THE BIURET TEST AS APPLIED TO THE ESTIMATION OF WHEAT PROTEIN[1]

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ABSTRACT

The biuret test developed by the author in 1949 for estimating protein content in wheat and flour has been modified to make it simpler and more accurate. Formerly, aliquots of cleared alkaline protein extracts were combined with measured amounts of the alkaline copper reagent. In the modified method the stabilized alkaline copper reagent is applied directly to the weighed sample for simultaneous extraction and reaction. Color intensities of the centrifuged test solutions, as determined in a spectrophotometer, are closely correlated with the protein contents of the samples as determined by the Kjeldahl method. Comparative tests indicate that sodium potassium tartrate is superior to glycerol as a stabilizer for copper in the biuret reagent.

During recent years a number of simple quantitative tests for protein in wheat and flour have been introduced. Among these, the biuret test as developed by the author (3) in 1949 has attracted favorable attention. In the original procedure, the protein extracted from the sample by shaking for 10 minutes with dilute alkali was treated with the alkaline copper reagent. In the procedure described in the present paper, the alkaline copper reagent serves also as the extractant, so that extraction and reaction proceed simultaneously. Thus, extraction is more nearly complete, and two volumetric measurements are eliminated. In older biuret methods, copper is held in solution by strong alkali for more or less complete reaction with the protein. But the copper tends to precipitate as the hydroxide before the reaction is complete. A more reliable result can be obtained if the copper is more completely stabilized by one of several possible agents. Ethylene glycol was proposed for this purpose by Mehl (2) in 1945. Sols (5) in 1947 employed glycerol. This agent was also used by the author in 1949 and in the present studies.

Jennings (1) has suggested sodium potassium tartrate as a copper stabilizer. This agent is used in the well-known Fehling test for sugar.

In this paper, there are presented the results of some biuret protein tests on samples of wheat and flour selected from the wheat classes hard red spring, hard red winter, soft red winter, white, and durum.

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These studies were designed to show the usefulness of the test in estimating protein when using glycerol or sodium potassium tartrate as the copper stabilizer in the biuret reagent. The protein content of the samples was determined by the usual Kjeldahl method.

Method

Apparatus: (1) Balance, analytical.
(2) Shaker; motor-driven rack which inverts stoppered centrifuge tubes about 60 times per minute.
(3) Centrifuge; Clay-Adams "Senior"[3]; speed, 4,000 r.p.m.
(4) Mill, Labconco.
(5) Spectrophotometer; Coleman Jr., Model 6A, fitted for ½-in. square cuvets.

Reagents:
A. Glycerol reagent. To 937 ml. distilled water, add 10 ml. of 10*N* potassium hydroxide solution and 3.0 ml. of glycerol. Add slowly with vigorous stirring, 50 ml. of 4% $CuSO_4 \cdot 5H_2O$ solution.
B. Sodium potassium tartrate reagent. To 930 ml. of distilled water, add 10 ml. of 10*N* potassium hydroxide solution and 20 ml. of 25% sodium potassium tartrate solution. Add slowly with vigorous stirring, 40 ml. of 4% $CuSO_4 \cdot 5H_2O$ solution.

In preparing the reagents, copper hydroxide may be precipitated if the solution is not stirred vigorously during addition of the copper sulfate. The reagents should be discarded if they are not perfectly clear and free of sediment.

Procedure: Weigh into an 80-ml. centrifuge tube (or other suitable container) 0.6 g. of sample for use with reagent A or 0.5 g. for use with reagent B. Mix with 1 ml. of carbon tetrachloride, and then add 50 ml. of the indicated reagent. Stopper and shake vigorously for 10 minutes, let stand 1 hour, and then mix thoroughly. Transfer a portion to a 20-ml. centrifuge tube and centrifuge until perfectly clear. Determine the color intensity at 550 mμ (3) in the spectrophotometer with the indicated reagent as the blank.

In establishing a curve or chart to convert biuret to protein values, a regression equation should be derived (4) from the biuret and Kjeldahl protein values of 35 or more samples selected to represent the normal protein range.

Experimental

Each of the reagents, A and B, was used in testing weighed portions of a sample of flour containing 17.5% protein. The weights of these portions were varied to represent the protein range 2 to 20%. In addition, a third reagent was used. This reagent, designated as "modified A," was similar to reagent A but contained only 40 ml. of copper solution per liter, as in reagent B. From the paired values, absorbance and percent protein, the regressions were calculated. These are shown in Table I.

