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Little Adjustment of Lowry and Biuret Methods to Get Better Absorbance of Proteins

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ABSTRACT

Copper sulfate has been staying as a major source of Cu^{2+} for copper based colorimetric protein determinations. Hemi-penta hydrated Copper nitrate furnish Cu^{2+} ions which can be used as an alternative to Penta hydrated Copper sulfate in Copper based colorimetric protein determinations. We describe herein the development of a new modified Lowry and Biuret procedures which replace penta-hydrated Copper sulfate of the original reagents ($Cu\ SO_4\ .5H_2O$) by hemi penta-hydrated Copper nitrate ($Cu_2(NO_3)_2\ 2.5\ H_2O$). Those methods were applied for measuring different standard samples of protein. We favorably compared the modified methods with the original Lowry and Biuret methods for reliability, assay time and resistance by interferences of non-proteinaceous compounds. Those modified methods showed better reliability, rapid assay time of color development and strong resistance of interfering substances. The modified procedures might be applicable as better options for the determination of protein in a biological sample.

Introduction

Proteins are the most abundant macromolecules in a biological system and their accurate quantification is an important part of any biochemical investigation in life sciences [1]. Many colorimetric dye-based methods are avilable for the measurment of protein concentration in solutions [2-4]. Out of those the most popular methods include Lowry [5-7], Biuret [8], Bradford [9] and Bicinconic acid [10]. However each of these methods has its own pros and cons with respect to sensitivity, reproducibility, rapidity and level of interference by non-protinaceous substances. For instance, deteregents interfere in the Bradford method [11], while chelating agents [12], Magnesium and Calceuim ions [13,14] and disulfite reagents [15] interfere in Lowry's method. Bicinconic acid method is susceptible for inhibition by thiols,lipids and reducing sugars [16,17]. Levels of interference are least in the Biuret method however its capacity to detect proteins at lower concentration is less in comparison with other assays [18]. Out of several methods designed for estemation of proteins, Lowry's assay ranks as the most higly sited method due to its simplicity and ability to detect proteins even in microgram conentrations [19]. Based on an uprising need to design a facile analytical procedure for protein estimation, various modifications have been made. Moreover, the expanding number of colorimetric assays for the quantitation of proteins has proceeded upgrade in accuracy and sensitivity with the onset of modern techniques. From DOC-TCA (Deoxycholate-Tricarboxylic acid) precipitation for avoiding interferences to comparison of separate methods for measuring specific standards were highly performed [20,21].

Recent modifications designed more sensitive methods to detect proteins even in Nano gram concentrations [22]. Although most of the modifications are focused to increase rapidity, sensitivity

and resistance to interfering substances, limited modifications try to modify measuring validity of the assay for numerous types of proteins. We suspected that possible replacement of Cu SO_4.$5H_2O$ of the Cu^{2+} based colorimetric protein determinations by Cu_2 $(NO_3)_2$ $2.5H_2O$ might advent a method that has better improvements. Relied on a difference in chemical and physical properties (Table 1), equal amounts of the two copper sources have caused varying activation on plant root-based peroxidases [23,24]. Those differences attracted our intension to perform and compare the dye-based methods inclusive of those chemicals. Among the mentioned four methods, we selected Biuret and Lowry procedures situated on better resistance to interfering substances and sensitivity respectively. Here we show the modified methods of Biuret and Lowry with replacement of Cu SO_4.$5H_2O$ of the usual reagents by Cu_2 $(NO_3)_2$ $2.5H_2O$ followed with testing of the modified methods for estimation of different standards including BSA, catalase, egg albumin and gelatin. Comparison of the new methods with the well-known procedures was conducted based on reliability, assay time and resistance to selected interfering substances (SDS, Tris buffer, Sodium sulfite, Cadmium sulfate, Cobaltous acetate, Sodium chromate and Tannic acid). DOC-TCA precipitation [7] was performed to eliminate recorded interferences from the non-proteinic substances and results are compared before and after the precipitation. Suitable wavelength scan of the new methods was also performed in between a range of 500-700 nm.

