine, mL	0	0,1	0,2	0,3	0,4	0,5	0,6	0,7	0,8	0,9	1,0
Diazonium salt	1 mL										

Thus, the formation of a red coloration was observed, with intensities proportional to the concentrations of the histidine solutions (Figure II.5.6). The spectra were then recorded both against water and against the blank sample.

Figure II.5.6. *Pauly reaction of histidine (concentrations 0–300 µg/mL) with diazotized sulfanilic acid.*

II.5.3. Determination of Proteins with Diazotized Sulfanilic Acid

Proteins that contain tyrosine and/or histidine react with diazotized sulfanilic acid to form calorimetrically detectable solutions. In a first stage, the UV-Vis spectra of proteins were recorded, using BSA as a reference. Naturally, in the spectral range below 240 nm, the blank sample exhibited strong interference. However, by using computer software, these errors can be corrected, and the spectra become significantly clearer. The maximum absorption of the formed azo dyes, relative to the blank, was observed in the wavelength range of 275–350 nm, i.e., in the ultraviolet domain (**Figure II.5.7**).

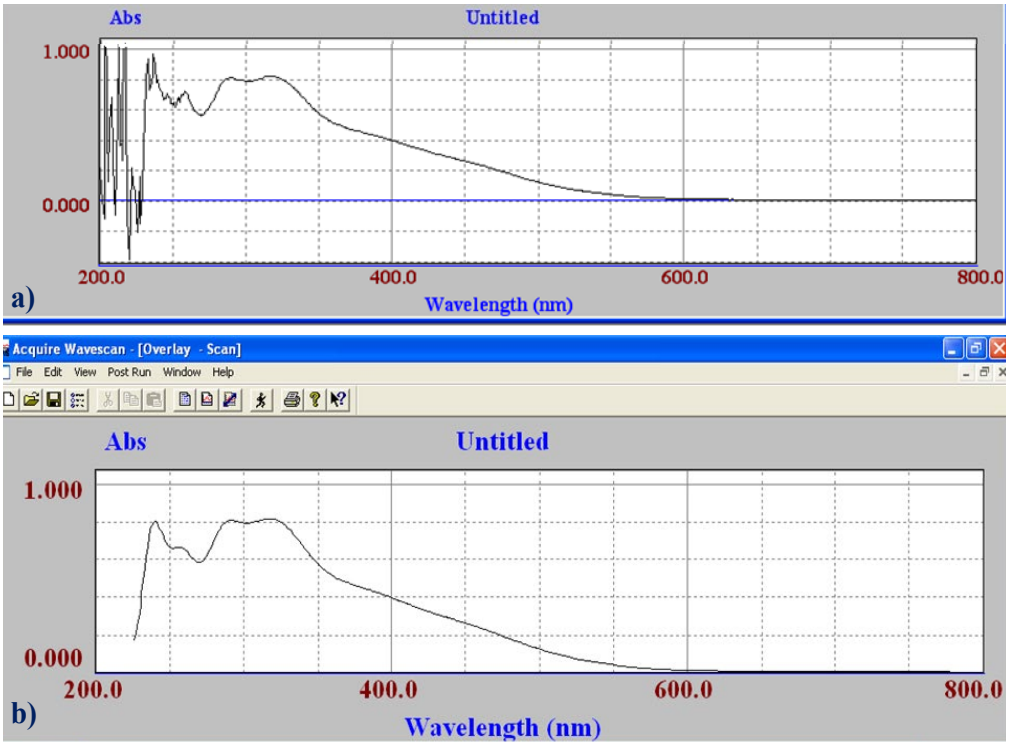

Figure II.5.7. *UV-Vis spectrum of BSA treated with the diazonium salt of sulfanilic acid:* ***a)*** *original spectrum showing errors caused by strong absorption of the blank sample in the far UV region;* ***b)*** *spectrum electronically processed by the spectrophotometer software, which automatically eliminates errors and improves the quality of the measurements.*

The absorption spectrum of BSA treated with diazonium salt solutions of sulfanilic acid showed that it is possible to measure protein concentration in the ultraviolet region at 365 nm with greater sensitivity than in the visible region (**Figure II.5.9**).

