A Modified Biuret Reagent for Determination of Protein[1]

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ABSTRACT

Addition of isopropanol in equal volume to the alkaline cupric tartrate biuret reagent of Jennings yields a stable solution which reacts rapidly with protein, and is readily filterable. This reagent can be used to estimate protein content in whole grain meal and starch-rich samples as well as in dispersions containing urea and/or acetic acid. Correlation coefficients, using the Kjeldahl method as a standard, exceed 0.995, with a standard error of 0.08 mg. protein. The reagent was used successfully to quantify the fractionation of the protein in wheat meal and flour by two solubility fractionation procedures. Each fraction had a slightly different specific biuret color. Using an apparatus capable of providing high rates of heat transfer, the time for color development can be shortened to 60 sec. and the analysis can be automated. In this form the method has potential for rapid determination of protein in grain samples.

The biuret method has been widely used by cereal chemists for the determination of protein content (1–7). The most common procedure is that developed by Pinckney (2,3) and modified by Jennings (1). Two recent reports (6,7) have described modifications which offer significant improvements in speed and in the filterability of the reaction mixture after completion of color development. This article reports further refinements of the method of Johnson and Craney (6) to improve the speed and convenience of use.

MATERIALS AND METHODS

Reagent

1. Dissolve 8.4 g. potassium hydroxide in 300 ml. distilled water and add 2.5 g. potassium sodium tartrate.
2. Dissolve 1.2 g. cupric sulfate (pentahydrate) in 200 ml. distilled water and add, with mixing, to the above alkaline potassium sodium tartrate solution..
3. Dilute the combined reagent with an equal volume of isopropanol (reagent grade).

Procedure

For Proteins in Solution. Add 10 ml. of the biuret reagent to a convenient (2 ml.) volume of solution, containing between 1 and 15 mg. protein. Mix thoroughly using a vortex mixer and incubate at 40°C. for 10 to 30 min. Centrifuge (13,000 × g, 10 min.) or filter through glass fiber filter (Reeve Angel, grade 943 AH) to remove any turbidity. Read the absorbance at 550 nm. against a blank containing all components but the protein sample. Standards and unknowns should be treated in an identical manner.

For Proteins in Meal and Flour. Weigh out a sample (ground to pass through 80-mesh screen) containing 2 to 15 mg. protein. Suspend the sample in 1.0 ml. AUC (0.01M acetic acid, 3M urea, and 0.01M cetyltrimethylammonium bromide) (8); add 10 ml. biuret reagent; mix; incubate; and centrifuge or filter as above.

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Rapid Procedure. Mix sample and reagent as described above and pump through a reaction coil at 60° C. The residence time in the coil should be exactly 60 sec. The color developed is stabilized by passing through a second short length of coil at 15°-20° C. Both coils can be conveniently mounted inside a single glass jacket providing inlet and outlet tubes for both heating and cooling water. Residence time in the cooling coil can be 15 to 20 sec. The stabilized reaction mixture is passed directly through the flow cell of a recording colorimeter, or if turbid, it is filtered through glass fiber filter prior to colorimetric assay.

Preparation of Gluten and Protein Fractions from Wheat Flour. The gluten was prepared from flour milled from Canadian hard red spring wheat (cv. Manitou) by the standard AACC method (9), except that oven-drying was replaced by freeze-drying. Flour proteins were fractionated into three fractions, by extracting consecutively with 0.5M NaCl (fraction I), 70% ethanol (II), and 0.1M NaOH (III) solutions. The extracts were dialyzed against cold distilled water, freeze-dried, and the protein content (N×5.7) determined by the Kjeldahl method. These three protein fractions were used to prepare standard curves for analysis of protein fractions obtained by two fractionation procedures (see next section).

