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Arunima S
Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Agricultural University, Raipur, Chhattisgarh, India

Satish Verulkar
Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Agricultural University, Raipur, Chhattisgarh, India

Corresponding Author
Arunima S
Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Agricultural University, Raipur, Chhattisgarh, India

Comparative analysis of different protein estimation methods

Arunima S and Satish Verulkar

Abstract
Protein quantification is a critical step in protein analysis, and it has several applications in clinical laboratory techniques as well as the study of biochemistry. There are numerous protein quantification methods. In this study, three protein quantification techniques (Biuret, Lowry, and Micro-Kjeldahl) were compared and found to produce consistent findings for samples with lower protein percentages. However, if the protein concentration is greater than 50%, the Lowry and Biuret procedures deviate from the Micro-Kjeldahl method. The sensitivity range of both the Biuret and Lowry techniques can be enhanced by dilution of samples prepared for spectrometric analysis without altering the standard curve. Protein estimation was performed at various degrees of dilution and reported to generate almost identical results to the Micro-Kjeldahl technique at 100 times dilution of the original sample solution prepared for analysis.

Keywords: Protein quantification, sensitivity, lowry method, biuret method, micro-kjeldahl method

Introduction
Proteins found in biological substances are extremely important since they offer nourishment for living organisms, act as genetic code, and play a vital role in cell metabolism (Syauqi *et al*, 2018). Fast, reliable and precise determination of nitrogen and hence crude protein content in food products are essential to analyze their nutritional quality (Beljkaš *et al.*, 2010) [1]. Protein quantification is a critical step in protein analysis, and it has several uses in clinical laboratory techniques as well as the field of biochemistry (Janairo *et al*., 2015) [5].

Protein concentrations in samples are estimated using various protein assay techniques, including as Dumas method, Nessler method, Biuret method, Berthelot's method, Lowry method, Kjeldahl method, Folin-Ciocalteu method, Dye binding, Direct alkaline distillation, Near infrared reflectance(NIR), Bradford method, Bicinchoninic acid (BCA) method (Plaza *et al*., 2013) [15] etc., which have been developed over past two decades (Okutucu *et al*., 2007) [10]. Although there are several approaches for quantifying protein, only a couple of them are commonly employed for protein quantification. Some conventional methods, such as Kjeldahl, biuret, and die-binding processes, are still used in current dietary protein analysis. The Lowry technique is more commonly used in biochemical analysis than in food analysis since it requires great sensitivity. UV absorption and chromatographic separation techniques are normally performed in combination, but IR spectrophotometry is significant for total protein quantification due to its quick and nondestructive approaches (Rayment *et al*., 2012; Rukke *et al*., 2010; Santos *et al*., 2012) [12, 18]. The effective use of various protein quantification techniques is possible with a deeper understanding of their advantages and limitations, taking into account factors such as the nature of the protein sample, the presence of interfering substances in the sample, as well as the speed, accuracy, and sensitivity of the assay. The sensitivity range of protein concentration and the linear response to standard solutions are essential considerations when selecting a protein quantification technique (Janairo *et al*., 2015) [5].

The biuret reaction was one of the first colorimetric protein assay techniques established for quantifying concentration of protein in a given sample (Gornall *et al*., 1949) [4]. The interaction of biuret reagent with peptide bonds produces a purple complex (Boyer, 2000) [2]. The coordination complex formed by the copper atom and the two nitrogen atoms from each peptide chain results in the development of coloured product (Switzer & Garrity, 1999) [20] at its peak intensity after about 15 minutes, and the complex formed is stable for a long period. The approach is reliable, and the error was determined to be less than 5%. According to Kingsley, 1939 [7], the biuret technique was shown to be in good agreement with the Kjeldahl

method in terms of total protein quantification. The biuret method is a popular choice in clinical labs due to its ease of use and speed, as well as its reliability when compared to approaches that rely on the presence of specific amino acids (Gornall *et al*., 1949) [4]. Biuret assay sensitivity is reported as protein samples with concentrations ranging from 0.0100 to 5.00mg/mL (Janairo *et al*., 2015) [5].

