The Different Methods of Estimating Protein in Milk.

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It is often desirable to estimate the proteids in milk other than the official method. This is especially true in cheese factories where it is desirable to know the percent of casein in milk, since it is the casein in milk that gives it its nutritive value, as far as the proteins are concerned. It is frequently desirable to know the protein content in milk for infant and invalid feeding. With the present method of determining the fat by the Babcock method, which is quite accurate and can be done in all creameries, a rapid method for estimating the percent of casein and fat in milk gives us the necessary data to control the ratio of casein to fat in milk for feeding. Frequently a chemist is requested to determine the fat and casein in human milk where a physician has reason to believe that there exists an unbalanced ratio of fats and proteids.

There are three methods for rapid estimation of casein or proteids in milk, all of which possess merits worthy of consideration and could be used in a great many laboratories that are equipped with the apparatus necessary to determine the proteids by the official method. Although such equipment is at hand, when only a few determinations are to be made, the methods reviewed in this paper save time and the results obtained are sufficiently accurate. For the volumetric estimations of milk proteids, two standard volumetric solutions are required, besides a few beakers and flasks, apparatus found in any laboratory, or if one wishes to fit up for this purpose only, the expense is quite nominal.

In discussing the different methods, the order in which they are taken up, is no indication of their priority. Since 1892 various attempts have been made in devising a volumetric method for the estimation of casein in milk, but most were unsatisfactory, either owing to the extensive equipment or to the complicated indirect methods used. The main characteristics that a method should possess are: first, it should be accurate; second, it should require only a short time in making an estimation; third, the apparatus should be simple; fourth, materials and apparatus used should be easily obtainable.

L. L. Van Slyke and A. W. Bosworth in 1909 published their volumetric method (Technical Bulletin, N. Y. Ag. Exp. St.). The method worked out in their publication mentioned is briefly as follows: "A given amount of milk, diluted with water, is made neutral to phenolphthalein by the addition of a solution of sodium hydroxide. The casein is then completely precipitated by the addition of standard acetic acid, the volume is then made up to 200 cc. by the addition of distilled water and then filtered. Into 100cc. of the filtrate a standard solution of sodium hydroxide is run until neutral to phenolphthalein. These solutions are so standardized that 1 cc. is equivalent to 1 per cent. casein, when a definite amount of milk is used. Therefore, the number of cubic centimeters of standard acid used, divided by 2 less the amount of standard alkali used in the last titration gives the percentage of casein in the milk."

This method is based on the well known facts in chemistry and shows quite clearly the casein molecule has a constant molecular weight. First, uncombined casein is insoluble in milk serum, water or very dilute acids. Second, it has properties of an acid and combines with alkalies to form definite chemical compounds, neutral to phenolphthalein.

Now, if we know the molecular weight of casein or its equivalent in terms of a standard alkali, we can at once devise a definite method for estimating the casein by titration. Casein exists in milk in a colloidal condition combined with bases, upon addition of an acid sufficient to combine with salts in combination with casein, free casein is formed, insoluble in the serum (it must be remembered that casein and other albuminoids are soluble in excess of acids, the solubility depends on the kind of acid and temperature). There exists a definite relation between the amount of acid required to form free casein and the amount of casein present. It has been found that one gram of free casein neutralizes 8.8378 cc. of $\frac{N}{10}$ sodium hydroxide, or 1 cc. of $\frac{N}{10}$ sodium hydroxide neutralizes .11315 grams of casein. From this data the molecular weight of casein can be calculated.

From the above facts it is easy to determine the quantity of milk required, so that each cc. of $\frac{N}{10}$ acid used shall correspond to percents or fraction of a percent. Since 1 cc. of NaOH neutralizes .11315 grams of casein, it must require an equivalent amount of acid to set free the casein from its original combination in milk. If we wish to know the quantity of milk to be taken so that 1 cc. of acid used to separate the casein from its combinaion shall equal 1 per cent. of casein, we make use of the above equivalent, i.e.

