A SIMPLIFIED TECHNIQUE FOR MILK PROTEIN DETERMINATION BY THE DYE-BINDING METHOD

by

MORIO KUBOYAMA

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APPROVED:

Professor of Dairy Technology

In Charge of Major

Head of Department of Food and Dairy Technology

Chairman of School Graduate Committee

Dean of Graduate School

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INTRODUCTION

It is well-known fact that milk protein is one of the most essential nutrients in the nutrition of human beings, especially in infancy. Other than human milk, the most important source of milk protein is the milk of the dairy cow. The nutritional and compositional evaluation of milk should be done most properly on the basis of the protein content.

Traditionally, the pricing of milk has been based on the milk fat content. The market price of butter has varied with conditions and its changeability has affected the pricing of milk. Even at present the price of milk still is determined by the milk fat content, although milk protein is much more valued than milk fat. This discrepancy in the evaluation of milk might be explained on the basis that butter has been a single manufacture of fully admitted nutritional and merchandizing value for a long period. The milk fat content of milk could be always determined readily by such well-established and comparatively simple devices as introduced by Babcock and Gerber.

Recent studies show that consumers, when given a preference, select milk containing a high percentage of solids-not-fat (SNF) (13, p.1743-1745). Also a close correlation between SNF and protein content in milk has been shown (8, p.70-79).

It is quite natural that the change in the nutritional value of milk fat, more recognition for milk protein, increasing consumption of cheese and fluid milk should demand a more simplified, reliable and rapid method of determining protein content in milk for mass testing in the field and dairy plant. The first criterion of a suitable method for field use is that it be simple and well within the range of understanding of those doing the work. Simplicity, ease and rapidity of determination, along with low investment in equipment usually go together. Simple methods when used by unskilled technicians are often more accurate under these conditions than so-called laboratory methods which are recommended because of greater accuracy.

A rapid test for protein is advantageous in a number of ways: Of immediate importance is the opportunity to more positively focus attention on milk's most valuable asset, the protein. Other uses involve selective breeding, pricing milk based on total composition, rapid determination of composition in certain manufacturing processes, and the emphasis on protein-lactose and protein-fat rations as they influence palatability of milk and the manufacture and palatability of processed products.

In the present study, a standardization of determining milk protein by the dye-binding method with Amido Black

dye and some modifications were tried with reference to field use.

REVIEW OF LITERATURE

In determining the protein content, it has become a widespread practice to regard milk protein as containing about 16 per cent nitrogen. The protein content is estimated by multiplying the nitrogen content (in per cent) by the factor 6.38. This forms the principle of Kjeldahl's method which has been employed as an official method for the determination of protein content of milk and milk products (20, p.682). This method is recognized as standard procedure for reference purpose (7, p.682). But this is not suitable for routine practice because of its laborious and time-consuming nature.

Recently, increased attention has been paid to routine methods for the determination of nitrogen or protein content of milk because of its importance in standardizing milk in processing and in determining the yield of cheese. Also protein testing is necessary in those pricing systems of raw milk which are based on the protein or solid-not-fat content (7, p.682).

The Biuret method of protein estimation which employs the Biuret reaction for colrimetric determination, and

the formol titration method have been suggested for this purpose. But the former lacks in repeatability, being affected by the turbidity caused by the lactose content. The general opinion is that the latter is a less satisfactory test because of the necessity of rigid standardization.

In 1950, Kofranyi (6, p.51-54) reported on a direct steam distillation method for the determination of the protein content of milk. In this method a sample of milk made strongly alkaline with NaOH is submitted directly without previous digestion, to steam distillation in a Parnas-Wagner micro-nitrogen distillation apparatus. The experimental conditions are arranged such that a consistent amount (approx. 11 per cent) of protein nitrogen is released. This nitrogen is mainly amide-nitrogen and a small amount is derived from alkaline hydrolysis of certain amino acids. The ammonia is received in 0.025 N NaOH with a mixed indicator (methylene blue-methyl red). Vanderzant et al. modified this method and obtained excellent agreement between the protein values as determined by this method and the official macro-Kjeldahl procedure (20, p.19-21). This method may be successfully conducted for mass analysis of milk in dairy plant other than field testing.

