# Protein determination based on the biuret absorption in the UV range and copper binding to peptides

Received for publication, April 25, 2007
Accepted, June 1, 2007

**R. GRADINARU,[*] MANUELA MURARIU,[**]**

**ECATERINA-STELA DRAGAN,[**] AND G. DROCHIOIU[*]**

[*] *Faculty of Chemistry, Al. I. Cuza University, 11 Carol I Iasi-705060, Romania, fax: 004032201313*

[**] *P. Poni Institute of Macromolecular Chemistry of Iasi, 41a Aleea Grigorie Ghica Vodă, Iasi-700487, Romania*

## Abstract

*Proteins can be assayed due to their biuret absorption in the ultraviolet region. However, copper ions may seriously interfere with the determination below 240 nm. In the present approach, proteins mobilize copper ions from insoluble salts at pH over 12 to form different copper–protein complexes, which can be measured in the range 237-250 nm. The stoichiometry of the copper–protein complexes measured directly with a mass spectrometer is pH dependent. To determine proteins, a 1.8% KOH solution containing 0–100 μg $ml^{-1}$ protein is treated with copper phosphate powder. The mixture is powerfully stirred, centrifuged, and the absorbance of the supernatant is measured at 237-250 nm in 1 cm quartz cuvette against a blank of the reagents. Bovine serum albumin has been used to plot calibration curves. This assay proved to be over 100 times more sensitive than the classical biuret procedure. Moreover, the method is highly selective and the determination is little affected by the presence of other substances, such nucleic acids or current additives in biological materials. The copper-protein complexes are also investigated by mass spectrometry.*

Keywords: protein assay; UV absorption; copper binding; ESI mass spectrometry.

## Introduction

The ability to easily and reliably quantitative protein content is paramount to many biological assays [1-4]. In a previous paper [5] a novel method for protein determination and characterization was described. This method is based on the biuret reaction of proteins at high pH values with copper insoluble salts to form copper-protein complexes, which can be measured in UV range. Although, the assay is very sensitive and almost completely free from interferences, special measures should be taken to avoid the interference of copper ions mobilized by proteins and which present an intense absorption in the far ultraviolet. On the other hand, nucleic acids, buffers, and other compounds usually found in the biological samples have a significant extinction coefficient around 260 nm, whereas aromatic amino acids have characteristic signals at 260-280 nm. Therefore, we scanned the UV wavelength range to find the most appropriate interval for protein determination using the biuret solution.

Therefore, this paper aims at describing a simple method to quantify the proteins in biuret solutions at higher wavelength (237-250 nm). In this range the copper-protein complexes do not interfere with those of nucleic acids or most of the common buffers. We have also investigated the resulted copper-protein complexes by mass spectrometry.

## Material and Methods

*Device.* A double beam UV-VIS spectrophotometer UVIKON 933-KONTRON with 1 cm matched cells of quartz was used for all spectral measurements. The pH values were measured with a CG 837-Schott pH-meter. Mass spectrometric measurements were performed on a prototype hybrid RF/DC quadrupole-linear ion trap mass spectrometer with axial ejection (Q trap, Applied Biosystems/MDS SCIEX, Toronto, Canada). The instrument was equipped with a nanoES source (Protana Engineering, Odense, Denmark). A TurboIonspray source (Ion Spray Voltage: 5000 V), a syringe pump (with 100 μl syringe) with a flow rate of 5μl/min were used. The instrument was working in positive mode and Analyst software from Applied Biosystems was used for data

acquisition. Before spectral measurements the samples were incubated using a water-bath and then centrifuged using a microfuge.

*Reagents.* All chemicals used were of analytical reagent grade and all solutions were prepared with Millipore grade water with R = 18.2 Ω. Copper phosphate was obtained by the reaction of copper sulfate with sodium phosphate tribasic dodecahydrate. Peptide Code 1017 (with no histidine residue and the following sequence: KDSVSQEGLMNTLEQNVT from RAPP Polymere, Tubingen, Germany was chosen to be investigated with the Q TRAP$^{TM}$ LC/MS/MS mass spectrometer. The formation of a copper-peptide complex and the ability of the investigated peptides to mobilize copper ions from their precipitates were followed.

*Procedure*. Amounts of 50 mg of copper phosphate powder were added to each 1 ml solution of 0-100 $\mu g \cdot mL^{-1}$ proteins in capped Eppendorf vials. A 1.8% solution of KOH was used to increase pH values over 12. The samples were shaken for 1 h, centrifuged and the supernatant was filtrated through a 0.22 μm filter. The spectra of the filtrates were recorded in a UV range from 215 to 275 nm using 1 cm quartz cuvettes. To investigate the interference of other compounds, including cell compounds as well as reagents, some samples with nucleic acids, amino acids, and buffers were also studied.