1 cc. $\frac{N}{10}$ acid = .11315 grams casein, or in other words .11315 grams of casein is capable of neutralizing as much alkali as 1 cc. of $\frac{N}{10}$ acid, so if we take 11.315 grams of milk we see from the relation above that every cc. of $\frac{N}{10}$ acid used equals 1 per cent. casein. By using different quantities of milk we need only change the normality of our acid.

If by using 11.315 grams of milk (or 11 cc.) where each cc. of $\frac{N}{10}$ acid corresponds to 1 per cent., by using a greater or larger quantity of milk the normality would have to be correspondingly less or greater. When we use 8.75 cc. or 9 grams of milk the normality would not be $\frac{N}{10}$ but 795 cc. $\frac{N}{10}$ acid plus water to make 1,000 cc. which equals $\frac{N}{12.56+}$. Upon the above facts the volumetric method of Van Slyke and Bosworth is based.

Procedure in carrying out in detail the volumetric estimation of casein: "A given amount of milk, diluted with water, is made neutral to phenolpthalein by the addition of a solution of sodium hydroxide. The casein is then completely precipitated by the addition of the standardized acetic acid; the volume of the mixture is then made up to 200 cc. by the addition of water, thoroughly shaken and then filtered. Into 100 cc. of the filtrate a standard solution of sodium hydroxide is run until neutral to phenolpthalein. The solutions are so standardized that 1 cc. is equivalent to 1 per cent. of casein when a definite amount of milk is used. The number of cc. standard acid used, divided by two (since only 100 cc. of the 200 cc. is used), less the standard alkali used in the last titration gives the percentage of casein in the milk examined." When 17.5 or 18 grams of milk are used the strength of acetic acid and alkali are made by diluting 795 cc. of $\frac{N}{10}$ to 1,000 cc. The same normality as was derived above. Since only 100 cc. of the 200 cc. were titrated this then represents the acid required to liberate the casein in 8.75 cc. or 9 grams of milk. Likewise by using 22 cc. or 22.6 grams of milk treated as above, then 1 cc. of $\frac{N}{10}$ acid equals 1 per cent of casein. By the use of a factor any convenient quantity can be used. Example, by the use of 20 cc. of milk and $\frac{N}{10}$ solution, adjustment is made by multiplying the final result by 1.0964.

Apparatus and reagents necessary to carry on the volumetric estimation of casein in milk are, first, two 50 cc. burettes, graduated to 1/10 cc. or better 1/20 cc., these must be accurate. One of the burettes should be supplied with a glass stop cock for the acid, and one with a pinch cock for the alkaline solution. Second, flasks, volumetric, holding 200 cc. At least two of these are needed and where a number of estimations are to be made more are required to do rapid work; ten to twelve are necessary for rapid work. The

necks of these flasks should have an internal diameter of at least three-fourths of an inch. The reason for this diameter is necessary if the milk is neutralized in the flask. This neutralization can be done in the beaker into which the milk is weighed, if weights are taken. Third, pipettes, a Babcock milk pipette accurately graduated to deliver 17.5 cc. of milk, when 17.5 cc. or 18 grams of milk are used. When 22 cc. or 22.6 grams of milk are used it will be necessary to have a volume pipette graduated to deliver the above amounts or a 25 cc. Mohr pipette graduated into 1/10 cc. will be required. Fourth, one 100 cc. pipette or a volumetric flask graduated to hold 100 cc. Fifth, beakers of convenient sizes holding at least 200 cc. Sixth, if standard solutions are to be made, measuring cylinders or volumetric flasks holding 1,000 cc. are needed.

In regard to the making of the solutions it is best to prepare both the sodium hydroxide and the acetic acid as tenth normal. The accuracy of the succeeding work depends primarily on the correctness of the standard alkali and acetic acid. When it is desirable to make dilutions for different quantities of milk it can be made from the tenth normal stock solution. The *phenolpthalein solution* is prepared by dissolving one gram of phenolpthalein powder in 100 cc. of 50 per cent. alcohol. This should be neutralized by the use of a few drops of $\frac{N}{10}$ NaOH to a very slight pink color.