Table 1: Physical and chemical properties of Copper (II) Nitrate and Copper (II) sulfate.

	Copper (II) Nitrate Hemi Penta Hydrate	Copper (II) Sulfate Penta Hydrate
Compound formula	$Cu_2(NO_3)_2$ 2.5 H_2O	Cu SO_4. $5H_2O$
Molecular weight	465.188g/mol	248.68g/mol
Appearance	Crystalline lumps	Crystalline solid
Melting point	115 ˚C	110 ˚C
Density	2.32g/cm^3	2.284g/cm^3
Solubility in water	Best	Best
Exact mass	463.86329	248.93415
Mono isotopic mass	463.86	248.93
Color	Light blue	Deep blue

Materials and Methods

Reagents and chemicals were obtained from the following sources: Egg albumin, Bovine Serum Albumin(BSA), FC reagent, Sodium carbonate, Gelatin powder, Potassium sodium tartrate, Casein, Cobaltous acetate, Cadmium sulfate, Sodium di chromate and Deoxycholate were obtained from British Drug House(BDH) British; Tri Carboxylic Acid(TCA) was obtained from May and Baker Company, England; Sodium Hydroxide was obtained from Fisher Scientific, United Kingdom; FC reagent was obtained from MERCK company, Germany; Tris Hydrochloride and Cupric Acetate were obtained from Labort Finch Chemical, India. Penta hydrated Cupric Sulfate was obtained from Osaka Hayashi pure Chemical Industries, Japan; Hemi Penta hydrated Copper nitrate, Sodium Sulfite, Sodium Dodecyl Sulfate (SDS) and Tannic acid were obtained from Sigma Aldrich, Chemicals, St. Louis, Missouri, USA.

Protein Estimation by the Standard and Modified Methods

Standard protein estimation was performed by Lowry's method according to Lowry et al. [5] within a linear range of 0-75 µg/ml, Biuret's method as reported by Gornall et al. [8] within a linear range of 1-5 mg/ml. Reagents for the modified Lowry and Biuret methods were prepared with substitution of Cu. SO_4 $5H_2O$ of the original reagents by Cu_2 $(NO_3)_2$ $2.5H_2O$. Briefly, modified Lowry's reagent was prepared by mixing 2% w/v of 1ml Cu_2 $(NO_3)_2$ $2.5H_2O$, 2% w/v of 1ml Sodium potassium (Na^+K^+) tartrate and 4% w/v of 98ml Sodium Carbonate. Modified Biuret reagent was prepared according to Gornall et al. [8] except for substitution of Penta hydrated Cu SO_4.$5H_2O$ of the standard by hemi penta-hydrated Cu_2 $(NO_3)_2$ $2.5H_2O$. Briefly, 1.5 g of Cu_2 $(NO_3)_2$ $2.5H_2O$ and 6g of Sodium potassium (Na^+K^+) tartrate were dissolved in 500 ml of water. While stirring the mixture, 300 ml of 10% w/v Sodium Hydroxide was added to it. Finally, 200 ml of deionized water was added and the mixture was kept for use.

Effect of Interfering Substances

Effect of interfering substances both in the standard and modified Lowry and Biuret methods was performed according to Brown et al. [17]. Interfering agents such as Tannic acid(0-20mM), Sodium sulfite(0-1mM), Tris buffer(0-0.5M), Sodium Dodecyl Sulfate/SDS(0-1Mm), Cadmium sulfate(0-1mM), Sodium chromate(0-1mM) and Cobaltous acetate(0-1mM) were checked for their effect on absorbance measurements.

Effect of Interference on Lowry and Modified Lowry Methods

On mixture of 0.5ml of interfering substance and 0.5 ml (35µg/ml) of BSA, 3ml of the reagent was added. While standing 30 minutes for the standard method, mixture was allowed to stand 23 minutes for the modified method. 0.5 ml of 1:1 diluted FC reagent was added and mixtures allowed to stand additional 10 minutes. Finally, the absorption was measured at 670nm (Figure 1).