Extraction Procedure Used in Protein Fractionation. Two different

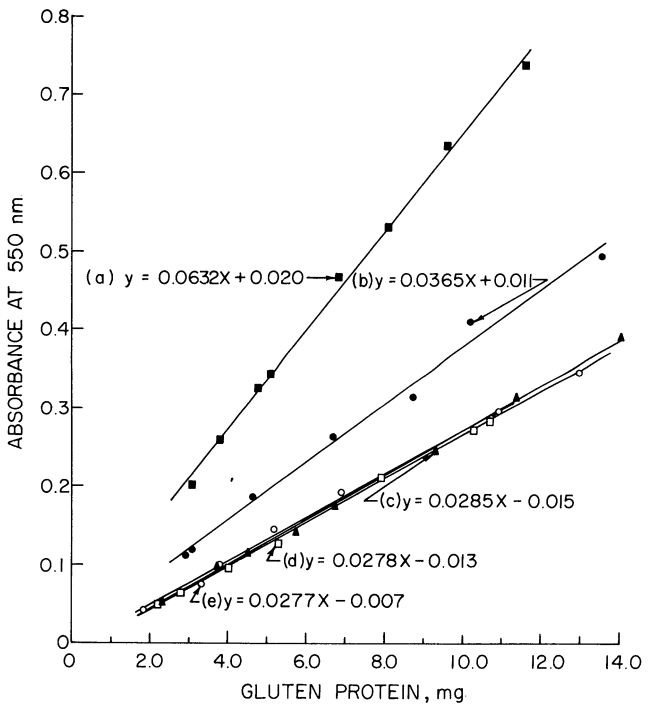

Fig. 1. Absorbance versus protein concentration for different methods of clarification: a) no clarification, b) filtration through Whatman No. 4 paper, c) filtration through Whatman No. 1 paper, d) centrifugation (10 min. at 13,000 × g), and e) filtration through glass fiber filter.

fractionation procedures, those of Chen and Bushuk (10) and Lee and MacRitchie (11), were used to test the new biuret reagent. The extraction part of the procedures in the two fractionation schemes were modified as follows. The sample (1 g.) was extracted three times (2 × 10 min., 1 × 5 min.) with 5.0 ml. solvent in an ice bath by homogenizing in a 15 ml. centrifuge tube with a specially fitted Plexiglas plunger. Each extraction was followed by 10 min. centrifugation at 20,000 × g. The three extracts were combined and the volume measured accurately. The solutions were used directly in the biuret assay. The residue was then similarly extracted with the other solvents sequentially to yield four soluble fractions and the final residue.

Standard Curves. For whole meal and flour samples, the method was standardized using the same materials of known protein content as determined by the Kjeldahl procedure. Alternatively commercial or laboratory-prepared, freeze-dried gluten of known protein content may be used as a convenient standard. For samples of wheat proteins in solution, the appropriate wheat protein fractions were used to prepare standard curves.

TABLE I. RESULTS OBTAINED WITH THE MODIFIED BIURET AND KJELDAHL PROCEDURES

Sample	Protein Content % as-is basis	Granulation	Biuret vs. Kjeldahl		
			Slope	Intercept	Correlation coefficient
Series A[1]					
Gluten	67.5	Powder	0.0287	+0.003	0.9947
Manitou wheat	16.8	40 mesh	0.0265	−0.001	0.9998
Manitou wheat	16.5	60 mesh	0.0275	+0.003	0.9998
Manitou wheat	16.1	80 mesh	0.0283	−0.002	0.9983
Manitou flour	14.0	100 mesh	0.0237	+0.008	0.9996
Series B[2]					
Gluten	67.5	Powder	0.0262	−0.001	0.9999
Fraction I	54.8	Powder	0.0330	−0.002	0.9981
Fraction II	90.6	Powder	0.0216	+0.002	0.9991
Fraction III	60.4	Powder	0.0252	−0.004	0.9994

[1]Determined by procedure for proteins in meal and flour.
[2]Determined by procedure for proteins in solution.

TABLE II. CORRELATION OF BIURET ABSORBANCE AND KJELDAHL PROTEIN FOR DIFFERENT CLASSES OF WHEAT

Variety	Class of Wheat	Protein Content % as-is basis	Absorbance[1] at 550 nm.
Pembina	HRS	16.5	0.431
Manitou	HRS	16.4	0.429
Winalta	HRW	14.5	0.369
Stewart 63	Durum	15.1	0.398
Talbot	SWW	12.3	0.310
A13	SWS	12.4	0.316
Lemhi	SWS	10.9	0.274

[1]Absorbance corrected for 0.1000 g. sample by multiplying the actual absorbance by 0.1000/sample weight.