Carrying out the operation. Weigh out 22.66 grams of milk, or measure out 22 cc., neutralize in the beaker in which the weighing has been made, using only enough alkali to give a very faint pink, then transfer to a 200 cc. flask and wash out beaker with 75 to 80 cc. of distilled water, free from carbon dioxide, shake and warm to 22° to 25° C. At this point observe the color of the diluted milk. Frequently on dilution the pink color becomes quite pronounced; if so, add a few drops of $\frac{N}{10}$ acetic acid to a light pink. Run in from a burette 25 cc. of a $\frac{N}{10}$ acetic acid, frequently shaking, for milk rich in casein it would require 30 to 40 cc. of acid. Then fill up to the 200 cc. mark, insert stopper and shake thoroughly. After standing for 5 or 10 minutes, filter, after filtration pipette or measure 100 cc. of the filtrate into a 250 cc. or 300 cc. beaker and titrate to a permanent faint pink color, record the cc. used. Since 25 cc. were added to the total volume and only one-half titrated, we only take 12.5 cc. into consideration. From what has been said a portion of the 25 cc. $\frac{N}{10}$ acetic acid has been used in forming free casein, therefore the difference between 12.5 cc. and the amount of $\frac{N}{10}$ NaOH used to neutralize the acid in the 100 cc. filtrate equals the number

of cc. acid used in liberating the casein. Since a quantity of milk has been taken so that each cc. of acid used equals 1 per cent. casein, then each cc. represents 1 per cent. of casein in the sample of milk. For example, it required 9.4 cc. to neutralize 100 cc. of the filtrate, and since it represented 12.5 cc. of the acid added to the 200 cc. of the diluted milk, we have $12.5-\frac{N}{10}\ 9.4 = 3.10$ per cent. casein.

Below are some of Van Slyke's results obtained by this method in comparison with the official method.

PERCENT CASEIN.

Volumetric Method (Van Slyke-Bosworth).	Official Method.
3.00	3.00
3.40	3.36
3.30	3.21
3.20	3.16
2.90	2.95
2.70	2.60

The second volumetric method which I wish to consider is that of E. B. Hart, of the University of Wisconsin, published in Research Bulletin, No. 10, 1910. For speed and accuracy this method offers no advantage over that of Van Slyke's and Bosworth's, just mentioned. However, the method is unique and sound in principle. The fact that free casein has the properties of an acid and can combine with an alkali in a definite proportion, it seems rational that if we dissolve casein in excess of alkali and the uncombined alkali is estimated by titration, using phenolpthalein as an indicator, we are in a position to calculate the casein equivalent per cc. of standard alkali used. This is true, and upon this principle rests Hart's volumetric method. Hart found the casein equivalent for each 1 cc. $\frac{N}{10}$ KOH to be .108 grams. Therefore, if we titrate the casein obtained from 10.8 grams of milk, we see that each cc. of alkali used must represent 1 per cent. of casein.

Details of the method. Measure 10.5 cc. or weigh 10.8 grams of milk into a 200 cc. Erlenmeyer flask, add 75 cc. of distilled water at room temperature and add to this 1 to 1.5 cc. of a 10 per cent. solution of acetic acid. The flask is given a quick rotary motion, usually 1.5 cc. of acetic acid gives

a clear and fast filtering separation, but if the milk is low in casein a little less acetic acid should be used. The separated precipitate is now filtered through a filter (9-11 cm. filter), the flask rinsed out thoroughly and poured on the filter, preferably cold. If a strong stream of water is directed against the filter, the casein washing is facilitated. About 250 to 300 cc. of water should pass through the filter to insure the removal of all traces of acetic acid. The precipitate, together with the filter paper, is now returned to the Erlenmeyer flask in which the precipitation was made. To this is now added 75 cc. of distilled water, *free from carbon dioxide*, and then a few drops of phenolpthalein and 10 cc. of $\frac{N}{10}$ potassium hydroxide. A rubber stopper is placed in the flask and the contents shaken vigorously. Complete solution is easily indicated by the disappearance of the white casein particles. After solution the stopper is rinsed off into the flask with carbon dioxide free water and immediately titrated with $\frac{N}{10}$ acid to the disappearance of the red color. It is necessary that a blank be run parallel with the determination. For example, suppose it required 7.20 cc. of acid to make the pink color just disappear and the blank amounted to .2 cc., the percent of casein would be 10 − 7.4 = 2.60 per cent. casein. *Precautions necessary.* First, water free from carbon dioxide, must be used. Second, the titration should be made as soon as solution of casein has taken place. This will be from half an hour to an hour after adding the $\frac{N}{10}$ alkali. Repeated shaking hastens solution.