Effect of Interference on Biuret and Modified Biuret Methods

On mixture of 0.5ml of interfering substance and 0.5 ml (1.5mg/ml) of BSA, 3ml of the reagent was added. While standing 30 minutes for the standard method, mixture was allowed to stand 23 minutes for the modified method. Finally, the absorption was measured at 570nm (Figure 1).

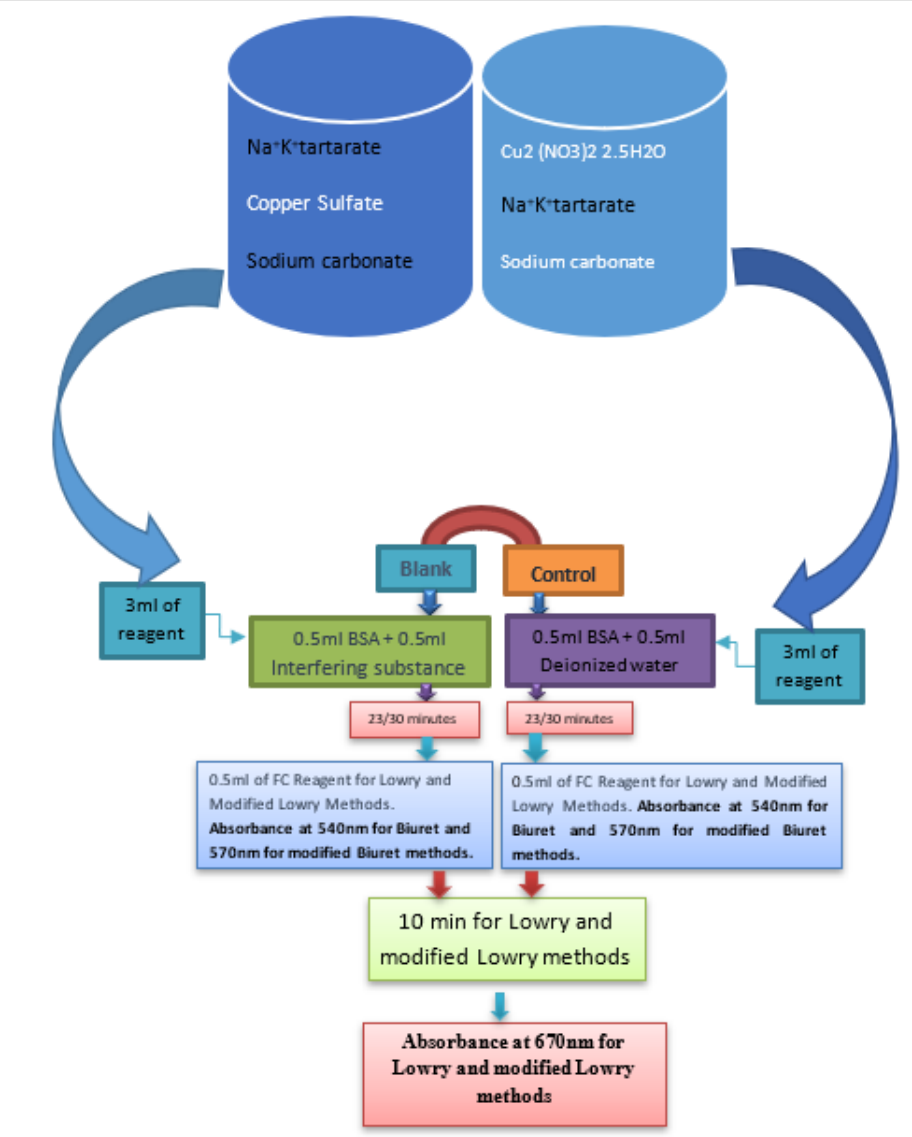

Figure 1: Procedure for the effect of interfering substances on the absorbance measurement by standard and modified Lowry and Biuret methods.

Concentration of Copper in the Original and Modified Reagents

As per the ratio given by Lowry et al. [5], concentration of Cu^{2+} ion in the original Lowry reagent was 80.42mM. With replacement of Copper source by Copper nitrate, concentration of Cu^{2+} in the modified Lowry reagent was 42.99mM. Based on the ratio described by Gornal et al. [8], concentration of Cu^{2+} ion in the original Biuret reagent was 60.318Mm. While replaced Copper source by Copper nitrate, concentration of Cu^{2+} in the modified Biuret reagent was 32.24mM.

