DIRECT COMPLEXOMETRIC DETERMINATION OF THE CALCIUM AND MAGNESIUM IN MILK (ERIOCHROMEBLACK T METHOD)

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SUMMARY

A method was developed to determine the total calcium and magnesium in diluted milk by direct titration with ethylenediamine tetraacetate (EDTA). The method yields complete recovery of the added calcium (99.86-100.02%) and values for total calcium and magnesium close to those obtained by the methods of Jenness and Bird et al. The test is not significantly affected by changes in dilution of the milk with water from a ratio of 1:10 to 1:30 or by differences in the amount of indicator added in the range of 0.025 to 0.075 ml. It was found that variations in the pH of the system from 8.5 to 10.0 did not significantly affect the results. Also, the test can be performed over a broad range of buffer strength (0.5 to 1.5 ml per 50-ml sample). The presence of the phosphates and proteins does not influence the results. The method is convenient, rapid, and reproducible, with a standard deviation of 0.20 meq/liter of milk or 0.264% of the total calcium and magnesium in the milk, which indicates sufficient precision for most purposes.

The importance and the similarity of the divalent cations, calcium and magnesium, in the milk, has resulted in the development of a variety of analytical procedures for their simultaneous determination. The work of Schwarzenbach and co-workers (2, 12, 13) for the determination of calcium and magnesium with ethylenediamine tetraacetate (EDTA) as the titrating reagent opened the way for its use in biological materials. Jenness (9) found that a milk serum prepared by acid precipitation, followed by treatment with an anion exchange resin in the acetate form, could be analyzed successfully in this manner for calcium and magnesium. Other workers have employed various methods for the preparation of the milk sample for titration: Van der Have (8) precipitated the proteins with trichloroacetic acid and Gehrke et al. (7) ashed the sample and removed the phosphates with anion exchange resin. Recently, Bird et al. (3) have developed a method for calcium and magnesium determination in milk serum prepared with potassium metastannate, according to the procedure suggested by Ling (11), by which proteins and phosphates are removed simultaneously.

These workers removed the proteins before titration, because of possible interference due to the turbidity of the sample system (8), and the phosphate because of the interference of this ion in the calcium determination with murexide (ammonium purpurate) as the indicator observed by Cheng and Bray (4), Willson (14), Gastler (6), and Jenness (9). However, recently Kamal (10) has determined the calcium and magnesium in the milk and blood plasma in the presence of the phosphates and proteins by back-titrating an excess of EDTA with Eriochromeblack T as indicator and artificial fluorescent light.

The interference with the test of such trace-metal ions as manganese, iron, aluminum, copper, nickel, and cobalt (5) has been eliminated by the incorporation of sodium sulfide in the borate buffer (1).

The purpose of the present paper is to describe a procedure for the determination of the total calcium and magnesium, which avoids the time-consuming and error-introducing steps of removing the proteins and phosphates from the milk. The test is performed in diluted milk in the presence of all its constituents by direct titration with EDTA and without fluorescent light. The limitations and the possibilities of the new method are investigated by a study of the various factors involved and results are compared with those of other methods.

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METHOD

Reagents

Standard EDTA solution. Dissolve 10 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate (TitraVer, Hach Chemical Company, Ames, Iowa) and 2 g of

sodium hydroxide pellets in water, and dilute to one liter with water. This solution has a titer of approximately 0.05 meq/ml and is standardized against the standard calcium solution.

Standard calcium chloride solution. Dry $CaCO_3$ (Analytical Reagent) at 100 C to constant weight and dissolve about 2.5 g (exactly weighed) in a minimum of hydrochloric acid. Transfer to a liter volumetric flask and dilute to volume with water. This solution contains approximately 0.05 meq Ca/ml, $\left(\frac{\text{g } CaCO_3}{100.09} \times 2 = \text{meq Ca/ml}\right)$.

Borate buffer. Dissolve (a) 3.332 g sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$) (A.R.) in 80 ml of water, and (b) 0.54 sodium hydroxide (A.R.) and 0.4165 g sodium sulfide (A.R.) in 10 ml water. Cool, mix the two solutions, and dilute to 100 ml with water.

Eriochromeblack T indicator solution. Dissolve 1 g of Eriochromeblack T powder in a mixture of 30 ml of water and 1 ml of 1 N sodium carbonate, and make up to 100 ml with 2-propanol. The indicator should be freshly prepared at least once a week.

Deionized water was used in the preparation of all reagents and throughout the study.

PROCEDURE

Twenty-five milliliters of milk is transferred to a 500-ml volumetric flask and diluted to volume with water.