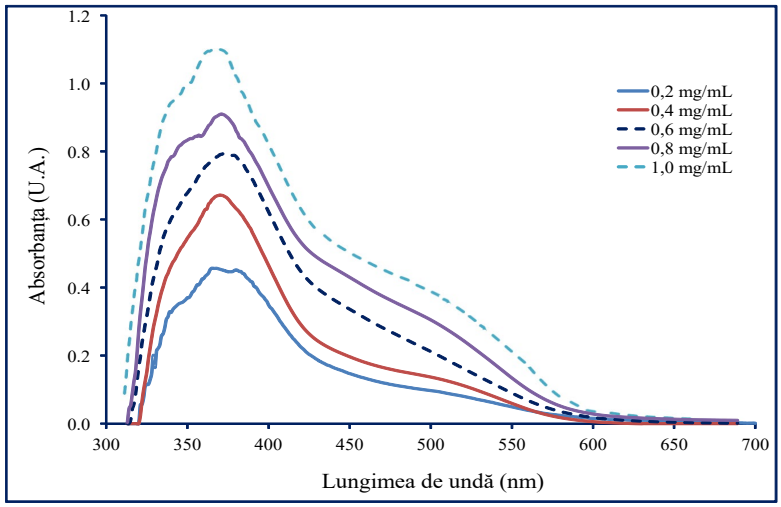

Figure II.5.9. *Absorption spectra of BSA treated with diazonium salt solutions of sulfanilic acid, with protein concentrations ranging from 0.2 to 1.0 mg/mL.*

Therefore, a calibration curve was constructed with BSA in the concentration range of 0.2–1.0 mg/mL at 365 nm (Figure II.5.10). The dependence of absorbance on concentration was linear within the 0.2–1.0 mg/mL BSA range, and the regression equation was $A_{365} = 0.6812 \cdot C + 0.3746$ ($R^2 = 0.9892$; $r = 0.994$), where A_{365} represents the absorbance at 365 nm, and **C** is the BSA concentration expressed in mg/mL.

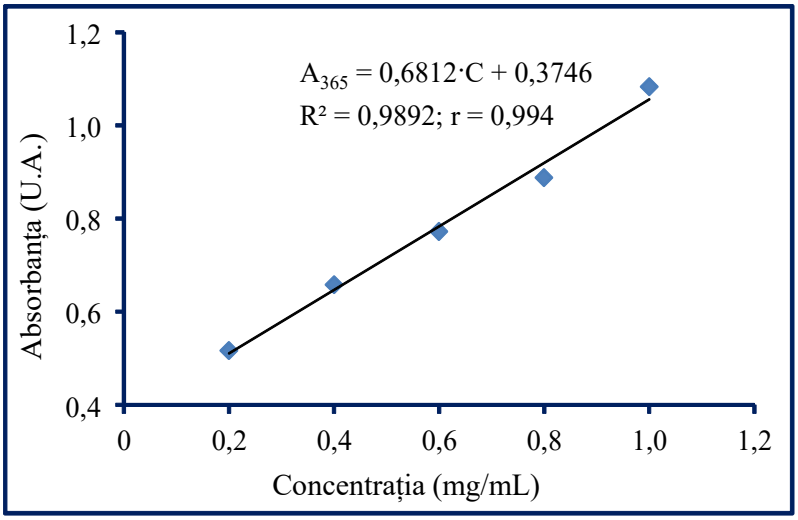

Figure II.5.10. *Calibration curve for protein determination using diazotized sulfanilic acid.*

II.6. FLUORIMETRY

Fluorimetric analytical methods offer the advantage of high sensitivity and selectivity, allowing the detection of compounds even at concentrations of 1–10 µg/mL. Initially, the emission spectra were recorded by fluorometric method for the protected peptide *N-CBZ-S-benzyl-L-cysteinyl-L-tyrosine (BCT)* and the amino acid *L-tyrosine*. To highlight the impact of iron ions on the peptide's fluorescence, spectral differences were measured between fluorescence readings performed in the presence and absence of these ions. A quenching of fluorescence was thus observed at the emission wavelength of 303 nm.

II.6.1. 3D Fluorescence Studies

Similarly, an 18 µM solution of the peptide *N-CBZ-S-benzyl-L-cysteinyl-L-tyrosine* was prepared as follows: a volume of 96 µL of peptide solution at a concentration of 0.187 mM, 4 µL distilled water, and 900 µL sodium acetate buffer at pH 7 were transferred into a quartz cuvette.

