Spectrophotometric Method for the Determination of Total Proteins in Egg White Samples

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The present study describes a modified version of the Biuret method, used for the determination of proteins. The Biuret reaction is based on the formation of a complex between cupric ions and protein. This procedure is developed for the determination of proteins in egg white samples, without addition of KI as reagent. KI is added to prevent auto-reduction and separation of cuprous oxide and to mask the presence of such impurities. The optimum conditions for proteins quantification, using ovoalbumin and BSA (Bovine serum albumin) as standard proteins were studied. Under the optimized experimental conditions, Beer’s law is obeyed over the concentration ranges of 0.22-1.98mg/mL and 0.15-1.35mg/mL for ovoalbumin and BSB, respectively. Method validation has been demonstrated through a variety of tests for linearity, accuracy, precision, LOD and LOQ. The obtained results from spectrophotometric and literature methods were statically compared.

Keywords: Spectrophotometric method, ovoalbumin, Bovine serum albumin, proteins, egg white samples

The protein content in foodstuff is an important and essential parameter to determine in order to ensure the quality and safety of food. Even if there are several methods used to quantify the proteins (soluble or insoluble) an increasing rigor is required for food products and the validation of the methods has become of high interest. Eggs are an important inexpensive source of high quality proteins and other nutrients. Egg white is a natural source of protein of recognized nutritional, biological and technological potential interest. Ovoalbumin, the major egg white component, corresponding to 54 g/100 g of the total egg white protein is a glycoprotein with coagulation and gelation properties. Due to unique functional properties of proteins such as gel and foam formation, hen egg white proteins have been extensively used as ingredients in processed foods [1-3]. Many total protein assays have a variable analytical response per unit of protein, depending on the type of protein [4-6]. The most quoted spectrometric methods for the quantification of soluble proteins are Biuret, Lowry’s and Bradford’s methods [1-2]. The Kjeldahl method, based on the nitrogen amount measurement, useful mostly for the insoluble proteins determination, even the oldest one, is still in use [7]. Because of its simplicity, sensitivity, reasonable accuracy and precision and cost-effectiveness, UV-visible spectrophotometer methods continue to be the used technique in laboratories [8-9].

The aims of this paper are:
- adaptation and simplification of the Biuret method and evaluation of its accuracy for egg white protein quantification;
- validation of the proposed method;
- pointing out the most simple and reliable method for soluble protein determination in foodstuff.

Experimental part

Equipment, methods and materials

All spectral measurements were made on UV/V-650 Jasco spectrophotometer (Tokyo, Japan) using standard 1.00 cm quartz cells.

Some commercial samples, including grade A eggs were purchased from the local market [10]. All reagents used were of analytical reagent grade. Double-distilled water was used throughout the investigation.

Standard solutions

Ovoalbumin. A standard solution containing 8mg/mL of ovoalbumin (Aldrich) was prepared by dissolving accurately 0.2 g of pure sample in 25 mL of double distilled water in a calibrated flask. Working standards were prepared by appropriate dilution of the stock.

BSA (Sigma) solutions were prepared in NaOH 0.1M and used as standard in all assays in the concentration of 10 mg/mL.

Spectrophotometric methods

Protein determinations were based on the Biuret reaction using a calibration curve builds by varying standard protein concentration in aqueous solutions [11].

Proposed method (A)

Different aliquots of working standard (albumin or BSA) solution ranging from 1.0 - 10 mL were transferred into a series of 25 mL serially numbered volumetric flasks by means of a micropipette. To each flask, 1.5 mL of 1% (w/v) copper sulfate, 1.0 mL of 2M sodium hydroxide and 6.0 mL of 1% (w/v) potassium sodium tartrate (which is used to stabilize the cupric ion in the alkaline solution) were added. The flasks were stoppered, contents were mixed well and kelp to room temperature for 5 min, for complete reaction. The volume was brought up to 25 mL with distilled water to get final concentration of 0.318 - 3.18 mg/mL. The absorbance of the resulting solutions was measured at 547 nm against the reagent blank which was prepared similarly except the addition of ovoalbumin.
Biuret method (B)

Different aliquots of working standard solution ranging from 1.0 - 10 mL were transferred into a series of 10 mL serially numbered volumetric flasks by means of a micropipette. To each flask, 4.0 mL Biuret reactiv solution were added. The flasks were stopped, contents were mixed and kept to room temperature for 5 min. for completion of reaction. The solution was brought up to the final volume with distilled water and the absorbance of the colored solution was measured at 456 nm against reagent blank.

