

## Comparative Study of References and Protein Quantifications Using Biuret-Spectrophotometric Method

Ahmad Syauqi\*, Muhamad Fuadi, Hari Santoso

Department of Biology, Faculty of Mathematics and Natural Sciences, Islamic University of Malang (UNISMA)

\*Corresponding author: [ahmad.amsyqi@gmail.com](mailto:ahmad.amsyqi@gmail.com)

DOI: <https://doi.org/10.24198/cna.v6.n2.19224>

**Abstract:** Comparison between the references and the quantification of biuret standard curves can be performed using statistical methods. The objective of this study was to select a method of protein quantification of the biuret-spectrophotometric based on the similar standard curve of the compound. The study used experimental methods in the laboratory and references from a comparative scheme of mean that has categories of statistics called academic and practical terms. Two standard curves were tested with the reference data. The academic way was performed with a comparison of 11.779-12.401% confidence, and the quantification of the results of the protein mean of 8.211% and 10.17% showed no significant difference. The practical methods were carried out with the original reference data of 10.8-12.8%, and the result of the test confidence values of 9.163-11.180% and 7.596-8.826% showed different accuracy results. The quantifications of the protein biuret method displayed different results on how to compare according to academic and practical ways. The quantification method using the Biuret-spectrophotometric practical way shows accuracy by a certain standard curve compared with the original values of references.

**Keywords:** standard curve, biuret-spectrophotometry, protein quantification

**Abstrak:** Perbandingan antara acuan dan kuantifikasi kurva standar biuret dapat dilakukan dengan metode statistika. Tujuan dari penelitian ini adalah untuk memilih metode kuantifikasi protein menggunakan metode spektrofotometri biuret berdasarkan kurva standar yang mirip dengan senyawa analit. Pada penelitian ini digunakan metode eksperimental di laboratorium dan acuan dari skema perbandingan rerata yang memiliki kategori statistika yang disebut cara akademik dan cara praktis. Dua kurva standar diuji dengan data acuan. Cara akademik digunakan dengan perbandingan 11,779-12,401% kepercayaan, dan kuantifikasi hasil rerata protein sebesar 8,211% dan 10,17%, menunjukkan tidak ada perbedaan nyata. Cara praktis dilakukan dengan data acuan asli pada 10,8-12,8%, dan hasil uji kepercayaan sebesar 9,163-11,180% dan 7,596-8,26% menunjukkan perbedaan hasil akurasi. Kuantifikasi protein dengan metode biuret menggunakan cara praktis menunjukkan akurasi dengan kurva standar tertentu dibandingkan terhadap nilai asli acuan.

**Kata kunci:** kurva standar, biuret spektrofotometri, kuantifikasi protein

### INTRODUCTION

A standard on chemical compound analysis is intended as a comparator, and it can provide consistency for repeat measurements known as reliability. The standard needs to be measured or tested in order to identify whether it really shows consistency. The determination method of selected protein compounds such as biuret and spectrophotometric techniques is very necessary to compare to the standard nitrogen content in the form of concentration. Protein compounds in biological substances are of prime importance such as the purpose of nutrition for the body energy of living things, a work of genetic code, or cell metabolism. The standard for comparison on protein quantity determination is desirable for only one and applies

appropriately to the scope of measurement using a spectrophotometer instrument.

A comparable standard appropriately provides repeatable measurement results and is believed to be a matter of validity and reliability (Sireci 2014). Knowing correctly the standard comparator through statistical techniques test is required in many procedures and necessarily used on proper and trusted results. The accuracy of spectrophotometric techniques associated with statistical techniques has not yet been widely addressed in particular to standard comparators, i.e. standard curve (Sireci 2014; Trihendradi 2007). Statistical techniques have been academically done in the analysis of a replication in order to identify whether it is an experimental unit or a sample taken. This is like a

standard procedure that follows the theory for knowing the value of variance, deviation, and standard error of mean (Sireci 2014). However, is it appropriate for the Biuret-spectrophotometric method? The limitations in this case are nullified systematical and human/random errors.

The true value in measurement is the mean in a series of data. Accuracy of a measurement is the closeness of the agreement in the result of a measurement and that is the qualitative concept about low or high, but in practice is understood as accurate to  $\pm X$ . The uncertainty was understood toward methods of calculating the value  $\pm X$  (Wolming & Wikström 2010).