The fluorescence spectrum (Figure II.6.4) was then recorded in the excitation range of 250–490 nm and emission range of 260–500 nm, using as a reference sample a mixture of 100 µL distilled water and 900 µL sodium acetate buffer at pH 7. From the 3D spectrum, a single emission maximum was observed at the spectral pair λ_{ex} / λ_{em} 275 nm / 301 nm.

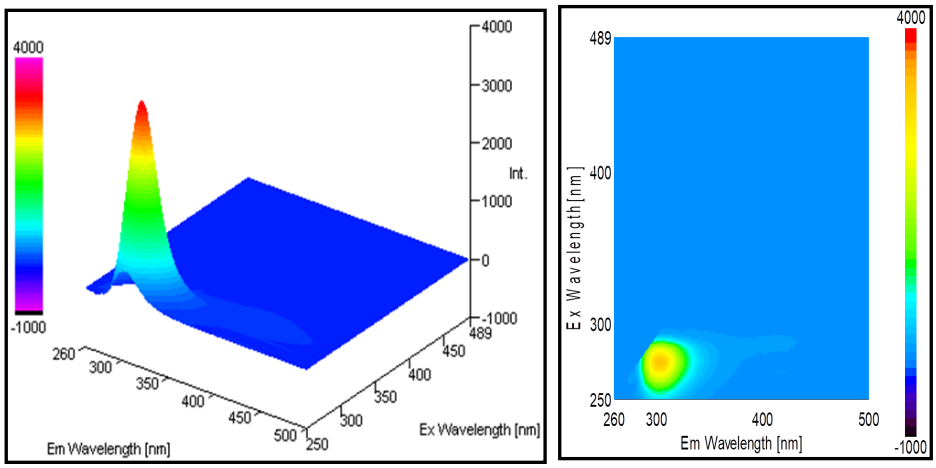

Figure II.6.4. *3D emission spectrum (left) of the peptide N-CBZ-S-benzyl-L-cysteinyl-L-tyrosine at a concentration of 18 µM and the projection spectrum (right) in 45 mM acetate buffer solution at pH 7.0.*

Similarly, the 3D spectrum of tyrosine was recorded using a 7 µM solution in acetate buffer at pH 7, within the same excitation and emission spectral range.

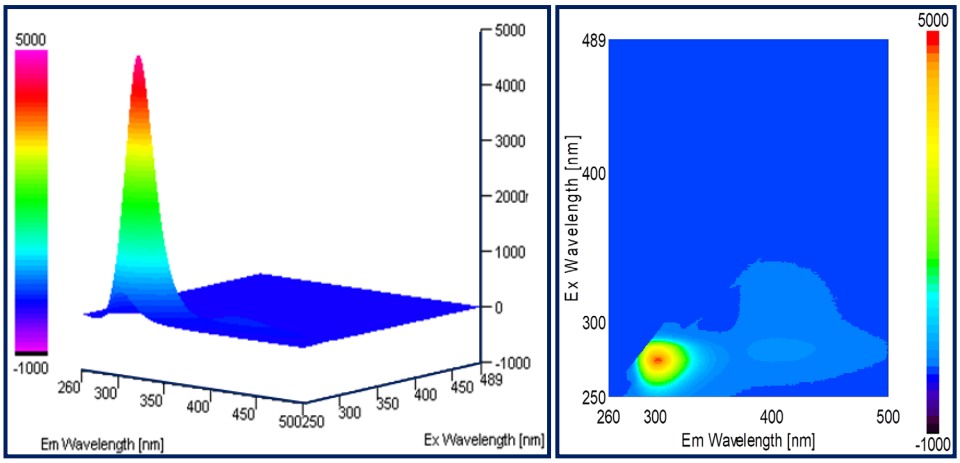

Figure II.6.5. *3D emission spectrum (left) of L-tyrosine at a concentration of 7 µM and the projection spectrum (right) in 45 mM acetate buffer solution, pH 7.0.*

II.6.2. Effect of Copper Ions

Fluorescence measurements were carried out using the peptide *N-CBZ-S-benzyl-L-cysteinyl-L-tyrosine* dissolved in acetate buffer, upon addition of Cu^{2+} ions. For this determination, a diluted peptide solution (4.5 µM) was used, and the acquisition parameters were adjusted so that excitation occurred in the 200–370 nm range, while emission was

recorded between 210 and 380 nm. The reference sample remained unchanged. The diluted peptide solution was prepared as follows: 24 µL of peptide at a concentration of 0.187 mM, 76 µL of distilled water, and 900 µL of 50 mM sodium acetate buffer at pH 7.0. **Figure II.6.6.** 3D fluorescence emission spectrum of the appropriately diluted peptide *N-CBZ-S-benzyl-L-cysteinyl-L-tyrosine* in 50 mM acetate buffer, pH 7.0.