In the both methods, the calibration graphs were prepared by plotting absorbance versus concentration of standard and the concentration of the unknown was read from the calibration graph or computed from regression equation derived from the Beer’s law data.

Kjeldahl method

The Kjeldahl method was applied as a standard method for nitrogen determination. Its value was multiplied by 6.25 to give the protein content [12].

Assay Procedure for eggs albumin

Commercial samples, including grade A eggs were purchased from the local market and were analysed for soluble protein quantification. Shelling and accurate separation of albumen from yolk were done manually. The egg albumin were homogenized and an aliquot of the solution equivalent to 2.0 g albumin was transferred to 25 mL calibrated flask and treated as per procedure described in the “Spectrophotometric methods” section.

Statistical analysis

The standard deviation (s), standard deviation of the mean (sx), t and F parameters and the coefficient of variation (CV%), were calculated in order to compare the methods. In order to check for the accuracy and precision of the proposed method, a sample of protein containing 2.5 mg/mL ovoalbumin was analysed by the both procedures. The result was expressed as the mean of six or less (as indicated) replicate analyses.

Results and discussions

Proposed method

Absorption spectra

In order to have minimum interferences, it was necessary to identify optimum wavelength for proteins determination in the proposed methods. This wavelength must be specific for the quantitative and specific monitoring of the complex over the range 400 – 800 nm with a UV-Vis spectrophotometer. Wavelengths of 547 nm were found optimum to get best results (fig. 1).

The effect of different experimental variables

Reagents concentration

The effect of the reagent concentration on the intensity of the colour developed at the selected wavelength was ascertained by adding different amounts of the reagents to fixed concentration of 2.5 mg/mL ovoalbumin.

The effect of copper sulfate reagent at 1% (w/v) solution in the range of 0.10 - 5.0 mL was studied to find the volume needed to get maximum colour intensity. The results indicated that 1.0 - 2.0 mL of the solution were necessary. Hence, 1.5 mL of 1% (w/v) solution in a 25 mL standard flask was taken.

Maximum intensity of the purple color was achieved in sodium hydroxide medium. The effect of NaOH concentration on the absorbance was studied; volumes from 0.5 - 3.0 mL of 2M NaOH were examined. The investigation showed that 1.0 - 1.5 mL of sodium hydroxide gave maximum absorbance and hence 1.0 mL of 2M NaOH was chosen for all measurements. Other alkaline solutions were tried, but best results were obtained by using sodium hydroxide.

The effect of varying the concentration of stabilization agent was studied using 1.0 - 10.0 mL of 1% (w/v) of potassium sodium tartrate. It was noticed that the maximum colour intensity and constant absorbance was formed with 6.0 mL of potassium sodium tartrate in final volumes of 25 mL.

Effects of temperature and standing time

The effect of temperature on coloured product was studied at different temperatures. It was found that maximum colour intensity developed within 5 min. at room temperature and remained almost stable for about 6 h. Increase in reaction temperature decreased the intensity of purple colour. Hence, a reaction time of 5 min. was selected for the analysis.

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Spectrophotometric method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured absorbance λ&lt;sub&gt;max&lt;/sub&gt;, nm</td>
<td>547</td>
</tr>
<tr>
<td>Liniarity range, M</td>
<td>10&lt;sup&gt;-3&lt;/sup&gt; - 7.10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Molar absorptivity, L/mol cm</td>
<td>1.3 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Regression equation</td>
<td>(Y = ax+b)</td>
</tr>
<tr>
<td>Sandell sensitivity, μg cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.017</td>
</tr>
<tr>
<td>Absorbance range</td>
<td>0.085 - 0.950</td>
</tr>
<tr>
<td>Correlation coefficient, r&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.9970</td>
</tr>
<tr>
<td>Detection limit LOD, M</td>
<td>1.8 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quantification limit LOQ, M</td>
<td>6.1 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(Y = ax+b), were Y is the absorbance and x is the concentration of ovoalbumin in mg/mL.