Fraenkel-Conrat and Cooper (5,p.43-55) developed the

microanalytical methods for the estimation of the number of acid or basic groups of proteins using Orange G. and Safranine O. They found the dye combined with the basic or acid groups of protein at a certain pH stoichiometrically. They also calculated the average of all values for the maximal amount of dye bound in mg, by multiplying a conversion factor in terms of acid or basic equivalents of dye bound per gm of protein. The experiment has shown the possibility to apply the quantitative dye-binding of protein for colorimetric determination of milk or other protein. Schober et al. (11, p.123-126) developed a simple colorimetric determination of milk protein based on the principle elaborated by Fraenkel-Conrat et al. They used Amido Black 10-B which had been successfully used for blood-protein and obtained good results. A diluted milk sample was added to Amido Black in citrate-phosphate buffer. After putting aside about 10 minutes, the mixture of milk and dye solution was centrifuged and the optical density of the supernatant was measured, following dilution with distilled water, at the wave length of 578 mμ using a spectrophotometer. Kjeldahl protein content was compared with corresponding optical density to obtain a good correlation. This method with Amido Black on milk protein has been studied further and modified by Steinsholt (9, p.259-264), Vanderzant (19, p.63-64) and Dolby (3, p.43-55). Schober et al. also

studied factors affecting results such as pH, buffer solution, presence of salts and others, and comparison with protein content by formol titration.

In this country, Udy (17, p. 190-197) started working on protein determination of wheat by this method with Orange G. He figured out a regression equation between optical density of unbound dye solution and protein content and obtained a fine correlation coefficient ranging between 0.992 and 0.997 with various group of wheat samples. This had been applied to the proteins of milk by Udy (16, p. 314-315), Ashworth (1, p. 614-623), Ashworth et al. (2, p.133-138), Dolby (3, p.43-55) and Treece et al.(14, p. 722).

To elaborate a simplified technique for determining milk protein by the dye-binding method, it has been necessary to review factors affecting the dye-binding of milk protein and related matters.

A. Dye Employed.

The above-mentioned reports by workers in Norway (9, p. 259-264, 279-284), Germany (11, p.123-126), the Netherlands (7, p.682), the United States (19, p. 63-66) and Australia (3, p. 43-55) indicated that the dye Buffalo Black (Amido Black) might have some advantages over Orange G. in determinations of the milk protein. Saturation of the protein

with this dye was accomplished much faster than with Orange G. Furthermore, the Amido Black-protein complex was very dense and was removed easily by centrifugation. The Orange G.-protein complex, on the other hand, was not dense and handling of the tube often caused incorporation of small amounts of precipitate into the supernatant fluid. Addition of calcium chloride, arginine and peptone to milk disturbed the relationship between the amount of Amido Black bound and the protein content of the milk sample (11, p.124-125). But the Amido Black might be more preferable for milk protein determination. Dolby referred to the purity of dye sample, saying that the dyes available may have a purity well below 100 per cent. European workers have used E. Merck's "Amido Black for electrophoresis," the dye content of which is stated by another worker to vary from 95 to 97 per cent between batches. Ashworth et al. have specified the use of a certified Orange G. dye which should assay at least 95 per cent (2, p. 133).

B. Choice of pH and Buffer Solution.

Fraenkel-Conrat specified a pH of 2.2 for binding acid dye with basic group of protein (5, p.240). Schober et al. found a pH between 3.5 and 1.9 applicable for this purpose, but at a pH above 3.5 dye-binding with Amido Black is not quantitative (11, p.124). Udy employed pH 2.2 for wheat

protein with Orange G. (17, p. 191). Ashworth (1, p.614) and Dolby (3,p.43) specified pH 2.0 on milk protein for Orange G. Vanderzant used pH 2.2 for milk protein with Buffalo Black (19, p.64). A citrate-phosphate buffer was used by all investigators except Ashworth (1, p. 614). He employed citric acid alone to give a pH of 2.0.

C. Mixing of Dye and Sample.

A thorough mixing of dye and sample is required. Mechanical shaking or tumbling of the mixture for 5 or 10 min was found preferable by Treece et al. (14, p.727) to improve agreement of duplicates. According to Ashworth et al. (2, p.138) the mixture can be left for 14 days without deterioration. It should be noted that these workers employed an Orange G. solution preserved with thymol. With solid samples such as casein the product should be finely ground (to say 100 mesh) and shaken with the dye for 12-16 hours (5, p.240). Sampling of milk fluid usually was done by pipetting, so specification of temperature of fluid milk sample must be important. While Vanderzant (19, p.64) specified that milk samples be warmed to 70° F, other researchers did not designate any temperature of milk sampled accurately. Before assaying milk sample with dye solution, some workers diluted it with distilled water but some not, which was differentiated with sample size. According to Ashworth (2, p.