Interference by urea and AUC was tested by making up a series of double-strength ovalbumin solutions in water and diluting with an equal volume of 4M and 8M urea, and double strength AUC. Final concentrations of urea in the three solutions were 2M, 4M, and 3M, respectively. The slopes and intercepts of the curve thus obtained were compared with those of the control to which an equal volume of water had been added.

RESULTS

The relationship between absorbance and protein concentration is both reproducible and linear, and is independent of the form in which the sample is presented. However the slope is strongly dependent on the method used for clarification of the reaction mixture. This is illustrated in Fig. 1 using gluten dispersed in AUC. Without any clarification, the solutions were faintly turbid,

TABLE III. CORRELATION OF BIURET PROTEIN CONTENT IN PRESENCE OF UREA AND AUC WITH KJELDAHL PROTEIN

	Biuret vs. Kjeldahl		
Solvent	Slope	Intercept	Correlation coefficient
Water	0.0323	+0.003	1.000
2M urea	0.0320	+0.003	1.000
4M urea	0.0327	+0.001	1.000
AUC	0.0323	+0.002	1.000

TABLE IV. RESULTS OBTAINED BY THE BIURET AND MICRO-KJELDAHL PROCEDURES ON PROTEIN FRACTIONS OF WHEAT FLOUR AND WHOLE WHEAT MEAL

	% Total Grain Protein[2]							
	Whole wheat meal			Flour			Std. dev.	
Solvent[1]	Biuret A[3]	Biuret B[4]	Micro-Kjeldahl	Biuret A[3]	Biuret B[4]	Micro-Kjeldahl	Biuret	Micro-Kjeldahl
(a) Modified Chen and Bushuk (10) fractionation								
H_2O + 0.05M NaCl	28.4	22.5a	27.2	17.9	14.2a	16.3	0.5	0.5
70% ETOH	27.2	33.0b	31.5	39.6	48.0b	46.6	1.1	1.5
0.05M Acetic acid	16.7	17.4c	16.9	22.0	22.9c	22.8	2.0	0.8
0.1M NaOH	19.0	19.8d	18.1	12.1	12.6c	11.6	2.3	0.4
Residue	0.0	0.0	5.8	0.0	0.0	1.7	0.0	0.4
Total recovery	91.3	92.7	99.5	91.7	97.7	99.0		
(b) Modified Lee and MacRitchie (11) fractionation								
H_2O + 0.01M Na pyrophosphate, pH 7.0	24.7	19.6[1]		14.5	11.5a	11.5[1]	0.9	
2M urea	20.1	24.4b		34.9	42.3b		1.5	
4M urea	7.2	7.5c		15.7	16.5c		1.1	
0.1M NaOH	36.1	37.7c		30.1	31.4c		2.0	
Residue	5.0	5.2c		0.0	0.0		2.2	
Total Recovery	90.2	94.4		95.2	101.7			

[1]Solvents used in succession.
[2]Mean of duplicate fractionations.
[3]Values based on standard curves for gluten.
[4]Values based on standard curves for protein fractions: a, fraction I, b, fraction II; and c, fraction III (see methods for description of fractions).

probably because of nonprotein impurities in the gluten. With clarification, the most reproducible results were obtained for solutions clarified by centrifugation or by filtration through Whatman No. 1 filter paper or the glass fiber filter. The latter method of clarification is particularly fast and convenient giving curves with a slope and intercept almost indistinguishable from those obtained by centrifugation. In each case the correlation coefficient against the Kjeldahl method as standard exceeded 0.995.

The modified biuret reagent was tested using laboratory-prepared gluten, wheat protein fractions, flour and whole wheat meal samples at different concentrations. The slopes, intercepts, and correlation coefficients against the Kjeldahl method are summarized in Table I. The results support the findings of Johnson and Craney (6) in that wheat and wheat flour differ appreciably in specific biuret color and the findings of Misra et al. (12) that different protein fractions (based on solubility) yield different biuret absorbances.