The pH values of all solutions used here were checked before and after biuret complexation and corrected using KOH. Each measurement was made in duplicate or triplicate, when necessary. The protein concentration was also measured using Lowry assay as standard method with bovine serum albumin (BSA) as a reference protein [6].

*Statistics*. The standard deviation, the overage ($s_x$) and other parameters were calculated in order to compare our results with those quantified using the Lowry method [7].

## Results and Discussion

Protein-copper complexes were well known to give a strong absorption at about 600 nm, which becomes more intense in the UV range [5]. Since in UV range below 230 nm (far UV) a strong background from the copper itself was observed, one option is to measure the absorbance of those complexes at higher wavelengths (> 240 nm).

The effect of pH on the absorbance of complexes in UV range was earlier investigated [5]. The maximum absorbance was found when the solution of biuret had pH values over 12 for all ranges of wavelength. Also, a significant absorption at around 260 nm by nucleic acids, guanidine, nucleotides etc was reported, whereas amino acids and buffers did not interfere [5]. However, they did not interfere with the measurements below 250 nm.

Therefore, in our study we used a variant of the biuret method in which the absorption is read in 240-250 nm region (Figure 1). Although the sensitivity decreased as compared with the protein determination at 226 nm, in the 240-250 nm range protein-copper complexes are better resolved. What the method lost in sensitivity, gained in its robustness. Moreover, nucleic acids and different buffer systems do not interfere in this region. Calibration curve was linear between 10 and 100 $\mu g \cdot mL^{-1}$ of the investigated proteins.

As shown in Figure 1, the highest values for the absorbance ratios were obtained for the wavelength range from 237 to 250 nm.

Normally, the crude extracts from different organisms contain beside proteins considerable amounts of DNA and RNA. Therefore, our method has the advantage to eliminate these interfering substances. Those complexes are given an insignificant absorption between 240 and 260 nm, but have rather high extinction coefficients at higher pH values and higher wavelength (> 260 nm). As a consequence this measuring interval represents a good compromise for both protein and nucleic acids.

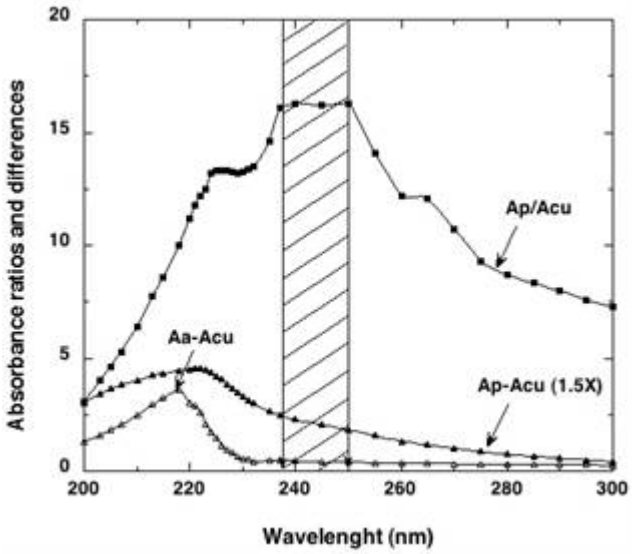


**Figure 1.** The feasibility of protein determination in UV range from 237 to 250 nm (dashed region). Here, Ap-Protein absorbance; Acu-copper absorbance; Aa-amino acid absorbance. Proteins absorb much below 226 nm, where copper ions and amino acids also absorb. In the range 237 – 250 nm, these substances do not interfere significantly.