DOC-TCA Precipitation

Based on recorded reliable interferences, DOC-TCA precipitation was conducted for the recovery of protein sample (BSA). The procedure was performed according to Peterson [7]. Briefly: mixture of 0.5 ml of interfering substance and 0.5 ml of BSA was centrifuged at 4800g for 10 min. With safe decantation of the supernatant, the pellet was kept for performing standard and modified measurements of it as per the description of Peterson [7]. Absorbance results were recorded and compared before and after precipitation.

Suitable Wave-Length Scan of the New Methods

Suitable wavelength scan was conducted for identifying best wavelength of both modified Lowry and Biuret methods. The scan was done by measuring absorbance of standard protein samples in at a wave length range of 500 to 700 nm.

Absorbance Measurement

Absorbance measurement of protein samples was performed by using Jenway 67 series spectrophotometer in the main laboratory of Department of Biochemistry, College of Health Sciences, and Addis Ababa University. All **results** were expressed in terms of Mean ± Standard Deviation.

Reliability of the Assay

Reliability of the modified assays was determined by variation of absorbance results between 24hrs/a day and between 168hrs/a week as per described by Pretty et al. [2,22,23]. Briefly, after the first absorbance measurement samples were kept at room temperature for 24hrs followed with the second absorbance measurement to determine a day coefficient of variation. Weekly coefficient of variation was also performed with the same procedure except keeping of the samples at a room temperature for a week. Reliability was expressed in terms of percent coefficient of variation. Comparison of coefficient of variation between the standard and modified methods was also performed.

Results and Discussion

The first step for methodological approval of the modified methods was checking linearity of the standard sample (BSA) absorbance measurement at a different wavelength. After measure-mental approval of both the modified Lowry (Figure 2A) and Biuret (Figure 2B) methods, the best linearity wavelength selection was followed. In both the methods, scan for selection was conducted at a wavelength rage of 500 to 700nm with a gap of 30nm in between (Figure 2). Though the modified Lowry method has shown a linearity in the selected wavelength ranges (Figure $5A_2$), the best curve of accuracy was recorded at 670nm with a similar wavelength to the original Lowry method [5]. To the nearest wavelength from the original Biuret method [8], the modified Biuret method has shown precise linearity at a wavelength of 570nm. Those observations confirm as the modified methods have validity to measure the standard protein at a specified wavelength. On the basis, tests remain to perform were conducted at 670nm for the modified Lowry method and at 570nm for the modified Biuret method. Rather than measuring concentration of BSA, validity of the new methods to measure different standard protein samples was performed with casein, gelatin and egg albumin (Figure 3). Both the modified methods manifest more linear curves for absorbance of the protein samples (Figure 3B & 3D). Since one of the ways using for demonstration of validity is testing consistency of the method for measurement of various standard samples, both our modified Lowry and Biuret assays displayed consistency with linear measurements of the mentioned standard proteins.

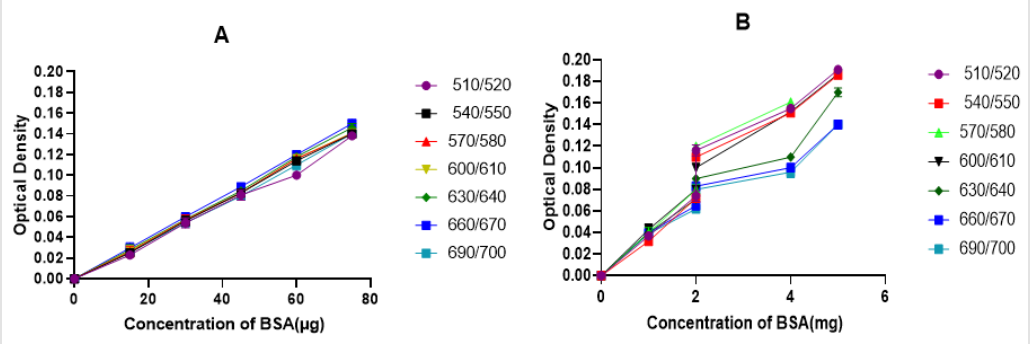

Figure 2: Scan for selection of best linearity wavelength for the modified Lowry (A) and Biuret (B) methods.