Practical terms such as the requirement of work standardization between laboratories have a comparative technique that essentially retests a measurement result (Yamamoto *et al.* 2009). Most protocols of laboratories have a high probability of experimental error that causes failure; uncertainty is less fraught to technology and needs flexibility in designing laboratory exercise that in practice can be pulled off with new confidence (MML.ILS 03 2001).

A good result of measurement in systematic error can be avoided by the calibration of instruments, but the variations of measurement techniques result in human/random error like reading the digit of absorbance at a certain voltage of spectrophotometer (Stiller 2006).

The consistency of a result value on each replication of measurement is important in determining how technical comparing has certain variations. Reliable yields are presented in the form of confidence interval values and for the scope of biological materials at a 5% error in general. The value of confidence interval is obtained by determining the standard error of mean, whether to use the technical academic or practical category above (Trihendradi 2007). The Biuret-Spectrophotometric method has the type of data diversity, first in the data of  $\text{Cu}(\text{NH}_3)_4^{2+}$  standard compounds of linear regression and second in the diversity of the protein quantity results from sample replication.

The variance of the standard  $\text{Cu}(\text{NH}_3)_4^{2+}$  compound data can be used to determine the standard error of mean for the observable protein value. The comparative reference of protein values can be calculated by variance and denoted confidence interval value with uncertainty toward reference protein value. The standard  $\text{Cu}(\text{NH}_3)_4^{2+}$  data in the form of two kinds of regressions have curve equations and needs to obtain one. That shows consistent test results through two kinds of protein values; the first is the observable value and second the reference. Therefore, the objective of the study is to select a method of protein quantification of the Biuret-Spectrophotometry based on the standard curve of the  $\text{Cu}(\text{NH}_3)_4^{2+}$  compounds that is similar in the samples.

## MATERIAL AND METHODS

### Materials

Visible Spectrophotometer (752 UV grating), stopwatch, analytical balance HWH DJ 6002 A, Pyrex glass volume, cupric sulfate pentahydrate (crystals), potassium sodium tartrate tetrahydrate (Merck), distilled water, sodium hydroxide (Merck), potassium iodide Merck. The Biuret reagent follows Robyt and White: 3 g of cupric sulfate pentahydrate and 9 g of potassium sodium tartrate tetrahydrate dissolved in 500 mL of sodium hydroxide 0.2 N, to which 5 g potassium iodide was added, and the entire mixture diluted into 1 L of water (Syauqi 2014).

### Methods

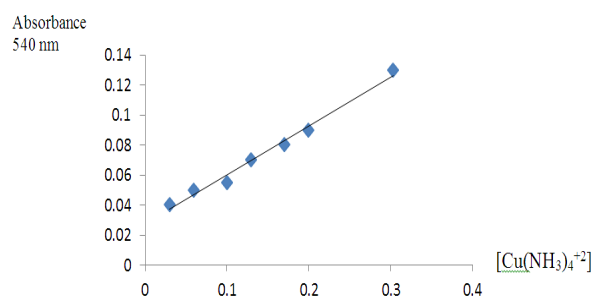
The study used experimental methods in the laboratory and references with a comparative scheme of mean that has categories of statistics called academic and practical terms. The variables of study are regression equations, namely:

- Variable I was standard curve I-reference;
- Variable II was standard curve II.

The controlled variable is the absorbance value of 0.243 at 540 nm. The analysis is designed to compare the quantitative value of protein confidence values of fish albumin at lower and upper limit mean values, egg white confidence values, and original values of references.

### Standard Curve I-reference

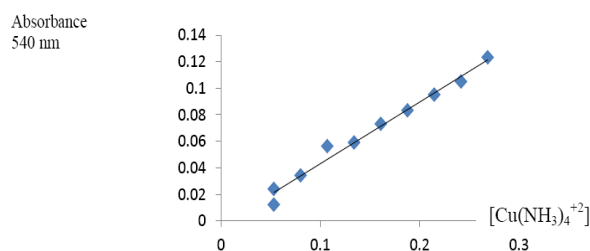
The data and regression equation were taken from reference (Figure 1) (Syauqi 2017).