The Lowry technique is one of the most sensitive and can detect protein samples ranging in concentration from 2 to 100 µg. The method is based on the biuret reaction, with the addition of the Folin-Ciocalteu reagent to enhance colour development. The protein treatment with alkaline copper sulphate in presence of tartrate is followed by addition of Folin-Ciocalteu reagent (Gordon *et al*., 2013) [13]. The copper interacts with peptide nitrogen atoms to generate a cuprous complex while FC reagent interacts with the cuprous ions, tyrosine, tryptophan and cysteine residues in the side chains and produces a blue green color that can be detected between 650 to 750 nm (Stoscheck, 1990) [19]. The major advantage of this method is that, it is 100-fold more sensitive than the original biuret reagent. Even though the method is highly sensitive, it is interfered by many common substances like Tris-HCl, K^+, Mg^{2+}, $NH4^+$, EDTA, carbohydrates, reducing agents etc. It is a two-stage reaction with an incubation time around 40 minutes and the FC reagent is reactive only for a short time period after its addition (Goldring, 2012).

Kjeldahl method was devised by Johan Kjeldahl for nitrogen estimation. It is one of the classical measurement in analytical chemistry, widely used over the past 130 years and still the primary reference method for protein analysis because of its precision and accuracy (Plaza *et al*., 2013a) [16]. It mainly involves three different steps: digestion of sample, distillation and ammonia determination by titration (Saha *et al*., 2012) [17]. Those samples having organic nitrogen are digested with sulfuric acid to ammonium sulfate, then liberated as ammonia by raising the pH and measured by titration. The Kjeldahl method is dependent on total organic nitrogen content in the sample, so that the accuracy of protein content will not be affected by protein structure (Wong *et al*., n.d.) [21]. Since it gives accurate protein results irrespective of the physical state of the sample, still remains the reliable reference method to standardize other methods (Bravo *et al*., 2009; Apenten, 2002; Rayment *et al*., 2012) [3, 11, 12].

Despite the fact that the Kjeldahl technique is regarded as a standard approach, the method is hazardous, time-consuming, and labor-intensive. It lacks analytical selectivity since it cannot discriminate between protein-based nitrogen and non-protein nitrogen. Because of the presence of non-protein molecules in the sample, there is always the possibility of overestimation of the actual protein content (Möller, 2010; Yuan *et al*., 2010) [9, 22]. The objective of this study is to examine the biuret and lowry techniques of protein estimation for highly concentrated protein samples with slight adjustments in the procedure and to see if they can totally replace the Kjeldahl method in conventional laboratory protein content estimation.

Materials

Kjeldahl Method

Instruments

Weighing balance**,** KEL plus digestion and distillation apparatus, Kjeldahl flasks, Micro Burette, Conical flasks, beakers, etc.

Chemicals

a) Catalyst mixture: K2SO4 & CuSO4.5H2O were mixed in the ratio of 5:1.
b) Boric acid solution (4%): 40g boric acid dissolved in 1litre of double distilled water.
c) Boric acid – indicator solution: Dissolve 0.099 g bromocresol green and 0.066 g methyl red in 100 ml of 95% ethanol. Add 20 ml of this indicator to 1 litre of 4% boric acid solution and adjust to pH 4.0.
d) Sulphamic acid (0.02 N): Dissolve 1.941 g of sulphamic acid in 1L double distilled water
e) NaOH solution (40%): 400g NaOH in 1 litre distilled water.
f) NaOH solution (15%): 150g NaOH in 1 litre distilled water for digestion unit.
g) Protein sample: fine powder of rice protein

Lowry's Method

Instruments

Weighing balance, test tubes, beakers, spectrophotometer

Chemicals

a) Reagent A: 2% Na2CO3 in 0.1N NaOH
b) Reagent B: 0.5% CuSO4.5H2O in 1% potassium sodium tartrate
c) Reagent C: Mix 50 ml of solution A&B prior to use
d) Reagent D: Folin-Ciocalteau reagent
e) Bovine serum albumin: for standard curve preparation.
f) NaOH (0.2N): 8g NaOH dissolved in 1litre distilled water
g) Protein sample: fine powder of rice protein