135) in the temperature range between 46° F and 130° F no significant effect of temperature on dye-binding could be observed. An improvement was developed by Udy which eliminated dilution procedure by using a flow-through cuvette (15, p.1360).

D. Standardization of Dye Solutions.

Ashworth et al. (2,p.620-621) adjusted their instrument to read an optical density of 0.500 on a 1:2 dilution of the dye solution.

E. Removal of Precipitate.

Centrifuging for 5 minutes at a speed of 2500-3000 r.p.m. has been generally recommended. The precipitate may be removed by filtration instead of centrifugation but a blank should be run to correct for dye adsorption by the filter paper. Ashworth et al. (1, p.618) found that filtering with a 9 cm S & S No. 595 paper gives a crystal clear filtrate and very little of the dye is absorbed by the paper. Also they reported that the value of dye absorption by filter paper was relatively constant for dye concentrations which varied from 0.3 to 1.5 mg per ml and consequently the percentage error would be greater for dilute solutions of the dye.

F. Colorimetric Measurement.

For Orange G. Ashworth et al. (2, p.134) used Beckman Model B Spectrophotometer at a wave length of 475 mμ and Schober et al.(11, p.123) employed a Zeiss-spectrophotometer at a wave length of 578 mμ, for Amido Black. Steinsholt (9,p.260) designated 612.5 mμ for Amido Black, and Dolby (3, p.45) obtained the peak absorption of Amido Black at 615 mμ. According to the Steinsholt (9, p.260), Beer's law is followed in Amido Black solution at concentration below 6.2 mg per liter. He therefore diluted the dye solution 1:40 with water before measuring the transmission. Ashworth et al. (2,p.133) have found that Orange G. solutions show deviations at concentrations above 0.5 g per liter. As their technique involved measurement of the absorption by undiluted solution, they recommended that the readings be corrected from a curve constructed for the purpose. Many workers have used 10 mm light path cuvette for colorimetric measurement. Udy (15, p.1360) worked out a new type cuvette with short light path and flow-through design, which successfully made unnecessary dilution after separating the unbound dye solution from a protein-dye complex.

G. Standard Curve and Regression Equation.

Udy (17, p.190-197; 16, p.314), Ashworth (1, p.614-623), Vanderzant (19, p.63-66) and Dolby (3, p.43-55) worked out standard curve between Kjeldahl protein and optical density of unbound dye solution, with referrence to different herds, various fractions of milk and milk products, and milk at different stage of lactation, and brought about results with higher correlation coefficient of 0.99 to 0.92.

The present work was planned to test the dye-binding method with Amido Black and develop a simplified procedure for determining milk protein.

EXPERIMENTAL

A. Milk Sampling.

Samples were obtained from (a) individual cows in Holstein and Jersey herds in Tillamook Country, Oregon, (b) homogenized and pasteurized milk samples from the Dairy Laboratory of Oregon State University. Each sample was tested for specific gravity, fat percentage, and total solids, also mastitis by the California method. The abnormal milk samples were rejected. The samples were iced immediately and stored under refrigeration at 40°F to 45°F. The protein content of the samples was determined within 2 to 3 days.

B. Preliminary Experiment.

Procedure. Amido Black 10-B was obtained from Farbenfabriken Bayer Leverkusen. A 0.001 M solution of Amido Black was prepared by dissolving 0.6165 g in 1 liter of citrate-phosphate buffer, pH 2.3. The milk sample was warmed to 102° F, mixed thoroughly, then cooled to 68° F. One-half ml of milk was pipetted into 20 ml of dye solution om a 125 ml Erlenmeyer flask, mixed thoroughly, and held for 5 min. at room temperature. Additional standing did not vitiate the results. The insoluble protein-dye complex was removed by filtration through Whatman No.2 filter paper (9 cm). A five ml aliquot of the clear filtrate was diluted with distilled water to 250 ml. The instrument, Coleman Junior Spectrophotometer 6 A, was standardized by adjusting absorbancy to 0,500 with 10 μM dye solution. Maximum absorbancy of dye solutions, ranging from 4 to 20 μM, was obtained at a wave length of 615 mμ.