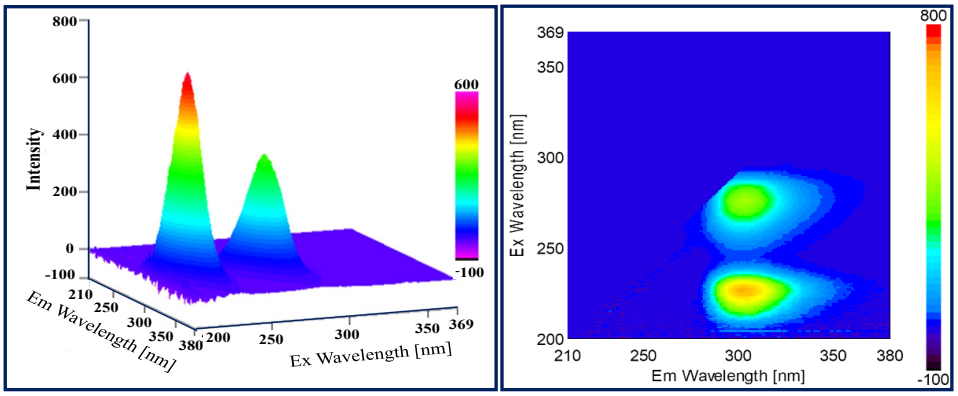

Figure II.6.6. *3D fluorescence emission spectrum of the peptide (4.5 µM, left) and its projection representation (right) in 45 mM sodium acetate buffer at pH 7.0.*

Upon the addition of copper ions, a slight change in fluorescente intensity was observed. The increase in fluorescence intensity may be attributed to the complexation of copper ions by the sulphur atom within the peptide, which would reduce intermolecular quenching of the fluorescence of the phenolic moiety (characteristic of tyrosine) by the sulphur atoms in adjacent peptide molecules (**Figure II.6.7**).

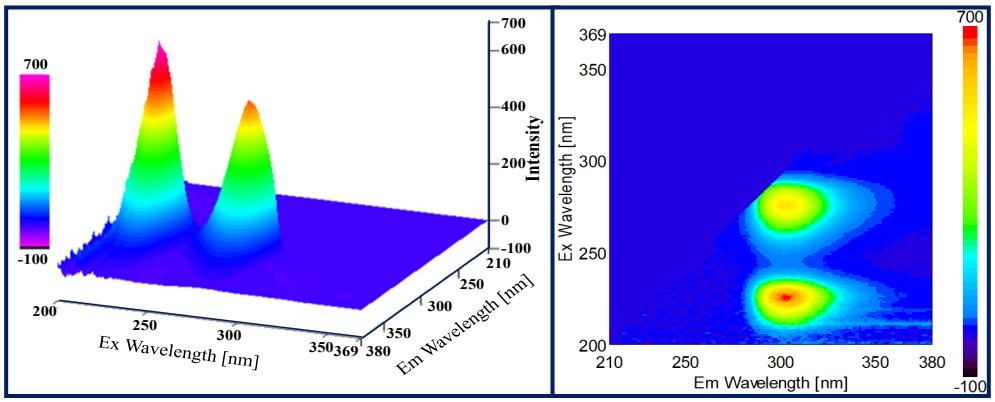

Figure II.6.7. *3D fluorescence emission spectrum (left) and projection spectrum (right) of the peptide N-CBZ-S-benzyl-L-cysteinyl-L-tyrosine (4.5 µM) following the addition of Cu^{2+} ions.*

The conclusion that can be drawn from these fluorescente measurements is that heavy metal ions, such as copper, influence the quenching of fluorescence

II.6.3. Comparative Fluorometric Determinations between Tyrosine and the Peptide

Fluorescence measurements were performed, recorded both as conventional emission spectra and as 3D fluorescence spectra. The excitation range was 250–490 nm, and the emission range was 260–500 nm. Measurements were carried out for both tyrosine and the peptide using the Jasco 8350 spectrofluorometer (Japan). The results are shown in **Figure II.6.8**. Although the concentration of tyrosine was higher (5.5 µM), the fluorescence intensity of the BCT peptide was significantly greater. This behaviour may be attributed to the presence of protective groups — carbobenzoxy (Cbz) and benzyl — within the peptide structure, which can enhance the fluorescence signal.