Results obtained by Hart as compared with the official method.

PERCENT CASEIN.

Official Method.	Volumetric Method (Hart).
3.78	3.75
3.12	3.05
2.87	2.85
1.90	1.85
2.30	2.25
2.37	2.30

The next volumetric method to be considered is the Formol titration method. This is perhaps the most rapid method of the three volumetric methods, for estimating the proteids in milk. It was pointed out in 1900 by Hugo Schiff that when formaldehyde was added to amino acids, the acid

properties of the acid were developed and could be titrated as any organic acid.

S. P. L. Sorensen worked out the details and made it possible to estimate amino acids quantitatively by means of formaldehyde. It is well known that amino acids, such as are formed by the hydrolysis of proteins, especially milk proteids, are neutral to phenolpthalein, have both an acidic group, carboxyl and a basic (amino) group. These exist in the same molecule and being the alpha amino acids neutralize each other, or in other words we have an amphoteric molecule, but as soon as formaldehyde is added, it reacts with the alkaline or basic group forming a methylene compound and leaving the acid group free to act.

For example:

$$CH_3 - CH\begin{cases} NH_4 \\ COOH \end{cases} + HCOH = CH_3 - CH\begin{cases} N = CH_2 \\ COOH \end{cases} + H_2O$$

(Alanine) (Formaldehyde)

$$CH_3 - CH\begin{cases} N = CH_2 \\ COOH \end{cases} + KOH = CH_3 - CH\begin{cases} N = CH_2 \\ COOK \end{cases} + H_2O$$

From Emil Fisher's researches on protein and polypeptids there is no doubt that the protein molecule is composed of amino acid units. The carboxyl group ($-COOH$) of one amino acid is combined with the amino group ($-NH_4$) of another amino acid, forming peptids, di, tri, etc., to polypeptids. For example, glycyl-glycine composed of two units of gylcine.

$$\begin{array}{l} CH_2 - CO\,OH \\ | \\ H_2N \end{array} \quad \begin{array}{l} CH_2 - COOH \\ | \\ H - N - H \end{array} = \begin{array}{l} CH_2\,CO - CH_2 - COOH \\ | \qquad\qquad | \\ H_2N \qquad\quad NH \end{array} + H_4O$$

(Glycine) (Glycine) (Glycil-Glycine)

Likewise different units may combine, as example, alanyl-glycyl-tyrosine From which we see that each peptid has one carboxyl group ($-COOH$) acidic and one amino group ($-NH_2$) basic. Now if the protein molecule is built up from amino acids, we can expect it to split up into simpler mole-

cules, by hyroloysis either with an acid or ferment into peptones, etc. Then we would expect the formol number to increase, double, if each protein molecule were split into two simpler ones. This is true, so formol titration gives a measure of the hydrolytic cleavage. We know that the proteids of milk are neutral to indicators, but on the addition of the formaldehyde become decidedly acid to these indicators.

Now if we can determine a factor or equivalent of the acidity produced on the addition of the formaldehyde to milk proteids, we can at once determine the percent of proteids in milk by titrating the acidity with a standard alkali.

In 1912, E. Holl Miller, of England, worked out a method for estimating the proteids in butter, and the same method is used in determining the proteids in milk.