Each sample was analyzed by the macro-Kjeldahl method and the protein per cent was calculated from the total nitrogen using the factor 6.38, which is commonly used to convert nitrogen to milk protein. Optical density of each sample was plotted against the per cent protein found by the Kjeldahl method. A linear regression equation was then derived by the least square method. The data were subjected

to statistical analysis. The data are shown in Table I (Appendix). The results of the statistical analysis were as follows:

No. of Samples	: 50
Mean Kjeldahl Protein	: 3.46 %
Mean Optical Density	: 0.415
Regression Equation	: Protein % = 7.13 - 8.85D
Regression Coefficient	: -8.85
Correlation Coefficient	: -0.92
Standard Deviation Coefficient	: 0.065

D= optical density.

C. Modification.

The above procedure would be satisfactory under laboratory conditions, and the results agree with those previously reported. For the purpose of adapting the procedure for field testing and increasing accuracy, the following modification was developed.

1) For pipetting a sample, a 500 lambda micropipette is employed to assure a correct sample (Figure 1).

2) Instead of using a 125 ml Erlenmeyer flask, a capped 40 ml polyethlene bottle was used for mixing the milk sample with the dye solution (Figure 1).

3) A clear filtrate was obtained by inserting a tube closed with a fritted glass disc through the neck of the

bottle into the mixture. A 50-fold dilution of clear filtrate was made by pipetting 0.2 ml of aliquot directly from the fritted glass tube with 200 lambda micropipette (Figure 1) into 9.8 ml of distilled water in a 10mm cuvette.

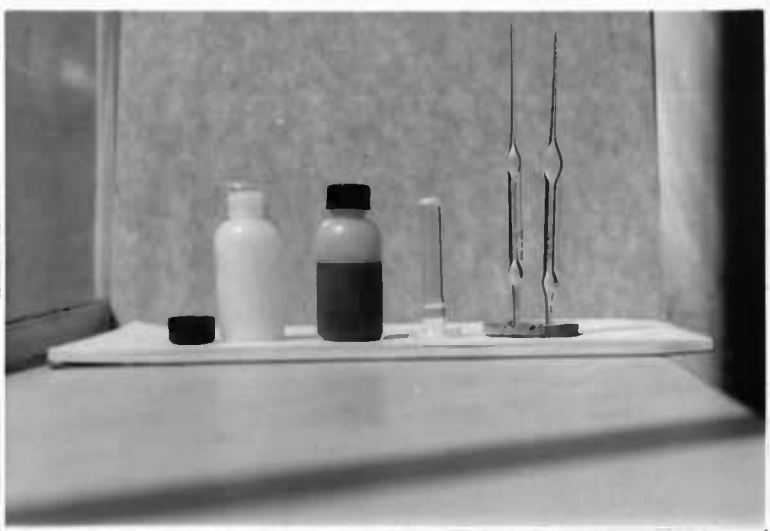

Figure 1. Illustration of apparatus: left to right, cap, polyethylene bottle with fritted glass filter tube inserted, a capped polyethlene bottle with 20 ml of dye solution, fritted glass tube (7 mm in diameter, 62 mm long and with a round sleeve at the top), and 200 and 500 lambda pipettes.

4) A syringe was used for measuring the dye solution and the distilled water.

RESULTS

1) The following experiments were conducted, employing the modified method, to develop a regression equation between Kjeldahl protein and optical densities of filtrates from milk of Holstein and Jersey cows. The sampling method, reagents and colorimeter used were as described for the preliminary experiments. The results obtained are shown in Table II (Appendix), summarized in Table 1 and plotted in Figure 2.

TABLE 1

Sample	No. of Samples	Mean Kjeldahl Protein(%)	Mean Optical Density	Regression Equation
Holstein	53	3.26	0.447	P = 6.77 - 7.82D
Jersey	45	4.04	0.303	P = 6.37 - 7.70D
Combined	98	3.65	0.375	P = 6.57 - 7.76D

Sample	Correlation	Standard Dev. from Regression
Holstein	-0.93	0.19
Jersey	-0.93	0.18
Combined	-0.93	0.18

P = per cent protein.
D = optical density.

2) Determination of protein in market milk. In order to study the effect of pasteurization and homogenization on dye-binding, 10 samples were obtained from a