Biuret Method

Instruments

Weighing balance, test tubes, beakers, spectrophotometer

Chemicals

a) Biuret reagent: 300mg CuSO4.5H2O and 900mg sodium potassium tartrate were dissolved in 50 ml 0.2M NaOH and 500mg KI was added in to that. The solution made up to 100ml by 0.2M NaOH.
b) Bovine serum albumin: for standard curve preparation.
c) NaOH (0.2N): 8g NaOH dissolved in 1litre distilled water.
d) Protein sample: fine powder of rice protein

Methods

Kjeldahl method

Procedure

Total protein content of the proteins extracted was estimated by modified micro-Kjeldahl method with the steps as follows:

i) Digestion process

About 0.5 g or less (0.2g) of sample was transferred into the digestion tube and 1-3g of K2SO4 and CuSO4 mixture was added (sample and K2SO4& CuSO4.5H2O in the ratio of 5:1). 10 ml of concentrated Sulphuric acid was added and digestion tubes were placed on the digestion block with temperature set at 400 ºC. After 2 to 3 hours when the colour of the contents in the digestion tubes turned light green, they were taken out of digestion block. The tubes were allowed to cool down to room temperature.

ii) Distillation process

Digested samples were subjected to distillation unit and distillation of samples was carried using 4% Boric acid and 40% Sodium hydroxide. The program was automatically adjusted to add 20 ml distilled water 30 ml boric acid indicator solution and 40 ml NaOH solution and distillation conducted for a time about 9 minutes. 10 ml of distilled water were added before starting of distillation process. The flask was beneath the condenser with the delivery tip immersed in the solution. The digested samples were transferred to distillation apparatus and 40% sodium hydroxide was added to it. The distillate was collected in a conical flask. A blank was always run containing the same quantities of the entire reagent but without the sample for every set of nitrogen determination.

iii) Titration process

The distilled samples were titrated against the 0.02 N Sulfamic acid until the colour changes from dark green to pinkish-purple as the end point. The titre value was used to calculate percent Nitrogen, which is then used to estimate total protein content by using conversion factor 5.95.

$$\% \text{ Nitrogen} = \frac{(V1 - V2) \times N \times 14 \times 100}{W \times 1000} \quad \text{Eq. (1)}$$

Where,
V1 = Vol. of Sulfamic acid
V2 = Vol. of blank
N = Normality
W = Sample weight (g)

Protein % = % Nitrogen x 5.95 Eq. (2)

Lowry's method

Procedure

1. Finely powdered protein sample (0.5 g) was dissolved in 10 ml 0.2 N NaOH solution.
2. One ml protein sample solution was taken in a test tube and 5 ml reagent C was added.
3. Mixed it properly and kept it for 10 minutes.
4. Reagent D of 0.5 ml also added, mixed and incubated in dark for 30 minutes.
5. The optical density of the solution measured in spectrophotometer at 660 nm.
6. The absorbance value compared with standard value and protein concentration was calculated from standard curve.

The standard curve prepared using different concentrations of bovine serum albumin prepared as per the same method of protein solution preparation and their absorbance values are measured. The standard curve used for Lowry's method is given in figure 1.