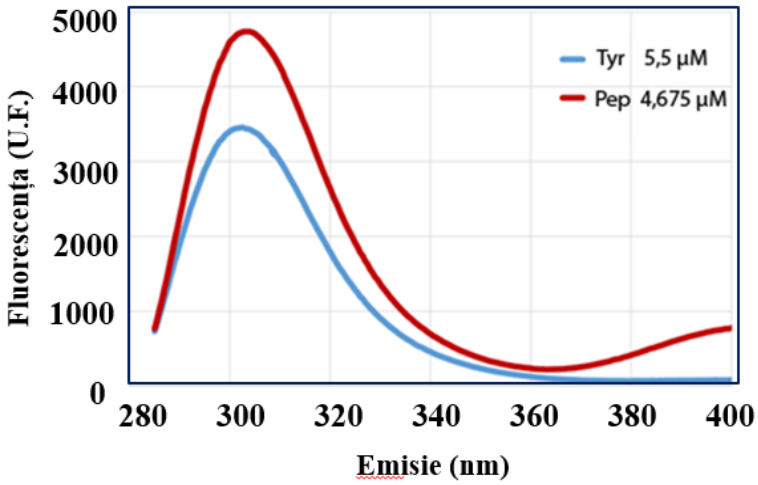

Figure II.6.8. *Emission spectra for tyrosine (5.5 µM, blue) and peptide (4.7 µM, red) in 45 mM acetate buffer, pH 7.0.*

II.6.4. Fluorimetry in the Study of Zeins

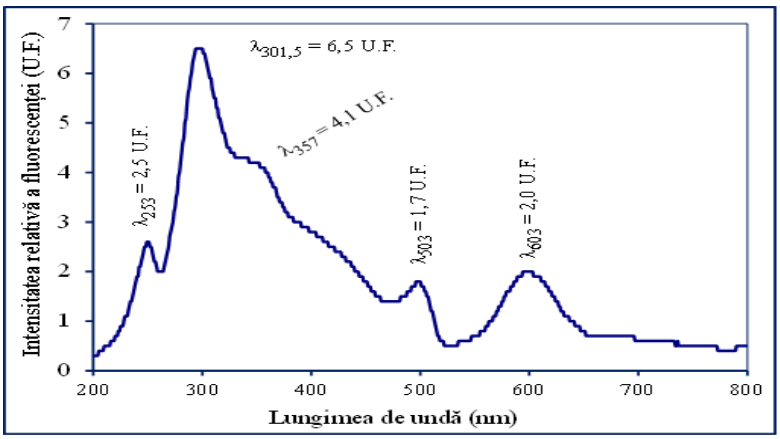

Figure II.6.9. *Fluorescence spectrum of a 70% alcoholic solution of zein (excitation at 250 nm, emission in the 200–800 nm range).*

Zeins are storage proteins derived from plant sources. Because zeins can be obtained in an almost pure state from alcoholic extracts, their fluorimetric behaviour was studied to enable the quantification of these proteins. For this purpose, spectra were recorded using different excitation wavelengths. Thus, using an excitation wavelength of 250 nm resulted in a weak fluorescence signal from zein, as expected, despite the presence of nine phenylalanine residues in the α-zein molecule (see Figure II.6.9). From this figure, several fluorescence bands can be observed, starting with a minor signal at 253 nm, likely caused by UV light scattering after absorption at 250 nm.

Despite its relatively low intensity, the most prominent fluorescence emission band was observed at 301 nm (6.5 fluorescence units). Additionally, a shoulder appeared at 357 nm with an intensity of 4.1 F.U. Two weaker emission signals were also observed at 503 nm and 603 nm, respectively. The latter signal can clearly and effectively be attributed to the fluorescence of zeins, likely due to phenylalanine residues, like the signal observed at 301.5 nm. This final emission band results from an electronic transition from a higher excited state to a lower excited state, followed by emission either directly to the ground state (301.5 nm) or via a two-step process, resulting in the emission of two light quanta at 603 nm. In **Figure II.6.11**, the fluorescence spectrum of the same zein solution is shown, this time following excitation at a wavelength of 280 nm.