Fig. 1. Absorption UV-Vis spectra (BSA and ovoalbumin-complex)
Order of addition of reactants

After fixing all other parameters, a few experiments were performed in order to ascertain the influence of the order in which reagents were added. The maximum absorbance and highest stability were obtained when the order of addition was: ovoalbumin - NaOH - Cu(II)-potassium sodium tartrate.

Validation of the Method

Linearity

Under optimum experimental conditions, a linear relation was obtained between absorbance and concentration of ovoalbumin. The regression parameters calculated from the calibration graphs data are presented in table 1. The linearity of the calibration graph was proved by the high value of the correlation coefficient (r) and the small values of the y-intercept of the regression equation. The molar absorptivity, Sandell sensitivity of the proposed method are also presented in table 1.

Sensitivity

Sensitivity of the method can be determined through the limit of detection (LOD) and limit of quantification (LOQ). The limit of detection and limit of quantification were determined using following equations [13]: LOD = 3s/a; LOQ = 10s/a, where s, is the standard deviation of replicate determination values under the same conditions as for the sample analysis in the absence of the analyte and a is the sensitivity, namely the slope of the calibration graph. The values of LOD and LOQ were given in table 1.

Precision and Accuracy

The validity of the method for the assay of ovoalbumin was examined by determining precision and accuracy. There were determined by analyzing seven replicates and the analytical results are presented in table 2. The low values of the relative standard deviation (% RSD) and percentage relative error (% RE) also indicate the high precision and good accuracy of the proposed method.

Comparison between the proposed method and the original Biuret method

Table 4 shows straight-line equations, range of ovoalbumin and BSA concentrations of work and relative specific absorbance (RSA), [RSA = specific absorbance of ovoalbumin/specific absorbance of BSA], for the following methods: proposed (A) and original Biuret methods (B). The selection of an appropriate standard is of critical importance because, the protein assays depend on the quality of the protein for the response. As shown in table 4, the correlation coefficients for all the straight lines were at least 0.9900, and the both methods showed almost the same sensitivity for proteins (range concentration of protein of work 0.22 - 1.98 mg/mL for method A and 0.15 - 1.35mg/mL for method B).

A good agreement for specific absorbance (RSA) between ovoalbumin and BSA for both methods was expected (0.9765 and 1.0196, respectively). These are indicating that ovoalbumin or BSA could be used as a standard. The RSA values showed that the reaction between the copper and the peptide bond depends on the molecular weight of proteins, and less on the composition of the amino acids in the proteins [14].

Evaluation of the proposed method with the original procedure showed they had similar precision when a sample of 1.0 mg/mL of ovoalbumin or BSA was analyzed

RSD (%) and RE (%) values obtained within the same day to evaluate repeatability (intra-day precision) and over five days to evaluate intermediate precision (inter-day precision).

Recovery

To study accuracy of the method, recovery studies were performed by the standard addition technique. For this, known quantities of pure ovoalbumin were mixed with definite amount of pre-analyzed samples (albumin) and the total amount was analyzed by the proposed method. The average percent recoveries obtained were quantitative (>95 %) indicating good accuracy of the method.

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The proposed method is reproducible and the color system was found to obey Beer’s law in established range of concentrations. However, by comparing the results of proposed method with original Biuret method (B), we observe the higher values for the absorbance of the first method. This difference probably can be due to the difference contents in free amino groups in solution.

**Analytical application**

The proposed method was applied to the determination of protein in commercially samples (grade A eggs) and the results are presented in table 6.

The performance of the method was assessed using the t-test for accuracy in comparison with the reference methods (Kjeldahl and B). The results showed that the t-test values are less than the theoretical value (t = 2.31) for 95% confidence level, suggesting that the proposed method (A) is comparable to the reference methods with respect to accuracy.

**Conclusions**

In this paper, a modified version of the Biuret method was proposed for protein quantification. In order to evaluate its accuracy, the proposed method has been validated with an LOQ of 6.1·10^{-5}M. This analytical method could be successfully applied to the determination of total proteins in egg albumin samples and of soluble proteins in foodstuff.

**References**


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