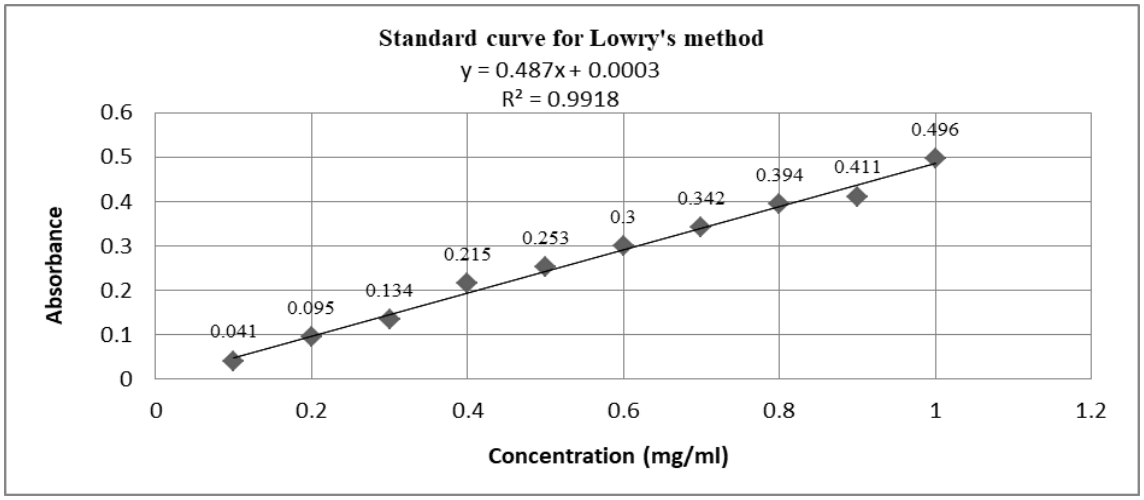

Fig 1: Standard curve prepared using a series of concentration of bovine serum albumin

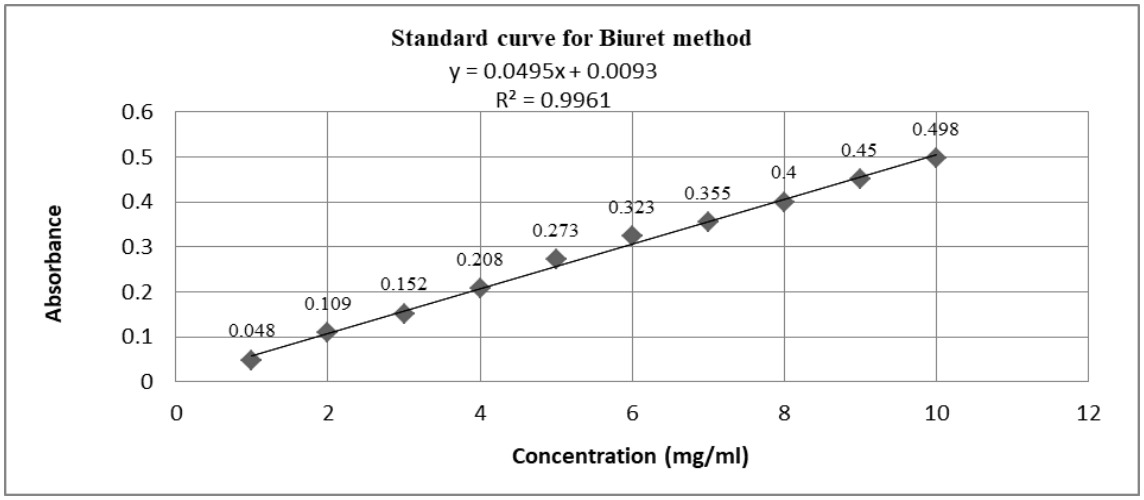

Fig 2: Standard curve prepared using a series of concentration of bovine serum albumin

Biuret method

Procedure

1. Finely powdered protein sample (0.5 g) was dissolved in 10 ml 0.2N NaOH solution.
2. Protein sample solution (1 ml) taken in a test tube and 3 ml of biuret reagent was added.
3. Mixed it properly by vertexing and kept it for 10 minutes at 37˚C.
4. The solution cooled to room temperature.
5. The optical density of the solution measured in spectrophotometer at 540 nm against blank.
6. The absorbance value compared with standard value and protein concentration was calculated from standard curve.

The standard curve prepared using a series of concentrations of bovine serum albumin prepared as per the same method of protein solution preparation and their absorbance values are measured. The standard curve used for Biuret method is given in figure 2.

Results

Protein quantification from controlled and processed rice was performed in an RBD design with single factor analysis at α=0.05 and an F-test were used to compare variance between the three distinct protein quantification methods. For each ANOVA table, a null hypothesis was proposed and the findings from the biuret and lowry procedures were compared to the results from the Kjeldahl method.