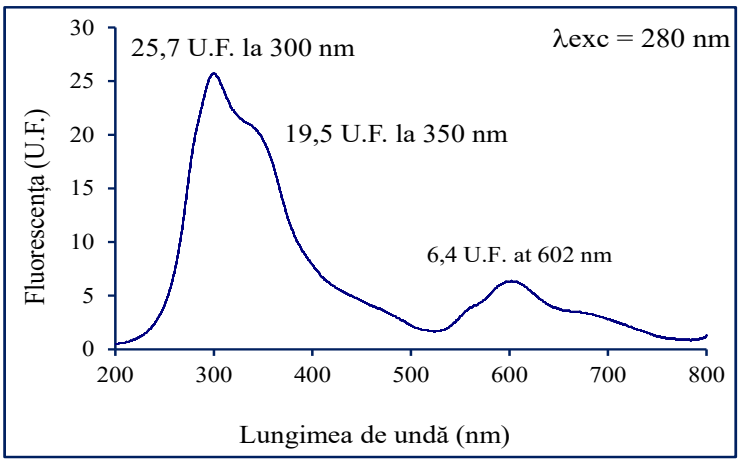

Figure II.6.11. *Fluorescence spectrum of a zein solution excited at 280 nm.*

In Figure II.6.12, the fluorescence spectrum of the zein solution excited at a wavelength of 260 nm is shown. The shoulder at λ263 = 8.3 RFU (Relative Fluorescence Units) may be characteristic of the scattering of the excitation radiation. Theoretically, tyrosine emission occurs at 301 nm. However, the emission maximum was observed at 296 nm (relative intensity of 21.1 RFU), while at 301 nm this intensity decreased to 96.2% of the maximum (20.3 RFU). Additionally, a specific shoulder was noted at 350 nm (10.3 RFU),

along with another one at 598 nm (5.8 RFU). This latter wavelength was expected to correspond approximately to double that of the main maximum.

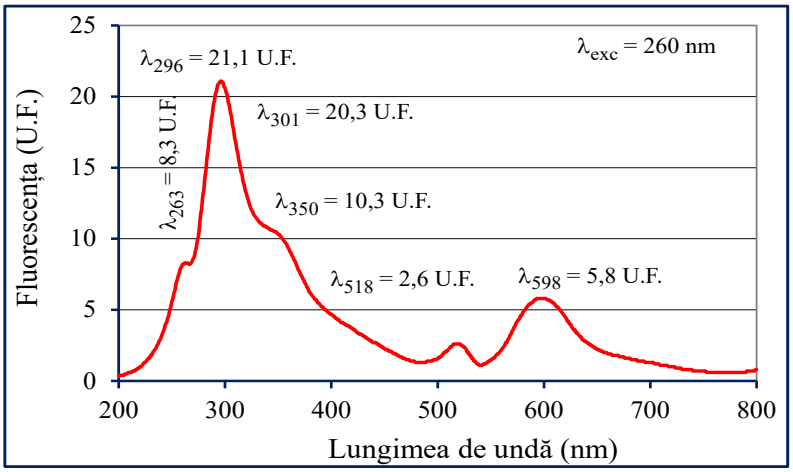

Figure II.6.12. *Fluorescence spectrum of a zein solution excited at 260 nm.*

Fluorimetric methods rely on the measurement of light emission by proteins and peptides following excitation with a specific wavelength. These methods are both sensitive and selective, providing valuable insights into the molecular structure and interactions of proteins and peptides, particularly in solution. Since zeins contain tyrosine residues within their molecular structure—and tyrosine could emit quanta of light at a wavelength around 300 nm—quantitative determination of these proteins can be achieved not only by using a single excitation wavelength (e.g., 274.2 nm), but also by employing multiple excitation wavelengths. This multi-wavelength approach helps to minimise potential interferences and improve the accuracy of the determination.