STANDARD CURVE BETWEEN KJELDAHL PROTEIN PERCENT AND ABSORBANCE OF UNBOUND AMIDO BLACK AT 615 Mμ

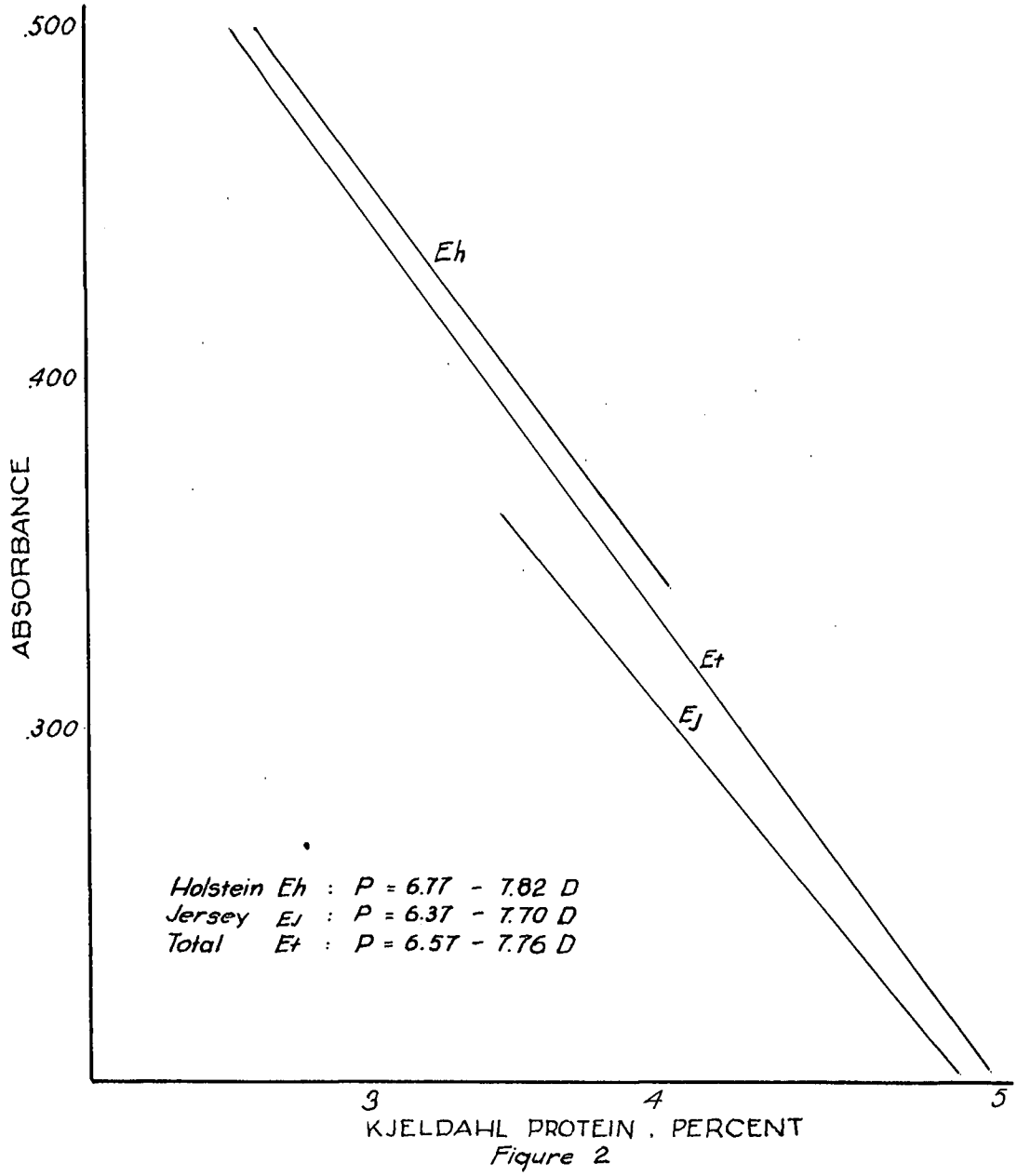

Figure 2

raw milk storage tank and the pasteurized homogenized products from the same lots. Protein determinations were made immediately after sampling using the modified technique. As shown in Table 2, no consistent differences were found between the raw and processed samples, although the results with the homogenized samples tended to be slightly higher.

TABLE 2.

Sample No.	Protein of Raw Storage Milk Per cent	Protein of Pasteurized and Homogenized Milk Per cent
1.	3.16	3.21
2.	3.20	3.31
3.	3.23	3.24
4.	3.44	3.44
5.	3.00	3.12
6.	3.03	3.15
7.	3.18	3.21
8.	3.21	3.23
9.	3.23	3.24
10.	3.23	3.24
Av.	3.19	3.24

3) Coleman Model 25 Photo-hemoglobinometer. (21) A portable colorimeter of sufficient sensitivity is necessary. The instrument was equipped with a 540 mμ filter and a light meter calibrated in per cent transmittance. With Amido Black solutions in the range of 5 to 10μM readings with the Model 25 agreed with those made with the Coleman Model 6 A, at wave length 615 mμ. They diverged at higher concentration of dye. As shown in Figure 3, the agreement

exists in the optical density range of 0.300 to 0.500. Since the readings of only 3 out of the 143 samples of milk tested were outside this range, the portable instrument appears to be be satisfactory. As a further test for the accuracy of the instrument, the protein tests of ten samples of milk were made. The results in comparison with those made with the Model 6 A are shown in Table 3.