Comparison with the original methods on the basis of standard measurements (Figure 3E & 3F) was also performed and the modified methods have shown similar consistency of measurements with the original methods (Figure 3A & 3B). Recent modifications [2,22,25,26] for the original protein estimation methods were focused on improving lower limit of detection (sensitivity), resistance to ionic compounds and reproducibility of the assay. We employed Copper nitrate-based modification of Lowry and Biuret methods for developing a little adjusted procedure showing better or comparable resistance of the interfering substances such as Tannic acid, Sodium sulfite, Tris buffer, Cobaltous acetate, SDS, Sodium chromate and Cadmium sulfate (Table 2). Some of the interfering substances resulted in a decline of absorbance including negative readings with significant formation of precipitates while others upraised absorbance to provide unreliable results. As clearly shown in Table 2, as low as 15µM of tannic acid was resulted to unreliable rise of absorbance reading for both the original (Figure 4) and modified Lowry and Biuret methods. Similar results on the Lowry method were reported by Xie et al. [27]. While elevating the absorbance reading for original and modified Lowry methods, as low as 0.3mM of Sodium sulfite did not affect Biuret and modified Biuret methods. Effect of sulfites on the Lowry method was also reported [28]. Although, 0.1M of tris buffer was deviated to over estimation of protein in Biuret and modified Biuret methods, it leaded to decline of absorbance reading for Lowry and modified Lowry methods. As low as 0.2mM of Sodium chromate, Cobaltous

acetate and Cadmium sulfate were resulted for the declined negative absorbance with a precipitate formation on the original Lowry method. However, they did not show any significant effect on the modified Lowry, original and modified Biuret methods (Table 2).

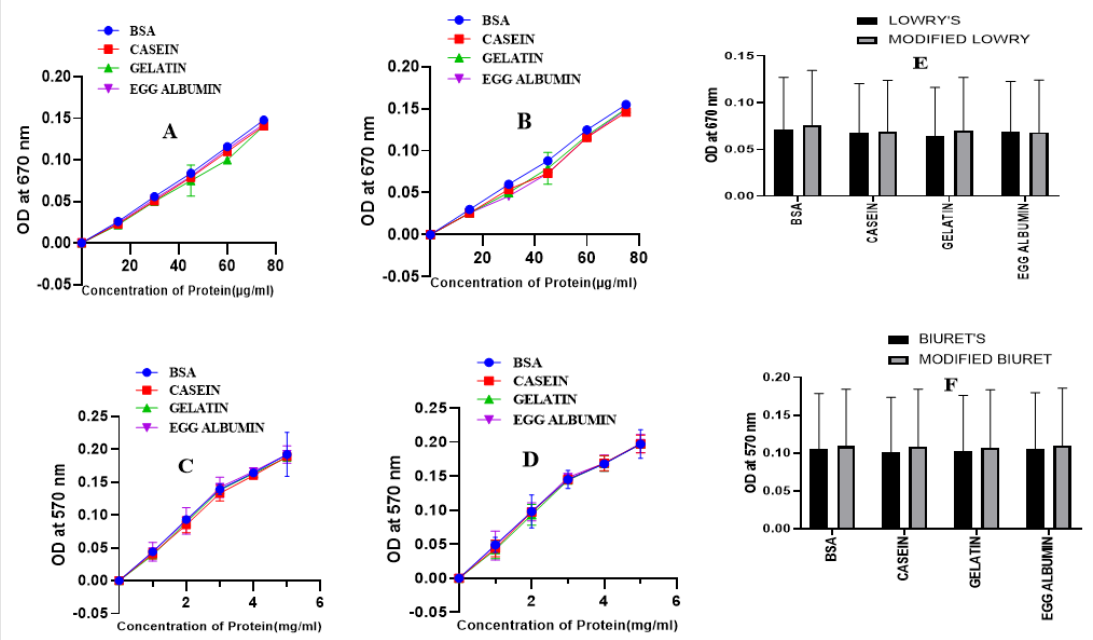

Figure 3: Standard curves of protein samples analyzed by Lowry's method (A), modified Lowry method (B), Biuret's method(C) and modified Biuret method (D). Comparison of Lowry and modified Lowry (F), Biuret and modified Biuret (G) methods for the measurement of standard proteins.