Table 1: Comparison of original protein content in different rice varieties (in %) by Lowry and Kjeldahl methods

S. No.	Variety	Protein content-Lowry (%)	Protein content-Kjeldahl (%)	Standard deviation
1	Swarna	7.99	6.64	0.95
2	R-RF-127	8.61	8.51	0.07
3	R-RF-105	7.95	6.64	0.93
4	MTU-1010	6.78	6.98	0.14
5	Broken rice	7.56	7.48	0.06

Table 2: Comparison of original protein content in different rice varieties (in %) by Biuret and Kjeldahl methods

S. No.	Variety	Protein content-Biuret (%)	Protein content-Kjeldahl (%)	Standard deviation
1	Swarna	6.93	6.64	0.21
2	R-RF-127	8.97	8.51	0.33
3	R-RF-105	6.93	6.64	0.21
4	MTU-1010	7.75	6.98	0.54
5	Broken rice	7.34	7.48	0.10

Table 3: Comparison of protein content with the help of heat map in processed rice samples having high protein percentage by Lowry and Kjeldahl methods using different dilutions

S. No.	Variety	Protein content-Lowry (%)	Diluted Lowry sample (50 times) (%)	Diluted Lowry sample (100 times) (%)	Protein content-Kjeldahl (%)
1	Swarna	20.85	41.25	64.44	65.21
2	R-RF-127	21.58	45.13	73.43	71.34
3	R-RF-105	19.16	42.34	69.21	69.66
4	MTU-1010	22.86	40.25	70.88	73.34
5	Broken rice	20.31	43.33	67.52	71.41

Table 4: Comparison of protein content with the help of heat map in processed rice samples having high protein percentage by Biuret and Kjeldahl methods using different dilutions

S. No.	Variety	Protein content-Biuret (%)	Diluted Biuret sample (50 times) (%)	Diluted Biuret sample (100 times) (%)	Protein content-Kjeldahl (%)
1	Swarna	21.54	44.16	64.08	65.21
2	R-RF-127	21.01	45.91	68.16	71.34
3	R-RF-105	22.24	41.15	72.24	69.66
4	MTU-1010	23.88	42.42	75.51	73.34
5	Broken rice	22.65	54.52	73.46	71.41

Discussion

Colorimetric analyses include spectrometric methods which operate in visible radiation. Spectrometric analysis are based on calibration curve and they are having a sensitivity range where the calibration curves are linear in nature (Janairo *et al.*, 2015) [5]. The calibration curves for Lowry and Biuret methods were created using standard BSA solution. Despite having linear responses with R^2 values of 0.9918 and 0.9961, respectively, both Lowry and Biuret standard curves covered the concentration of unknown samples up to a specific limit. Higher protein content samples were unable to exhibit a linear relationship with absorbance. As a reference approach, the Micro-Kjeldahl method was used. Lower protein percentage samples (less than 10%) had extremely consistent readings across all three techniques. The F-test was performed by comparing it to the Micro-Kjeldahl technique, and the estimated f-values were negligible, implying that the null hypothesis was accepted, indicating that there was no significant difference between the three methods.

However, neither the Lowry nor the Biuret techniques were able to precisely measure those samples with a greater protein percentage (above 50%). The calculated protein values were significantly lower and calculated f-values were significantly higher. Hence rejected null hypothesis and accepted alternative hypothesis that there was significant difference between both lowry, biuret methods and reference Micro-

Kjeldahl method. For standardisation of lowry and biuret methods, the protein sample prepared were diluted just prior to take spectrophotometric reading, by adding 0.2N NaOH solution. The dilution conducted at different levels and here included the data of 50 times and 100 times diluted protein samples (table 3 and table 4). Protein amount values from a 50-fold dilution were similarly inconsistent with reference data. Because the computed f-values were considerably higher, the null hypothesis was rejected. However, the f-test for 100 times diluted samples of lowry and biuret exhibited consistent results with the reference Micro-Kjeldahl technique.

The study could broaden the range of the linear calibration curve for greater protein-containing samples with minimal modifications in sample preparation and no changes to the standard curve. The results revealed that both the lowry and biuret techniques, with such adjustments, could nearly completely replace the reference Micro-Kjeldahl method, that had complicated procedures.

Conclusion

The study demonstrates that the three protein quantification techniques (Lowry, Biuret, and Micro-Kjeldahl) are adequate for measuring the protein content in lower percentage samples. Although the lowry and biuret techniques of protein measurement are adequate for quantifying samples with low protein percentages, they are only highly sensitive up to specified limits. Above such limits, both techniques deviate considerably from the standard Micro-Kjeldahl method. This work improved the biuret and lowry procedures in order to quantify higher protein in samples and broaden their application with significant f-values.

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