**Figure 1.** Standard Curve I-Reference for determination of Nitrogen samples using Biuret-Spectrophotometry has slope of 0.327

### Standard Curve II

Took biuret reagent at 10 mL and added 10 mL of  $\text{NH}_3$  2 M and 5 mL of distilled water. The biuret reagent was replicated for 9, 8, 7, 6, 5, 4, 3, 2, and 1 mL, respectively.  $\text{Cu}(\text{NH}_3)_4^{2+}$  complex compound concentrations in 25 mL volume was calculated respectively and read the absorbance in spectrophotometer at 540 nm spectrum. The standard curve regression equation was calculated by Excel Office of Windows operating system. The result is shown in Figure 2.



**Figure 2.** Standard Curve II for determination of Nitrogen samples using Biuret-Spectrophotometry has slope of 0.464

### Quantitative Value of Protein Test of Fish Albumin

Albumin molecule preparation by Marmon & Undeland (2010): Blended 5 g of fish meat with distilled water at 1:6 ratio and dropped 10% NaOH until 11.5 pH. Centrifuged the fish meat paste was done at 10.000 rpm for 10 minutes. Cooled the precipitation in the refrigerator for 5 hours and dropped supernatant 10% H<sub>2</sub>SO<sub>4</sub> until 5.5. Cooled for 5 hours and centrifuged at 10.000 rpm for 10 minutes. The precipitation of 0.5 mL was added with distilled water and stirred using a magnetic stirrer for 5 minutes. Took 3 mL, added 3 mL of biuret reagent, incubated at 37°C in a water bath for 10 minutes, and read the absorbance in spectrophotometer at 540 nm. The quantity of the albumin is:

$$\% \text{ Albumin} = [(v \cdot N \cdot P) / 10s] \times 6.25$$

Where  $v$  = result of precipitate [mL]  
 $N$  = Nitrogen quantity result of standard curve [mg/mL]  
 $P$  = Factor of albumin precipitate dilution  
 $s$  = weight of sample [g]

### Quantitative Value of Protein Test of Egg White

The protein value and absorbance value of 0.243 at 540 nm spectrum taken from reference (Syauqi 2017).

### Reference Mean Confidence and Test

The standard error of mean  $e_m$  of the sample has an accuracy value of  $\pm s/(n^{1/2})$ , which is an estimation for  $\sigma_m$  data population and follows the normal distribution named *Empirical Rule*:

$$P[m - (2s/\sqrt{n}) \leq \mu \leq m + (2s/\sqrt{n})] \geq 0.95.$$

Using t-factor according to WS Gosset (Mursyidi 1984) has the formula (Syauqi 2015):

$$P[m - (t_{0.05(1)} s) / \sqrt{n} \leq \mu \leq m + (t_{0.05(1)} s) / \sqrt{n}] \geq 0.95.$$

The proposed hypothesis is  $H_0: \mu = a$ ; and  $H_1: \mu \neq a$  at  $\alpha = 95\%$  ( $P=1-\alpha$ ). The test used the *two-tailed*

*test*. The computation was done using Excel Office application of Windows 10 operating system. The formula of the calculated t-value ( $t_s$ ) is:

$$t_s = |a - \mu| / (s/\sqrt{n}) \quad \dots\dots\dots (1)$$

$$t_s = |\mu_I - \mu_{II}| / \sqrt{1/n(s_I^2 + s_{II}^2)} \quad \dots\dots\dots (2)$$

Where  $t_s$  = calculation of t,  
 $a$  = the determination protein value by standard curve  
 $\mu$  = mean of protein data population  
 $\mu_I$  = mean of data by variable I  
 $\mu_{II}$  = mean of data by variable II  
 $s$  = standard deviation of data  
 $s_I^2$  = variance of variable I  
 $s_{II}^2$  = variance of variable II  
 $n$  = population number of data

### Confidence of Nitrogen Concentration Determination and Test

The accuracy procedure (Wolming & Wikström 2010; Syauqi 2015) or the limit of concentration uncertainty  $\pm [t \cdot (V_C)^{1/2}]$  was calculated and done using the DOSBOX application in Windows 10 operating system.