TABLE 3.

Comparison of Absorbancies between Coleman Model 25 Photohemoglobinometer and Model 6 A Junior Coleman Spectrophotometer.

Sample No.	Per cent Transmittance Model 25(540 mμ)	Junior 6 A(615 mμ)
1.	49.0	49.0
2.	45.0	45.0
3.	50.0	50.0
4.	51.0	51.0
5.	52.0	51.0
6.	53.0	52.0
7.	28.0	30.0
8.	35.0	35.0
9.	24.0	26.0
10.	29.0	31.0

COMPARISON OF ABSORBANCIES AT VARIOUS DYE CONCENTRATIONS BETWEEN COLEMAN "25" PHOTO-HEMOGLOBINOMETER AND COLEMAN JUNIOR SPECTROPHOTOMETER 6A.

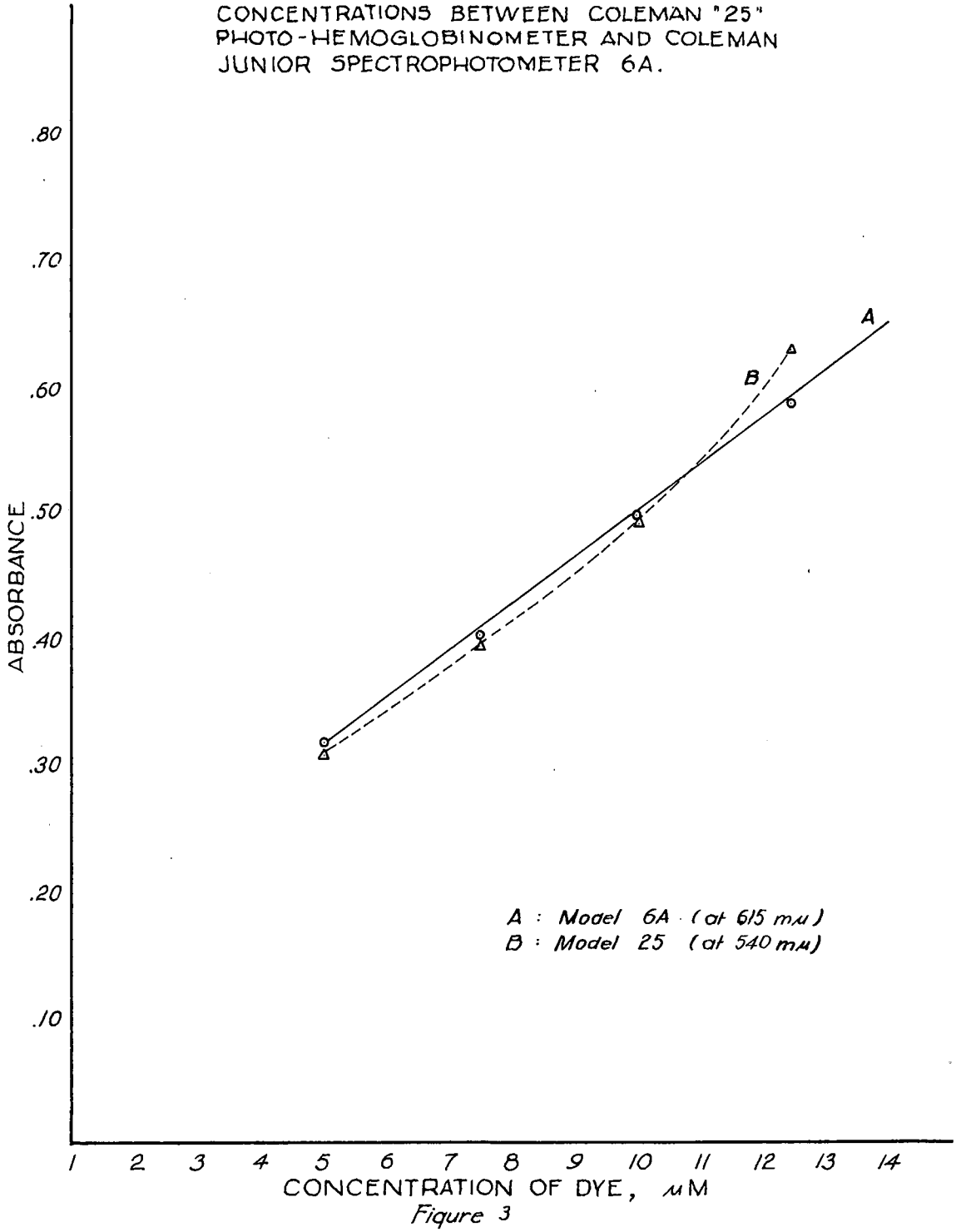

Figure 3

DISCUSSION

The results of the present study indicate that the dye-binding method, using Amido Black, for the determination of the protein content of milk is feasible for testing raw milk under field testing conditions as well as in the laboratory. The values of the unbound dye correlate closely with the Kjeldahl values. An analysis of the data indicates that the published Amido Black method can be used to determine the protein content of milk samples in the range of 2.5 to 4.88 per cent protein. For example, with raw milk samples from individual cows in Jersey herds, the values for Kjeldahl protein and the optical density, at 615 mμ, of the filtrates show a correlation of -0.92. This is identical with the results obtained by another worker (19, p.65). Closer correlations between Kjeldahl protein and the optical densities in the case of a single breed were obtained by means of a modified technique.

Covariance analysis of the data for Holstein and Jersey milks shows no significant difference in the slope or elevation of the regressions (Table 2).

Modifications to simplify the procedure were made, especially in the dilution and filtration steps and the treatment of sample. A fritted glass filter tube, 7 mm in diameter and 62 mm long, was designed and constructed for the present study. It is inserted into the polyethylene

bottle containing the dye-milk mixture, the lower end being immersed in the milk dye mixture. The filtrate enters the tube through the coarse fritted glass disc. In the case of homogenized milk, it is preferable to include a thin layer of glass wool over the fritted glass in order to assure a clear filtrate. To accelerate filtration, one may press the body of the polyethylene bottle to raise the pressure on the solution. This method also eliminates the absorption of dye particles, which usually happened with filter paper. The simplified procedure permits making an analysis in less than seven minutes without sacrificing accuracy. In order to further simplify the test an accurate, compact, portable colorimeter was sought. Preliminary results with a modified Coleman Model 25 Photo-hemoglobinometer appear very promising. If further tests and modification of the instrument prove its value, then the entire technique will find application in field, laboratory and processing plant.