GENERAL CONCLUSIONS

In this doctoral thesis entitled *"Rapid and Sensitive Methods for the Determination of Proteins and Peptides"*, various methods for protein and peptide determination were studied and improved. The research aimed to enhance the methodology for protein and peptide analysis using bioanalytical techniques that are inexpensive, robust, rapid, and sensitive. New UV-Vis spectrophotometric and fluorometric techniques were proposed for the determination of proteins, peptides, and their constituent amino acids, in comparison with reference methods described in the scientific literature. The protein determination achieved through these new methods was compared with a standardised method—specifically, the

micro-Kjeldahl method, which measures the nitrogen content in the samples under analysis—or with values obtained using known spectrophotometric methods.

The research carried out during the doctoral studies also led to a better understanding of the interactions between copper ions and proteins, with the aim of identifying potential applications in the chemical analysis of proteins.

The study of rapid and sensitive methods for the determination of proteins and peptides yielded experimental conclusions relating to the development of new working approaches, with the research focusing on the following key areas:

1) Improving the Methodology of Protein Determination via the Biuret Method

The reaction conditions of proteins with copper ions were studied, and it was shown that replacing the reagent containing copper (II) sulphate and sodium-potassium tartrate significantly improved the stability, speed, and especially the sensitivity of determinations using the biuret method.

The formation of the biuret complex and its ultraviolet absorption were studied. It was demonstrated that the complex is stable only in alkaline medium, while it decomposes easily in acidic conditions and even upon dilution with water—diluting the solution containing the complex leads to its breakdown.

The interference of amino acids in the proposed biuret method was examined in the visible range. It was found that histidine causes strong interference, whereas other amino acids, such as tryptophan, showed only minor interference. It was also observed that amino acids form complexes with different absorption maxima, meaning that the interference caused by a given amino acid at 545 nm may differ from that at 560 nm.

2) Studying Protein Determination Using Their Reaction with the Diazotised Salt of Sulphanilic Acid

The Pauly reaction was studied using the diazonium salt of sulphanilic acid (SA) and the amino acids tyrosine and histidine. It was found that these amino acids form azo compounds that absorb both in the visible and ultraviolet spectral regions.

The Pauly reaction with proteins was also examined, and it was observed that the azo derivatives formed absorb significantly across a wide range of wavelengths, including the ultraviolet domain.

A method for protein determination based on the Pauly reaction was proposed, wherein calibration curves are constructed using the proteins of interest.

3) Determination of Proteins in the Ultraviolet Range

The ultraviolet absorption spectra of peptides and proteins revealed characteristic maxima corresponding to peptide bonds. Peptides and proteins containing aromatic amino acid residues in their molecules display a specific absorption maximum between 274–280 nm.

Methods for protein determination in the far-UV region were explored, and it was found that high purity is required to avoid errors caused by multiple interferences. Purification can be achieved using trichloroacetic acid (TCA), as recommended in the literature, and HPLC-based separations allow for such determinations.

4) Investigation of Peptide Determination Using Sensitive, Rapid, and Selective Spectrophotometric Methods in the UV and Visible Domains

Glutathione, a tripeptide with a protective role in living cells, was determined using the biuret method proposed in this thesis—both in the visible domain and in the UV at 228 nm. The sensitivity of the measurements at 228 nm was approximately 44 times higher in the UV than in the visible range.

Another model peptide, containing ten amino acid residues and a high copper-binding capacity, was used to study the binding of copper ions to peptides at various pH values. It was found that this peptide binds copper ions strongly, owing to the presence of both sulfhydryl groups and copper-coordinating amino acids. Furthermore, the intensity of absorption of the complex formed between the peptide (P10) and Cu^{2+} increased with rising pH of the solution.

Several peptides were studied, including N-CBZ-S-benzyl-L-cysteinyl-L-tyrosine (BCT), seryl-glycine, and glycyl-tryptophan. It was established that each of these peptides can be determined in the UV at specific wavelengths, and for each, a calibration curve and molar absorption coefficient must be established.

5) Fluorimetric Studies on Peptides and Proteins, Particularly Zeins

The fluorescence intensity of various peptides, amino acids, and proteins was measured to explore the potential for quantifying peptide compounds. Research on the BCT peptide indicated that its fluorescence is not significantly influenced by other amino acid residues or protective groups but is affected by contaminants such as potassium ferricyanide and copper ions.

Zeins can be determined fluorimetrically due to the relatively high content of tyrosine residues in their molecules, despite the absence of tryptophan. These proteins can also be obtained in a nearly pure state by alcoholic extraction. Since zein contains tyrosine residues that emit light around 300 nm, excitation at multiple wavelengths is possible to minimise interferences. The most intense fluorescence was observed at 270 nm, suggesting that fluorimetric determination of zeins should be performed at this excitation wavelength.