The nitrogen concentration variance ( $V_C$ ) value used the formula (Mursyidi 1984):

$$V_C = (V_A / b^2) \{1 + 1/N + [(A_{\text{sample}} - A_{\text{standard}})^2 / b^2] \cdot [1 / \Sigma(C_{\text{standard}} - C_{\text{standard}})^2]\} \quad \dots\dots(3)$$

Where  $V_C$  = the limit of observable concentration accuracy  
 $V_A$  = variance of Standard Absorbance 540 nm  
 $N$  = population number of data  
 $b$  = regression parameter as slope of curve  
 $C$  = Nitrogen concentration of Cupric tetramine complex ion [Cu(NH<sub>3</sub>)<sub>4</sub>]<sup>2+</sup>  
 $A_{\text{sample}}$  = observable of sample absorbance value  
 $A_{\text{standard}}$  = mean of standard absorbance value  
 $\Sigma(C_{\text{standard}} - C_{\text{standard}})^2$  = result value that used operational formula of  $\Sigma(C^2) - [\Sigma^2 C] / N$

Hypothesis test was used to plot values (Yamamoto *et al.* 2009; Syauqi 2015).

## RESULTS AND DISCUSSION

This research used assumptions; the data variance of the  $\text{Cu}(\text{NH}_3)_4^{2+}$  standard compound in the process of calculating the parameters of linear regression equation and the variance of reference data protein values could not be used for the analysis of two average (two populations). The uncertainty of protein determination using a standard curve was given to one value and not the average population of the data. The analytical methods were categorized into academic and practical ways. The academic way corresponded with the general habits in the study, and analysis was done to compare the two prevailing values. Meanwhile, the practical way designated practical needs that applied for laboratory calibrations.

### Academic Way

The quantification results of fish albumin from the standard curves of I and II were consistent at the level of significance. The standard curve for the albumin quantification in statistical analysis was addressed to each replication. The confidence of all data replication was obtained from the uncertainty of standard curve,  $\pm [t \cdot (V_C)^{1/2}]$ , which is accuracy value (Morey *et al.* 2016). Tables 1-3 provide the lower and upper limit confidence values as the data population. The lower and upper limit patterns were used for comparative tests. Standard curve I had a significant value of  $P > 0.05$ , while standard curve II showed the similarity and vice versa when  $P < 0.05$ . The use of standard curves I and II showed the equal confidential results.

**Table 1.** Significant Value of Two Sample Tests toward Percentage Data Population of Fish Albumin Quantification from Lamongan

Kinds of Standard Curve Equation	Data Population of % Fish Albumin Quantification from Lamongan	$t_s$ Value	Assuming Equal Variances and Two Tails ( $P=1-\alpha$ )
I-Reference II	Lower Limit of mean	0.081	0.837
I-Reference II	Upper Limit of mean	- 0.069	0.946

**Table 2.** Significant Value of Two Sample Tests toward Percentage Data Population of Fish Albumin Quantification from Lumajang

Kinds of Standard Curve Equation	Data Population of % Fish Albumin Quantification from Lumajang	$t_s$ Value	Assuming Equal Variances and Two Tails ( $P=1-\alpha$ )
I-Reference II	Lower Limit of mean	0.164	0.782
I-Reference II	Upper Limit of mean	- 0.145	0.887

**Table 3.** Significant Value of Two Sample Tests toward Percentage Data Population of Fish Albumin Quantification from Lamongan and Lumajang

Kinds of Standard Curve Equation	Two Populations of % Fish Albumin Quantification from Lamongan and Lumajang	$t_s$ Value	Assuming Equal Variances and Two Tails ( $P=1-\alpha$ )
I-Reference	Lower Limit of mean	2.899	0.015
	Upper Limit of mean	2.275	0.021
II	Lower Limit of mean	2.944	0.014
	Upper Limit of mean	2.687	0.022