Homogenization and pasteurization in ordinary commercial practice do not affect the dye-protein reaction. The method should prove useful for testing and standardizing market milk for protein.

SUMMARY AND CONCLUSION

1. A preliminary investigation of the dye-binding method, with Amido Black 10-B, for milk proteins was conducted under laboratory conditions. The regression equation relating protein per cent and optical density was established and various statistical constants was calculated.

2. Some modifications such as the employment of micropipettes, syringe, capped polyethylene bottle, and a newly-designed fritted glass filter tube were made to simplify the procedure and were successfully applied.

3. Preliminary trials with a simple, compact, portable photo-colorimeter have been made.

4. The following simplified procedure for testing whole milk for protein by the dye-binding method using Amido Black, may be recommended:

Reagent: 0.001 M Amido Black (M.W. 616.5) in 0.1 M citrate buffer (pH 2.3): 17.28 g of citric acid, 2.375 g $Na_2HPO_4 \cdot 2H_2O$ made to 1 liter with distilled water.

Procedure: Into 20 ml dye solution in a 40 ml polyethylene bottle, pipette 0.5 ml milk sample, at 20° C, using 500 lambda micropipette. Stopper, shake thoroughly and allow to stand for 5 min. Filter by inserting a glass

tube with fritted glass end into the bottle. Dilute an aliquot of the clear filtrate 50 times by adding 0.2 ml of filtrate, pipetted directly from the glass tube, to 9.8 ml of water, using a 200 lambda micropipette for the filtrate and a syringe for distilled water. Determine the optical density of the diluted filtrate at 615 mμ. The instrument is standardized by adjusting the reading of a 100-fold dilution of the original dye solution to an absorbancy of 0.500. Obtain the protein percentage from a conversion table constructed from the regression equation:

Protein % = 6.57 + 7.76D (D = optical density)

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TABLE I (APPENDIX)

Kjeldahl Protein Percentage and Optical Density Values of Holstein and Jersey Milks from Individual Cow