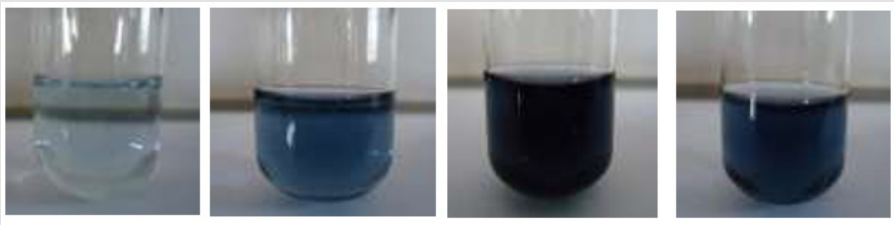

Figure 4: Photographs of BSA alone and BSA +tannic acid solutions prepared by Lowry procedure. The deep blue color development in the tannic acid concentration dependent manner; lane 1, BSA alone, lanes 2-4, 15, 20, and 25 µm of tannic acid mixed with BSA, respectively.

Table 2: Effect of different concentrations of interfering substances on the optical density measurement by Lowry, modified Lowry, Biuret and modified Biuret methods. Interferences are identified for 35µg of BSA in Lowry and modified Lowry methods while for 1.5mg of BSA in Biuret and modified Biuret methods.

Protein sample	Interfering substance	Concentration of interfering substance	Lowry method (Copper sulfate) 670nm	Lowery method (Copper nitrate) 670nm	Biuret method (Copper sulfate) 570nm	Biuret method (Copper nitrate) 570nm
BSA	**+ Tannic acid**	**0.0µM**	**0.069±0.00153**	**0.069±0.0057**	**0.079±0.0005**	**0.079±0.0005**
		5.0µM	0.07±0.00115	0.069±0.0037	0.08 ±0.0005	0.079±0.0005
		10µM	0.071±0.0005	0.069±0.0041	0.08±0.011	0.084±0.0001
		15µM	0.26±0.01	0.19±0.015	0.11±0.01	0.092±0.0001
		20µM	0.51±0.01	0.41±0.01	0.11±0.01	0.098±0.0001
	+Sodium sulfite	**0.0 mM**	**0.069±0.00153**	**0.069±0.0005**	**0.079±0.0005**	**0.079±0.0005**
		1mM	0.071±0.001	0.07±0.0005	0.079±0.0005	0.08±0.00
		2mM	0.12±0.01	0.097±0.001	0.079±0.0005	0.08±0.00