**Table 1.** Copper binding to peptide at pH 10.30.

| Species [a] | % | m/z | | Species [a] | % | m/z | |
|---|---|---|---|---|---|---|---|
| | | Found | Calcd.[b] | | | Found | Calcd.[b] |
| $Pep{\cdot}Cu^{2+}$ | 15.4 | 1028.6 | 1028.5 | $Pep{\cdot}Cu^{2+}{\cdot}Na^{+}$ | 5.4 | 1040.2 | 1039.5 |
| $Pep{\cdot}2Cu^{2+}$ | 18.0 | 1059.2 | 1059.1 | $Pep{\cdot}2Cu^{2+}{\cdot}Na^{+}$ | 6.2 | 1070.1 | 1070.2 |
| $Pep{\cdot}3Cu^{2+}$ | 11.8 | 1089.6 | 1089.6 | $Pep{\cdot}3Cu^{2+}{\cdot}Na^{+}$ | 3.6 | 1100.6 | 1100.6 |
| $Pep{\cdot}4Cu^{2+}$ | 7.1 | 1120.6 | 1120.1 | $Pep{\cdot}4Cu^{2+}{\cdot}Na^{+}$ | 1.6 | 1131.8 | 1131.6 |
| $Pep{\cdot}5Cu^{2+}$ | 3.6 | 1150.5 | 1150.6 | | | | |
| $Pep{\cdot}6Cu^{2+}$ | 2.0 | 1181.2 | 1181.1 | | | | |
| $Pep{\cdot}nCu^{2+}$ | 57.9 | | | $Pep{\cdot}nCu^{2+}{\cdot}Na^{+}$ | 16.8 | | |
| $Pep{\cdot}Cu^{2+}{\cdot}2Na^{+}$ | 0.7 | 1049.8 | 1050.6 | $MetOx{\cdot}Cu^{2+}$ | 2.8 | 1036.5 | 1036.9 |
| $Pep{\cdot}2Cu^{2+}{\cdot}2Na^{+}$ | 2.8 | 1080.7 | 1081.2 | $MetOx\cdot2Cu^{2+}$ | 3.4 | 1067.7 | 1067.2 |
| $Pep{\cdot}3Cu^{2+}{\cdot}2Na^{+}$ | 3.4 | 1111.8 | 1111.5 | $MetOx\cdot3Cu^{2+}$ | 2.0 | 1097.5 | 1097.6 |
| $Pep{\cdot}4Cu^{2+}{\cdot}2Na^{+}$ | 4.5 | 1139.0 | 1141.1 | $MetOx\cdot4Cu^{2+}$ | 2.2 | 1128.5 | 1128.6 |
| $Pep{\cdot}5Cu^{2+}{\cdot}2Na^{+}$ | 2.4 | 1169.6 | 1169.5 | $MetOx\cdot5Cu^{2+}$ | 1.1 | 1156.6 | 1157.5 |
| $Pep{\cdot}nCu^{2+}{\cdot}2Na^{+}$ | 13.8 | | | $MetOx{\cdot}nCu^{2+}$ | 11.5 | | |

[a] Elimination of protons was not shown.

[b] m/z was calculated with the equation, m/z = (1994+63n+23m-2n–m+2)/2 = 998+30.5n+11m, where 1994 means the average mass of the peptide, 63 that of copper isotope, and n and m correspond to the number of $Cu^{2+}$ ions and $Na^{+}$ ions, respectively.

However, further investigation is needed to establish the other analytical parameters and interferences.

Figure 2 shows the electro spray ionisation mass spectrum of the pure peptide and its copper-complexes at pH 10.70. The spectrum is more complicated than the other at lower pHs.

The interactions between $Cu^{2+}$ and a synthetic peptide with no histidine residue were investigated by mass spectrometry under a variety of pH conditions. Thus, the peak at m/z 997.5 in the spectrum of peptide solved in copper-free water was attributed to double protoned peptide.

ESI-MS method has the advantage to quantify speciation of the Cu-protein complexes. At higher pH value all the copper was found in the complex. 1-3 $Cu^{2+}$ ions have been found to be trapped by more then 60%

of the total complexed protein (Table 1). However the percentage of high complex peptide (4-6 $Cu^{2+}$/peptide) was significant reduced under these conditions. Thus the content of the complex is invert proportional with the number of Cu ions present in the complex.

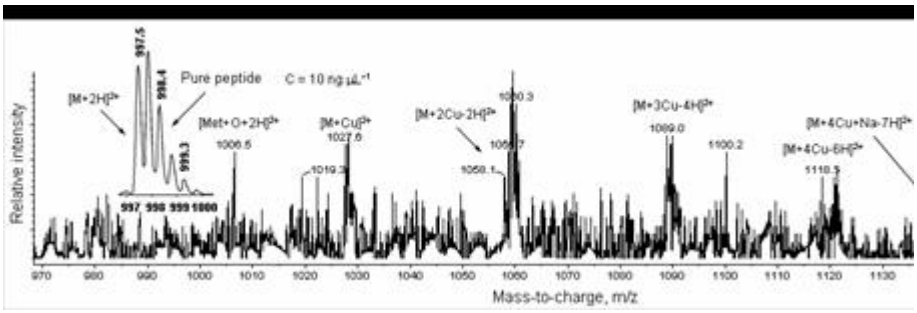


**Figure 2.** ESI mass spectra of peptide-copper complexes. In the spectrum no free peptide is observed but mono, di-, tri-, tetra-, penta- and hexacopper bound peptide have characteristic peaks. Hexacopper-peptide complex was omitted for editorial reason. The inset represents the spectra of peptide (without any histidine residue) before complexation at pH 10.7.

## Concluding remarks

Proteins can be assayed in the ultraviolet range from 237 nm to 250 nm without any significant interference. This assay based on the biuret absorption in the ultraviolet region is relatively simple, highly selective and sensitive. Copper-peptide complexes formed by mobilizing copper ions from their insoluble precipitates can be measured by mass spectrometry.

**Acknowledgements**
This paper was supported by CNCSIS Grant 1451.

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