The starch in wheat and flour samples adsorbs some of the copper. This reduces the color of the reagent and the observed absorbance

[3]Mention of specific trade names or instruments is made for the purpose of identification and does not imply any endorsement by the United States Government.

of the test solution. In order to determine the magnitude and net effect of this factor, the reagents were applied to testing purified wheat starch. A portion of each reagent was diluted with 10*N* potassium hydroxide solution, three parts to one part of reagent. Portions of the starch weighing 0.4 g. were then tested with each of the reagents, diluted and undiluted. In determining the color intensities in the spectrophotometer, a water blank was used. The results are shown in Table II.

In the previous paper (3), it was found that about 85% of the total protein (Kjeldahl) in wheat and about 95% of the total protein in flour were extracted in the test. In applying the modified method, the protein concentration in the test solutions of eight samples of wheat and four samples of flour were determined by the Kjeldahl

TABLE I

REAGENT COLOR INTENSITY AND RESPONSE

Reagent	Water Blank: Reagent Color Intensity Absorbance [a]	Reagent Blank			
		Regression Equation: Absorbance × % Protein	Absorbance at 14% Protein Level	r	Sy.x
A	0.170	y = 34.6x — 0.5	0.419	0.9994	± 0.21
Modified A	0.150	y = 40.5x — 0.2	0.350	0.9996	± 0.16
B	0.050	y = 38.3x — 0.4	0.376	0.9995	± 0.19

[a] Protein calculated on the assumption 0.6-g. sample for reagent A or 0.5-g. sample for modified A and for reagent B corresponds to 17.5, the percent protein in sample. Thus, 0.1-g. sample = 1/6 × 17.5 = 2.9%, for A, or 1/5 × 17.5 = 3.5% for modified A and for B.

TABLE II

ADSORPTION OF COPPER BY STARCH

	Glycerol Reagent		NaK Tartrate Reagent	
	Undiluted	Diluted	Undiluted	Diluted
Reagent absorbance	0.170	0.051	0.053	0.023
Starch test absorbance	0.141	0.024	0.048	0.021
Difference	0.029	0.027	0.005	0.002
Equivalent protein, %	1.5	1.4	0.5	0.4

TABLE III

PERCENTAGE OF THE TOTAL PROTEIN (KJELDAHL) EXTRACTED BY THE REAGENTS

	Wheat				Flour	
	Hard Red Winter		Durum		Hard Red Spring	
	Reagent A	Reagent B	Reagent A	Reagent B	Reagent A	Reagent B
	88.4	89.4	86.0	89.2	99.6	98.9
	89.5	91.0	87.6	90.8	100.0	100.0
	89.7	92.2	88.8	91.0	100.0	100.0
	89.2	92.9	90.4	92.3	100.0	100.0
Average	89.2	91.4	88.2	90.8	99.9	99.7

method. These values are shown in Table III as percentages of the total protein of the samples.

A statistical summary of the results obtained in applying the modified biuret test to the samples selected from the five wheat classes is presented in Table IV. For comparison, certain values from the previous paper are also included.

The errors in the predicted protein values of the 24 hard red winter wheat samples selected for the present study and of the 100 hard red winter wheat samples in the previous work (3) have been calculated and classified as shown in Table V.

TABLE IV

STATISTICAL SUMMARY OF THE RESULTS OBTAINED IN TESTING SAMPLES OF THE FIVE MAIN CLASSES OF WHEAT AND OF FLOUR OF HARD RED SPRING WHEAT