Directions for estimating the proteids in butter. Weigh into a tared beaker exactly 10 grams of butter, which is placed in a water bath at 60° to 70° C. until the butter is completely melted. Twenty-five cc. of carbon dioxide free water is then added at about 60° C. and 1 cc. of phenolpthalein solution. The contents are well agitated. Run in $\frac{N}{20}$ NaOH until a faint permanent pink color is formed. It is found that the end point is masked by the yellow color of butter fat, the contents of the beaker should be allowed to settle and the bottom aqueous layer observed, and the addition of alkali continued until the pink tint is obtained. Five cc. of formaldehyde (40 per cent.) is added. The formaldehyde must either be neutralized before addition or its acidity equivalent for 5 cc. obtained and afterwards deducted. After the formaldehyde has been added the beaker is well shaken and again $\frac{N}{20}$ NaOH run in until a permanent faint pink color is produced in the aqueous layer. The number of cc. $\frac{N}{20}$ alkali used in the second titration less the amount equivalent to the acidity of the formaldehyde. No deduction is necessary if the formaldehyde was neutralized before being added to the butter. Now the number of cc. $\frac{N}{20}$ alkali used to neutralize the acidity produced on the addition of the formaldehyde is proportional to the protein present. One cc. of $\frac{N}{20}$ alkali is equivalent to .01355 grams of protein nitrogen or .0864 grams milk protein, assuming a definite proportion of casein and albumen. Then to calculate the percent of protein we have $\frac{.0864 \times 100 \times \text{cc.}}{10}$ = percent protein if 10 grams of butter were taken.

The following table shows the percent protein in butter by the Formol titration and official method:

Official Method.	Formaldehyde.
.65	.59
.48	.47
.46	.42
.48	.50
.60	.68
.45	.42
.42	.40
.41	.41
.49	.52

Procedure to estimate the protein in milk. To estimate the proteids in milk, weigh out 10 or 20 grams, preferably 20 grams, in a tared beaker, about 150 to 200 cc. capacity. Add 1 cc. of phenolpthalein solution, then run in from a burette $\frac{N}{20}$ NaOH until decided pink color is produced, a little practice will enable one to carry the shade of color in mind. Then add 10 cc. of neutralized formaldehyde, stir with a glass rod, when well mixed add $\frac{N}{20}$ NaOH until the same shade of pink is produced as that before the formaldehyde was added (note this last addition of alkali). For example, if 7 cc. of $\frac{N}{20}$ NaOH were required to neutralize the acidity produced on addition of formaldehyde to 20 cc. of milk, then as in the case of butter:

$$\frac{.0864 \times 100 \times 7}{20} = \text{percent protein} = 3.024$$

If we wish to estimate the casein alone and assuming the casein and albumen are in proportion of 3 per cent. casein and .5 per cent. albumen, then by using the equivalent of .075, we have as above:

$$\frac{.075 \times 100 \times 7}{20} = \text{percent casein} = 2.62$$

The following table gives the results of the three volumetric methods compared with the official methods on the same sample of milk:

PERCENT CASEIN.

Official.	Van Slyke-Bosworth.	Hart.	Formol Titration.
2 98	3.05	2 95	2 99
2 96	3.05	2 90	2 98
2.45	2.45	2.40	2 50
2 40	2 40	2.35	2 48
1 79 (d)	1 80	1 80	1 85
1 77 (d)	1 75	1 85	1 83
3 28	3.25	3 18	3 18
3 29	3 20	3 15	3 20
2 46	2 49	2 40	2 46
3 77	3.80	3 65	3 70
2 90	2.90	2 80	2 96
2.47	2.50	2 45	2 48
3 71	3 70	3 70	3 71
2 85	2 85	2 85	3 01
2 80	2 71	2 70	2 76
2 89	2 85	2 90	2 91

Note.—The two samples marked (d) were diluted milk.
Samples were taken on different days from the same source.

The above table shows the relative accuracy of the different methods. For the estimation of casein in milk the choice of the methods mentioned depends on the purpose for which the analysis is made. If total proteids are to be estimated, the Van Slyke-Bosworth and Hart methods must be excluded, unless an assumption is made as to the average amount of albumen in milk. This could be done on the same basis as that for the formol method and which would introduce only a slight error for normal milk and from a mixed herd.

In reviewing these methods and considering speed, and ease of carrying out the work, the formol titration method is to be preferred. In all three volumetric methods it is very essential that the water used for dilution should be free from carbon dioxide. Very little distilled water found in laboratories is free from carbon dioxide. This factor alone may introduce errors to vitiate the results. Titration after the addition of the formaldehyde should be carried to a sharp pink color and remain so for at least five minutes.

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