Sample No.	Kjeldahl Protein per cent	O. D.	Sample No.	Kjeldahl Protein per cent	O. D.
1.	3.25	.437	26.	4.18	.359
2.	3.23	.437	27.	4.12	.357
3.	3.73	.367	28.	3.25	.420
4.	3.55	.399	29.	3.30	.410
5.	3.30	.458	30.	3.71	.398
6.	2.84	.342	31.	2.37	.562
7.	4.29	.321	32.	3.14	.441
8.	4.50	.394	33.	2.94	.484
9.	4.07	.396	34.	3.26	.453
10.	3.80	.396	35.	4.17	.342
11.	2.49	.476	36.	2.54	.550
12.	2.94	.440	37.	2.45	.492
13.	3.01	.431	38.	3.19	.456
14.	3.91	.331	39.	2.71	.481
15.	3.26	.424	40.	3.65	.399
16.	2.99	.434	41.	3.69	.401
17.	3.33	.430	42.	3.29	.478
18.	3.09	.466	43.	4.39	.316
19.	2.90	.447	44.	3.17	.446
20.	3.41	.386	45.	3.36	.421
21.	3.88	.430	46.	2.88	.503
22.	3.25	.432	47.	3.50	.444
23.	3.62	.420	48.	4.88	.260
24.	4.26	.324	49.	4.18	.337
25.	4.13	.369	50.	3.92	.347
			Av.	3.46	.415

TABLE II (APPENDIX)

Kjeldahl Protein Percentage and Optical Density Values of Holstein Individual Cow

Sample No.	Kjeldahl Protein per cent	O. D.	Sample No.	Kjeldahl Protein per cent	O. D.
1.	3.60	.380	28.	2.80	.453
2.	2.73	.457	29.	3.41	.413
3.	3.51	.432	30.	3.36	.430
4.	3.24	.425	31.	3.25	.410
5.	3.47	.433	32.	3.14	.450
6.	3.14	.445	33.	3.13	.440
7.	3.71	.475	34.	2.87	.450
8.	3.43	.419	35.	3.56	.428
9.	3.54	.413	36.	3.36	.430
10.	3.76	.383	37.	3.01	.470
11.	3.19	.429	38.	2.74	.469
12.	3.54	.413	39.	2.74	.470
13.	3.76	.383	40.	2.96	.470
14.	3.19	.429	41.	3.56	.430
15.	3.51	.413	42.	2.95	.477
16.	3.48	.383	43.	3.61	.380
17.	2.96	.530	44.	3.68	.382
18.	3.86	.382	45.	3.12	.440
19.	3.01	.470	46.	2.98	.475
20.	2.91	.455	47.	2.77	.470
21.	2.97	.448	48.	2.33	.617
22.	3.81	.398	49.	3.86	.382
23.	3.04	.469	50.	2.99	.480
24.	3.95	.361	51.	2.95	.483
25.	3.24	.363	52.	2.70	.465
26.	3.44	.435	53.	3.15	.445
27.	3.45	.404			
			Av.	3.26	.447

TABLE III (APPENDIX)

Kjeldahl Protein Percentage and Optical Density Values of Jersey Individual Cow

Sample No.	Kjeldahl Protein per cent	O. D.	Sample No.	Kjeldahl Protein per cent	O. D.
1.	3.95	.300	24.	4.29	.292
2.	4.32	.270	25.	4.07	.324
3.	3.95	.300	26.	4.26	.305
4.	3.16	.350	27.	4.17	.300
5.	4.22	.270	28.	3.92	.323
6.	4.17	.288	29.	4.39	.293
7.	3.81	.325	30.	4.32	.270
8.	4.23	.305	31.	3.91	.325
9.	4.35	.286	32.	4.20	.285
10.	3.96	.310	33.	3.58	.330
11.	4.53	.215	34.	4.06	.304
12.	3.54	.349	35.	4.17	.300
13.	4.20	.285	36.	4.22	.270
14.	3.89	.330	37.	4.17	.295
15.	4.84	.272	38.	4.07	.323
16.	4.37	.290	39.	3.96	.310
17.	3.10	.410	40.	4.35	.286
18.	4.21	.278	41.	4.53	.215
19.	3.58	.330	42.	4.32	.270
20.	3.55	.350	43.	3.10	.410
21.	4.06	.304	44.	4.26	.305
22.	4.23	.295	45.	4.07	.324
23.	3.73	.307			
			Av.	4.04	.303