REAGENT AND MATERIAL	N	REGRESSION	r	Sy.x	s (PROTEIN)
Glycerol (a)					
Wheat: HRS	32	$y = 31.4x + 0.4$	0.99	± 0.28	2.4
HRW	24	$y = 32.8x + 0.7$	0.97	± 0.21	0.8
SRW	24	$y = 30.9x + 0.9$	0.99	± 0.12	1.1
White	19	$y = 28.1x + 2.0$	0.94	± 0.27	0.8
Durum	30	$y = 38.9x - 1.6$	0.98	± 0.28	1.4
Durum[a]	30	$y = 37.5x - 1.8$	0.99	± 0.21	1.4
Flour: HRS	24	$y = 27.7x + 1.6$	0.99	± 0.20	1.7
NaK Tartrate (b)					
Wheat: HRS	32	$y = 39.0x - 0.7$	0.99	± 0.21	2.4
HRW	24	$y = 40.9x - 0.9$	0.98	± 0.18	0.8
SRW	24	$y = 38.8x - 0.2$	0.99	± 0.15	1.1
White	19	$y = 36.3x + 0.3$	0.95	± 0.26	0.8
Durum	30	$y = 47.2x - 2.5$	0.99	± 0.18	1.4
Durum[a]	30	$y = 41.8x - 1.1$	0.99	± 0.16	1.4
Flour: HRS	24	$y = 39.5x - 0.9$	0.99	± 0.23	2.0
Glycerol, 1949[b] (Wheat HRW)	100	.....	0.96	± 0.38	2.0

[a] Shaken 15 minutes.
[b] From previous paper, 1949 (ref. 3).

TABLE V

PERCENTAGE OF SAMPLES IN EACH OF FIVE SELECTED RANGES OF ERROR IN PREDICTED PROTEIN CONTENT (HARD RED WINTER WHEAT)

PROTEIN ERROR RANGE	PERCENTAGE OF SAMPLES IN EACH RANGE		
	Reagent A	Reagent B	1949 Method
0.0 – 0.19%	70.8	79.2	41.0
0.2 – 0.39%	20.8	16.6	35.0
0.4 – 0.59%	8.4	4.2	17.0
0.6 – 0.79%	0.0	0.0	6.0
0.8 – 0.89%	0.0	0.0	1.0
Sum	100.0	100.0	100.0

Discussion

The data in Table I indicate that the amounts of copper in the reagents as applied are adequate for the protein range 0 to 20%. Similar experiments have shown that less than two-thirds of the copper is taken up by the protein at the 20% level.

As observed by Jennings (1), the color intensity of the glycerol reagent is greater than that of the sodium potassium tartrate reagent (Tables I and II). Consequently, the adsorption of copper by starch causes a greater decrease in the color intensity of the glycerol reagent than in that of the tartrate reagent (Table II). The concentration of the copper in the diluted reagents (Table II) is less than that of the residual copper in the test as ordinarily applied to a sample containing 24% protein. Thus, for each of the reagents, A and B, the starch effect is nearly constant throughout the protein range.

The differences in color intensity change from this cause are probably reflected by the differences in a values ($y = bx + a$) of the regressions involving the two reagents (see Table IV).

In Table I, the difference in average absorbances for the two series seems to indicate a greater color response for the sodium potassium tartrate reagent, as stated by Jennings (1). The test solution contains two color components: the protein-copper complex and the uncombined copper. The color intensity of the latter is diminished from its original value by the transfer of the copper to the protein. The instrument zero, adjusted with a reagent as the blank, is in error by an amount proportional to the amount of protein in the sample tested and also proportional to the color intensity of the reagent used. The absorbance of each test represents less than the total of the color intensity of the biuret complex by the amount of this error. Therefore, the color responses of the two reagents may be nearly identical.

From Table III, it is evident that the protein of durum is slightly more resistant to extraction than is the protein of wheat other than durum. Apparently, the tartrate reagent is slightly the more effective in extracting the protein from wheat.

In Table IV the regressions of the classes other than durum are similar within each reagent series. The regressions for the class durum are quite different. The longer shaking time of the second durum series produced regressions which are more nearly in line with those of the other classes. The change in the regression slope for the glycerol series is comparatively slight, but it is accompanied by corresponding changes in r, Sy.x, and average absorbances. The latter value increased from 0.450 to 0.472 for the glycerol series and from

0.391 to 0.408 for the tartrate series.

The modified method is more accurate than the original method (3), as shown by the more favorable r and Sy.x values and error distribution (Table V). By the same standards, the sodium potassium tartrate reagent is preferable to the glycerol reagent.

The modified biuret method gives a means of estimating wheat protein in a fairly satisfactory manner. It is simpler than the original method, and the results are more nearly in line with the Kjeldahl values. The method is empirical, and a strict time schedule must be followed to get good results.

The advantages of the tartrate reagent over the glycerol reagent largely result from the lesser reagent color intensity.

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