### Test on Fish Albumin

The standard curve consists of 2 types that were used to calculate the percentage value of the protein, and the results were analyzed according to the statistical procedure. The mean difference analysis of the two populations of data (Table 1-2) was done to each of the lower and upper limit values. The standard deviation was derived from the variance of  $\text{Cu}(\text{NH}_3)_4^{2+}$  standard compounds data. The variance is the result of regression analysis as mentioned in the above method. Each value of data protein has a relative uncertainty depending on the reading of absorbance by the spectrophotometer and Formula (3). The uncertainty value contains two elements: first is the standard curve, and second from reading the value of light transmittance (%T) as an intrinsic character of spectrophotometer instrument (Sooväli *et al.* 2006). The light transmittance at 99% has an uncertainty value of 4%, which means that %T is greater than 80 in value or lower than A at 0.1 (Demertzis *et al.* 2012). The transmittance of light is 20-80% or the absorbance interval 0.1-0.7 of the 540 nm spectrum, giving the value of uncertainty at 1%. According to formula (1), the analysis result acceptance of the null hypothesis that the standard curves of I-reference and II were not different significantly ( $P = 0.05$ ). Similarly, it was applied to test the standard curve toward lower and upper limit values based on different sampling locations (Table 3). The results of the analysis showed consistency of significant differences ( $P = 0.05$ ) for each of the two lower and two upper data populations. This means that the two standard curves are not different in producing the quantities of albumin protein.

The next test toward variables was based on the knowledge of interval of means and how the analysis should be done. Interval of means refers to the values between the lowest and the highest with the trend at true value, namely average or median. The data are in the form of intervals, as in this case, so that each data in the data sets is the result of determination based on the standard curve that can provide the lowest and highest values. The analysis of the data to identify the two standard curve variables is known as an interval mean (Syauqi 2015). The purpose of data interval

analysis is the use of a value that is imposed to the true value (mean) in a data set. Consideration to the cause of the interval is the relative uncertainty generated by a standard curve. If a set of data and each datum has a relative uncertainty, that additional procedure of uncertainty becomes unsuitable because it will give the relative uncertainty of measurement an accumulation and the value becomes high percentage.

Understanding the interval in this case is the relative uncertainty of each datum in a set of data. Therefore, the relative uncertainty value in the comparative analysis is proposed with the first assumption that the value is continuous and not a ratio. Secondly, the data set can be searched for its middle value (mean) as the center of the relative uncertainty tendency. Both assumptions state that the mean value can be given to the measurement average as a mean's relative uncertainty of a data set. Table 4 shows the two-tailed t-test (Formula 2) was done toward two means of two population data, and the result is consistent with the Lamongan data, in which the relative uncertainty of the standard curve between reference and experiment is significantly different ( $P=7.238\text{E-}08$ ), while the Lumajang data set is not ( $P=0.067$ ). The parameter of data variance on Lumajang is higher than Lamongan. The mean with the higher variance is the absorbance values of the samples that are lower than 0.1.

### Test on Chicken Egg White

Table 5 shows that the reference data were analyzed according to the t-distribution at 95% confidence and the accuracy of value was  $(11.6 \pm 0.801)\%$ . The comparison method with one (true) value of the determination result of standard curve, i.e. I-references is 8.211 and II is 10.17%. Based on the reference data, the variance showed no significant differences through the difference analysis test of true value from the standard curve and mean of reference. In other words, the null hypothesis is accepted and the alternative is rejected. Between the reference mean and protein quantified value of the two standard curves is equal ( $P = 0.05$ ).

**Table 4.** Two-tailed t-Test: Two Samples Assuming Equal Variance toward Variables

Parameters	Lamongan Data		Lumajang Data	
	I-Reference	II	I-Reference	II
Mean	9.0466	7.646	13.213	10.573
Variance	0.01978	0.03678	7.2415	2.2547
Observations	6	6	6	6
Pooled Variance	0.02828		4.7481	
Hypothesized Mean Difference	0.05		0.05	
df	10		10	
t Stat	13.9028		2.05872	
P(T<=t) two-tailed	7.23818E-08		0.06652	
t Critical two-tailed	2.2281		2.2281	

**Table 5.** Protein Quantification Values based on Standard Curve and Statistical Mean Value from Reference

Kinds of Standard Curve Equation	Absorbance Value at $A_{540}$ nm of Egg White (Syauqi 2017)	True Value of Protein (%)	Confidence Interval of Protein (%) in Reference <sup>8</sup>	Calculation of t	Significance (P)
I- Reference		10.170		0.761	>0.05 (0.488)
II	0.243	8.211	(11.6 ± 0.801)	1.804	>0.05 (0.145)

**Table 6.** Protein Quantification Values based on Uncertainty of Respective Standard Curve and Protein (%) of Reference Original Values

Kinds of Standard Curve Equation	Absorbance value at $A_{540}$ nm of Egg White (Syauqi 2017)	Measured Accuracy of Protein (%)	Protein (%) of Reference Original Value <sup>8</sup>	Equalization
I-Reference		(10.170 ± 1.007)		equal
II	0.243	(8.211 ± 0.615)	10.8-12.8	not equal