6) Determination of Real Samples

The research carried out in this doctoral thesis confirmed that it is possible to determine both proteins and key amino acids from a single sample weighing. The proposed methods are simple, cost-effective, and provide reliable results for the sensitive, selective, and rapid determination of proteins and peptides in a wide range of biological environments. The proposed methods were validated according to international standards for selectivity, linearity, accuracy, precision, robustness, limit of detection, and limit of quantification (Araujo, 2009; Sahoo et al., 2018).

The research produced valuable data that not only improved protein and peptide determination methodology but also resulted in publications in peer-reviewed international journals and presentations at national and international conferences. Specifically, three scientific papers with an impact factor were published or accepted for publication (two already published). Additionally, two papers were published in international conference proceedings, and others were presented at conferences in abstract form only.

In addition to the novel methods developed and presented in this thesis, existing analytical methods for protein and peptide determination were refined. Furthermore, efforts were made to increase sensitivity and broaden the concentration range by employing modern spectrophotometers and spectrofluorometers. These instruments support determinations in the far-UV region, and spectral data can be processed directly on the devices using features such as smoothing (removal of spectral noise). The data can then be further analysed in Excel.

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PUBLISHED WORKS AND PARTICIPATION IN SCIENTIFIC EVENTS

A. Full-Length Scientific Articles Published in Web of Science Indexed Journals with Impact Factor

1. Drochioiu, G., **Mihalcea, E**., Lagobo, J., Ciobanu, C. I. Rapid tryptophan assay as a screening procedure for quality protein maize. *Molecules*, *29*, 4341, 2024.

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https://doi.org/10.3390/molecules29184341

2. Mihalcea E., Enache A.C., Grădinaru R.V., Drochioiu G., Simultaneous determination of protein and essential amino acids in biological samples, *Acta Chemica Iași*, 32(1), 2024.

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3. **Mihalcea, E**., Drochioiu, G. Improved protein assay based on the biuret reaction: interference of proteinogenic amino acids. *Revue Roumaine de Chimie*. Acceptată la publicare 2025.

Impact factor (IF) 0,4 Q4

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B. Scientific Papers Published in Proceedings of International Conferences

4. **Mihalcea, E**., Drochioiu, G., Jitaru, S. C., Mangalagiu, V., Gradinaru, R.V. **2022**. Protein and peptide determination based on the modified biuret procedure: implications for various biotechnologies. *International Multidisciplinary Scientific GeoConference: SGEM*, *22*(6.1), 113-120.

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5. Darie-Ion, L., Drochioiu, G., Pui, A., **Mihalcea, E**., Gradinaru, V. R., **2021**. Zein conjugates of biomedical interest: synthesis and characterization by MALDI-ToF mass spectrometry and Fourier transform infrared spectroscopy. *International Multidisciplinary Scientific GeoConference: SGEM*, *21*(6.1), 155-162.

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C. Presentations at International Scientific Conferences

1. Darie-Ion, L., Drochioiu, G., Pui, A., **Mihalcea, E.,** Grădinaru, V. R., Zein conjugates of biomedical interest: synthesis and characterization by MALDI-ToF mass spectrometry and Fourier transform infrared spectroscopy. 25. Advances in Biotechnology, Albena, Bulgaria, XXIth International Multidisciplinary Scientific GeoConference SGEM 2021

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3. Drochioiu G., **Mihalcea E.,** Lagobo Z.M., Grădinaru R.V., Simultaneous determination

of protein and two essential amino acids in maize,International Paris Congress on Medical and Health Sciences, Paris- France, 24-26 October, 2023.
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D. Presentations at National Scientific Events

4. Mihalcea E., Amiri N. Peptide complexes with heavy metal ions: P10 peptide and its relationship with copper ions, poster, Scientific communication session for students, masters and doctoral students in open to knowledge frontier chemistry, 11th edition, Iași, 29-30 October 2020.
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5. Mihalcea E., Enache A.C., Drochioiu G. Simultaneous determination of protein and essential amino acids in biological samples, poster, IașiCHEM 5-MIT 2023, Faculty of Chemistry Conference, Iași, Romania, 26-27 October 2023, abstract in Book of Abstracts IașiCHEM 5-MIT 2023, p.24.
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