		3mM	0.198±0.001	0.164±0.0015	0.079±0.0005	0.08±0.00
		4mM	0.199±0.0005	0.171±0005	0.08±0.00	0.08±0.00
		10mM	2.497±0.001	2.15±0.01	0.08±0.00	0.08±0.00
	+Tris Buffer	**0.0M**	**0.069±0.00153**	**0.069±0.0005**	**0.079±0.0005**	**0.079±0.0005**
		0.1M	0.063±0.0015	0.067±0.0005	0.10±0.0015	0.082±0.0009
		0.2M	0.061±0.00095	0.064±0.0005	0.10±0.00095	0.0880±0.0005
		0.3M	0.05±0.0032	0.058±0.0005	0.10±0.00055	0.0930±0.0009
		0.4M	0.041±0.001	0.049±0.0005	0.10±0.0005	0.099±0.0005
		0.5M	0.033±0.0019	0.048±0.0005	0.10±0.0005	0.10±0.0095
	+Cobaltous acetate	**0mM**	**0.069±0.00153**	**0.069±0.0005**	**0.079±0.0005**	**0.079±0.0005**
		0.2mM	-0.001±0.00005	0.011±0.001	0.058±0.001	0.07±0.0005
		0.4mM	-0.002±0.0001	0.001±0.00011	0.037±0.00057	0.05±0.0015
		0.6mM	-0.002±0.00011	-0.001±0.00017	0.033±0.00057	0.04±0.001
		0.8mM	-0.004±0.00001	-0.002±0.00005	-0.025±0.00057	0.034±0.001
		1.0mM	-0.006±0.00001	-0.002±0.0001	-0.023±0.00057	-0.021±0.00057
	+SDS	**0.00 mM**	**0.069±0.00153**	**0.069±0.0005**	**0.079±0.0005**	**0.079±0.0005**
		0.2 mM	0.0686±0.00153	0.069±0.0005	0.079±0.0005	0.079±0.0005
		0.4 mM	0.069±0.00153	0.0688±0.0005	0.079±0.0005	0.079±0.0005
		0.6 mM	0.0688±0.00153	0.069±0.0005	0.0788±0.0005	0.0789±0.0005
		0.8 mM	0.069±0.00153	0.0687±0.0005	0.079±0.0005	0.0789±0.0005
		1.0 mM	0.069±0.00153	0.069±0.0005	0.0789±0.0005	0.079±0.0005
	+Sodium Chromate	**0.00mM**	**0.069±0.00153**	**0.069±0.0005**	**0.079±0.0005**	**0.079±0.0005**
		0.2mM	-0.019±0.00057	0.028±0.001	0.056±0.00057	0.073±0.00057
		0.4mM	-0.02±0.001	0.019±0.001	0.039±0.00057	0.056±0.001
		0.6mM	-0.041±00057	0.02±0.0005	0.04±0.00	0.051±0.00057
		0.8mM	-0.053±00057	-0.016±0.001	-0.039±0.00057	0.012±0.00057
		1.0mM	-0.053±0.001	-0.019±0.0015	-0.039±0.00057	-0.011±0.056
	+Cadmium sulfate	**0.00mM**	**0.069±0.00153**	**0.069±0.0005**	**0.079±0.0005**	**0.079±0.0005**
		0.2mM	-0.0019±0.00001	0.019±0.00057	-0.011±0.0005	0.012±0.0013
		0.4mM	-0.0029±0.00057	0.01±0.00023	-0.011±0.001	0.010±0.00057
		0.6mM	-0.012±0.001	-0.011±0.00057	-0.016±0.054	-0.011±0.0005
		0.8mM	-0.099±0.00057	-0.013±0.0001	-0.016±0.001	-0.012±0.00017
		1.0mM	-0.099±0.00057	-0.042±0.00057	-0.016±0.0015	-0.012±0.0011

But effects with negative readings were observed for all the methods when the concertation raised to 0.6mM. Specially, over 0.6mM of cobaltous acetate was given rise to shifting of the solution color from blue to bluish cloudy light (Figure $5A_4$). Instead of been a cause for negative absorbance reading by Lowry and Modified Lowry methods, as low as 0.6mM of sodium chromate was also resulted for shifting of solution color to light green (Figure $5A_1$). Up to 1mM of SDS was not shown any potential effect on the absorbance measurement by both original and modified Lowry and Biuret methods. In comparison, we found both the modified methods have comparable or better resistance to interferences by all the mentioned chemicals. Assay time for color development of the modified methods is compared with the original Lowry and Biuret methods (Table 3). Both the modified methods have shown development of deep blue color within 23 minutes. However, the original methods used an incubation time of 30 minutes for a light blue color development [5,8]. One of the expected hypotheses might be the fastest reaction formation capacity of nitrate ion to provide an easy access of copper ion for complex formation with the protein. The other hypothesis with ideal similarity of Pretty et al. [23] is when the concentration of Cu^{2+} reduced in the reagent, compactness of Cu^{2+} in the solution was also reduced and leaded to fastest movement of Cu^{2+} to form complex with the protein. On the basis, our modification confirms domination of rapidity over the original Lowry and Biuret methods. Reliability of the modified methods was employed based on daily and weekly percent coefficient of variations. For a reliability checkup, the determination of variation was performed with standard samples of BSA, Casein, Gelatin and Egg albumin.