### Practical Way

The accuracy is indicated by the confidence interval of the reference curve of I-reference 9.163-11.180% (Table 6), and there are several values of 10.8-11.180 equal to the original reference data at interval of plot values of 10.8-12.8%. However, the confidence value of standard curve II compared with the original reference data is none of the equal value. The values of 7.596-8.826 are below the value of 10.8 of the reference data. This is known as the accuracy of protein quantification results of two standard curves showing differences. In other words, there are no values that are equal with the interval of 10.8-12.8%.

### CONCLUSIONS

The academic way of the two standard curves of I-reference and II shows consistency in the fish albumin protein and egg white protein quantification. The two kinds of standard curves of I-reference and II showed differences in the practical way. Uncertainty in biuret-spectrophotometric methods was derived from the data variance of standard  $\text{Cu}(\text{NH}_3)_4^{2+}$  compounds. The quantification method of Biuret-spectrophotometric practical way showed accuracy (exactness) by a certain standard curve compared with the original values of references.

### REFERENCES

- Demertzis, M.A., Palios, A.I., Calin, A.V., Vijdeluc, C.I.M., & Chrysafis, G.A. (2012) spectrophotometric error due to uncertainty in reading the signal. *Journal of Chemistry and Chemical Engineering*. 6(7): 585-590.
- Marmon, S.K. & Undeland, I. (2010). Protein isolation from gutted herring (*Clupea harengus*) using pH-shift processes. *Journal Of Agricultural and Food Chemistry*. 58(19): 10480-10486.
- MML.ILS 03. (2001). Publication 3, Material 3 Theory of Uncertainty, in The Technical Implementation Unit of Balai LIN-LIPI-Indocal Laboratory System Limited Co., Quality Management Training on Laboratory of SNI 19-17025-2000 third Batch; June 20<sup>th</sup>-22<sup>nd</sup>, Indocal Laboratory System Limited Co., Bandung, 12
- Morey, R.D., Hoekstra, R., Rouder, J.N., Lee, M.D., & Wagenmakers, E.J. (2016). The fallacy of placing confidence in confidence intervals. *Psychonomic Bulletin & Review*. 23(1): 103-123.
- Mursyidi, A. (1984). *Statistika Farmasi dan Biologi*. Ghalia Indonesia. Jakarta.
- Sireci, S.G. (2014). Validity Theory and Applications. In Balakrishnan, N., Colton, T., Everitt, B. Piegorsch, W., Ruggeri, F., & Teugels, J.L. (eds), Wiley StatsRef: Statistics Reference Online.
- Sooväli, L., Rõõm, E.I., Kütt, A., Kaljurand, I., & Leito, I. (2006). Uncertainty sources in UV-Vis spectrophotometric measurement. *Accreditation and Quality Assurance*. 11(5): 246-255
- Stiller, J.W., & Coggins, T.C. (2006). Teaching molecular biological techniques in research context. *The American Biology Teacher*. 68(1): 36-42.
- Syauqi, A. (2015). Bio statistic Quantification of Statistical Parameter Survey and Biological

- 
- Experiment, Faculty of Mathematics and Natural Sciences, Islamic University of Malang (UNISMA), Malang.
- Syauqi, A. (2014). Biochemical Technique Theory and Practice, Central Laboratory of Islamic University of Malang (UNISMA), Malang.
- Syauqi, A. (2017). Standard Curve of Biuret Method-Spectrophotometry Using Ammonia for Protein Molecule, An unpublished Article.
- Trihendradi, C. (2007). *Kupas Tuntas Analisis Regresi: Strategi Jitu Melakukan Analisis Hubungan Causal*. Penerbit Andi. Yogyakarta.
- Wolming, S. & Wikström, C., (2010). The concept of validity in theory and practice. *Assessment in Education: Principles, Policy & Practice*, 17(2): 117-132.
- Yamamoto, T., Yamamoto, M. & Miyahara, K., (2009). Accuracy of Standard Blocks for Hardness and Uncertainty of Hardness. XIX IMEKO World Congress Fundamental and Applied Metrology on September 6-11, Lisbon, Portugal,
-