The effect of sample granulation on the biuret protein determination was tested by analyzing samples of hard red spring wheat ground on a Wiley mill to pass through 40-, 60-, and 80-mesh screens. The results (Table I) showed a shift in slope toward the gluten slope value with decreasing particle size. This suggests that the reaction with the biuret reagent is incomplete for the coarser samples. For routine analysis, all samples should be ground to pass an 80-mesh screen to minimize the effect.

To evaluate the biuret procedure for practical use, whole wheat meals of different classes of wheat varying widely in protein content were analyzed to compare the modified biuret and Kjeldahl procedures. The results are summarized in Table II. A correlation coefficient of 0.9983 with a slope of 0.0284 was obtained from the regression line between biuret absorbance and milligrams of Kjeldahl protein. It is concluded that the modified biuret reagent can be used for routine protein determination in wheat samples.

The reproducibility of the method was checked on eight replicate (10-mg.) samples of gluten. The mean absorbance and a standard deviation were 0.450 and 0.005, respectively, without clarification, and 0.174 and 0.002, respectively, with clarification by centrifugation. These standard deviations correspond to 0.079 and 0.072 mg. of protein, respectively. It should be noted that they include errors of weighing as well as those associated with the method.

Table III gives the slopes, intercepts, and correlation coefficients obtained for ovalbumin with the modified biuret reagent in the presence of urea and AUC. In each case interference was found to be negligible and could be entirely eliminated by using the appropriate solution in the blank.

To further test the versatility of the reagent, it was applied to the estimation of protein in fractions obtained by two different solubility fractionations. Table IV summarizes the results obtained. The total protein was determined for each extract and the results are expressed as percent of total grain protein (as determined by the Kjeldahl procedure). The zero or extremely low biuret protein value for the residue suggests that a very small proportion of the total protein cannot be solubilized by the fractionation procedure used nor by the biuret assay. This insoluble protein may be intimately associated with the bran and starch components of the whole meal and flour. Specific protein fractions appear to be more suitable standards than the dry vital gluten since only the protein value of the salt-soluble fraction differed significantly from the micro-Kjeldahl protein.

This difference may be due to the presence of nonprotein nitrogen in this fraction which would be analyzed by the Kjeldahl procedure but not by the biuret procedure.

DISCUSSION

The procedure described in this article offers the following improvements over the method of Johnson and Craney (6) on which it is based:

1. It requires only one reagent which is readily prepared and is stable.
2. No additional weighing of solid cupric carbonate is required.
3. Color development does not depend on interaction with an insoluble reagent component.
4. The method can be adapted for rapid analysis.
5. It can be applied to insoluble samples; the resulting suspensions can be easily filtered through Whatman No. 1 filter paper and glass fiber filter.
6. Analytical repeatability is high with a standard deviation of 0.08 mg. protein.
7. The sample size in the biuret tests can be reduced to 50 to 100 mg. (for wholemeal and flour) without losing any accuracy.
8. The protein values by the modified biuret reagent were linearly correlated with those obtained by the Kjeldahl method for all classes of wheat tested.

The versatility of the method is further demonstrated by applying it to protein fractions in various solvents (Table IV). In these analyses, the samples were presented as solutions in water, pyrophosphate buffer, 70% ethanol, 2M and 4M urea, 0.1M NaOH, and a starch-rich residue. All solutions clarified readily, and recoveries of protein averaged above 92%.

The exact time of incubation is not critical except in the rapid method, where it must be accurately controlled. Incubation (in test tubes) up to 30 min. at 40° C. did not affect the absorbance readings. The longer time is necessary if protein suspensions are analyzed. For proteins in solution or dispersion, 10-min. incubation was usually adequate. For highly accurate work, both standards and unknowns should be incubated at the same time. Provided it is stored in the refrigerator, the developed color is stable for at least 24 hr.

In the rapid method, complete color development is probably not achieved in the short (60 sec.) incubation period. The exact time of incubation is therefore critical for high reproducibility. This is best achieved by pumping the mixed sample and reagent solutions or suspensions through heating and cooling coils. This modification of the biuret method is currently under development as a rapid procedure for protein determination.

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