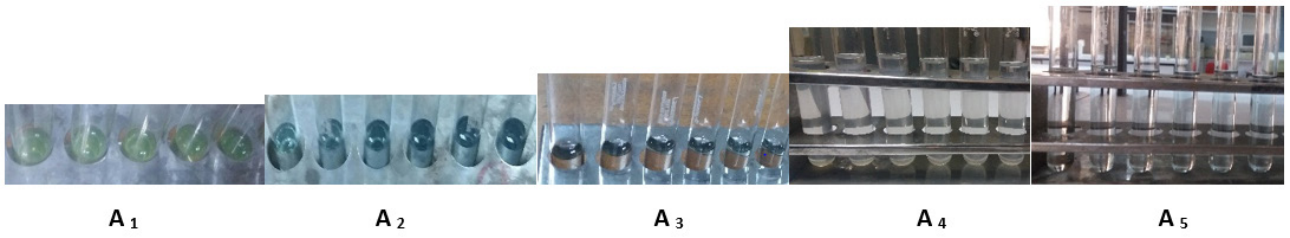

Figure 5: Photographs showing effect of Sodium chromate in Lowry Protein assay (A_1), Standard measurement of BSA by Modified Lowry assay (A_2), effect of cadmium sulfate on the modified Lowry assay (A_3), effect of cobaltous acetate on the Lowry assay (A_4) and effect of sodium sulfite on the modified Lowry assay (A_5).

Table 3: Effect of substitution of Copper nitrate by Copper sulfate on the incubation time of Lowry and Biuret methods.

Assay method	Source of Cu^{2+}	Incubation period(min)
Lowry	Copper sulfate	30
	Copper nitrate	23
Biuret	Copper sulfate	30
	Copper nitrate	23

As shown briefly in Table 4, both the modified Lowry and Biuret methods were been recorded with low amounts for coefficient of variation. Even in comparison with the original methods reliability of the new methods reflect better or comparable results. DOC-TCA precipitation was conducted to purify protein samples from the recorded interferences by the interfering substances (Table 2). Most of the interfering substances were abolished by the precipitation. However, threshold amounts of Sodium chromate, Cadmium sulfate, and Sodium sulfite carried over from the precipitation were caused significant reduction of absorbance measurement by Lowry and modified Lowry methods (Table 5). So, effect of other interfering substances was efficiently avoided by DOC-TCA precipitation. Though, substances carried over from the precipitation caused significant interference, our farther study will continue on checking of additional purification methods for adequate abolishment of those substances. In comparison, after DOC-TCA precipitation, both the modified methods have shown dominated resistance over the original ones. Especially the modified Biuret method was manifested better resistance for threshold amounts of interfering substances which bypassed DOC-TCA precipitation.

Table 4: Daily and weekly based coefficient of variations for the standard and modified Lowry and Biuret methods.

Protein	Daily based coefficient of Variation (%)				Weekly based coefficient of variation (%)			
	Lowry	Mod. Lowry	Biuret	Mod. Biuret	Lowry	Mod. Lowry	Biuret	Mod. Biuret
BSA	0.91	0.86	0.95	0.90	0.85	0.80	0.91	0.87
Casein	0.894	0.81	0.93	0.88	0.83	0.79	0.90	0.85
Gelatin	0.90	0.87	0.93	0.89	0.87	0.81	0.91	0.88
Egg albumin	0.892	0.82	0.91	0.871	0.87	0.81	0.89	0.84

Conclusion

Hemi-Penta hydrated Copper nitrate-based modification of Lowry and Biuret methods resulted for a better validity and reliability with a reduction of assay time in both methods by 7 minutes. The modification also increased resistance of the methods for interferences by SDS, Tris buffer, Sodium sulfite, Cadmium sulfate, Sodium chromate, Cobaltous acetate and Tannic acid.

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