Fifty-milliliter aliquots of the diluted milk are transferred to 125-ml Erlenmeyer flasks, 1 ml of borate buffer and three drops of Eriochromeblack T indicator are added, and the sample is titrated with EDTA solution to a light blue end point. A microburet, 5-ml capacity, calibrated in 0.01-ml divisions, provides satisfactory accuracy of volume measurement.

To determine the end point accurately, the Erlenmeyer flask with the sample should always rest on a white background. The titration is continued until, with further addition of EDTA solution, no change in the color takes place. It is helpful to develop completely the color of the end point in one of the aliquots by overtitration. Then with this color as a guide, the titration is continued until the colors match. Since the color of the solution used for comparison changes gradually with time, it should be replaced at intervals.

Concentration of the calcium and magnesium in the milk is calculated as follows:

$$\text{meq(Ca + Mg)/liter} = \frac{\text{Milliliter EDTA solution employed} \times \text{normality of EDTA solution} \times 1{,}000}{\text{Milliliter of milk in the sample titrated}}$$

RESULTS

Comparison between direct and back titration. Although Kamal (10) was able by means of back titration to satisfactorily recover calcium and magnesium added to milk and blood plasma, this method is not as simple nor as potentially accurate as direct titration. Therefore, the direct and back titration methods were compared. The direct titration was performed as described above, whereas in the back titration 5 ml EDTA solution was added to the dilute sample and standard calcium chloride solution was used to back-titrate the sample to the light purple color.

Results (Table 1) indicate no significant difference between the two methods of titration and, therefore, direct titration was employed.

TABLE 1

Comparison of the direct and back titration method for the determination of total calcium and magnesium in milk

	Calcium and magnesium content (Average of four replicates)			
Milk Sample No.	1	2	3	4
	(meq/liter)			
Direct titration	76.45	74.75	74.05	69.25
Back titration	76.26	74.53	73.84	69.15

Analysis of variance

Source of variance	Degrees of freedom	Mean square	Significance [a]
Method (M)	1	0.263	N.S.
Milk (m)	3	75.033	V.S.
M × m	3	0.006	N.S.
Error	24	0.064	

[a] V.S. = Very significant; N.S. = not significant.

Comparison of the proposed method with other methods. To help establish that prior treatment of the milk sample is unnecessary for the accurate determination of total calcium and magnesium in milk, the proposed method was compared with the procedures of Jenness (9) and Bird et al. (3). Results of the comparison with Jenness' procedure (Table 2) show that for routine analytical purposes the methods provide reasonably close results; never-

TABLE 2

Comparison of the proposed method with the method of Jenness for the determination of total calcium and magnesium in milk

Milk Sample No.	Calcium and magnesium content (Average of four replicates) 1	2	3	4	5
	(meq/liter)				
Proposed method	77.57	74.67	76.46	79.70	72.49
Jenness' method	76.81	73.98	75.80	78.70	71.49

Analysis of variance

Source of variance	Degrees of freedom	Mean square	Significance [a]
Method (M)	1	6.947	V.S.
Milk (m)	4	60.611	V.S.
M × m	4	0.015	N.S.
Error	30	0.039	

[a] V.S. = Very significant; N.S. = not significant.

theless, the differences are real, as shown by the analysis of variance.

The Jenness method always yields lower values than the direct method. To determine if calcium and magnesium is lost in the precipitate in Jenness' method, 10 ml of milk was adjusted to pH 4.0-4.1, transferred to a 100-ml volumetric flask, diluted to volume, and filtered into a 100-ml volumetric flask. The amount of the serum collected was measured by addition of water to the mark and the missing volume of material was considered as consisting of the precipitated protein and occluded serum. The volume of the precipitate was estimated from reasonable values, as shown in Table 3. The difference between the missing volume and the volume of the precipitate is considered as the amount of serum occluded in the precipitated proteins. The total calcium and magnesium in the serum was determined by Jenness' procedure. The precipitate with occluded serum was dispersed with 0.1 N NaOH, transferred to a 100-ml volumetric flask, and diluted to volume with water. The total calcium and magnesium in this dispersion was then determined according to the proposed method.

A comparison by the "t" test of the total calcium and magnesium content of the precipitate and the occluded serum with the calculated values for the occluded serum (Table 3) indicates that the precipitate contains calcium and magnesium (0.1% significance level). However, correcting the values obtained by Jenness' method for both the volume of the precipitate and the calcium and magnesium content of the precipitate does not completely account for the difference observed between the two methods. It is recognized that if the estimate of the value of the precipitate was too great, the amount of calcium and magnesium calculated to be in the precipitate would be less, but even

TABLE 3

Influence of protein precipitation upon the total calcium and magnesium determination by Jenness' method

Sample No.	Ca and Mg content (Jenness' method) Obs.	Cor.[a]	Filtrate collected *(10 ml milk)*	Serum occluded in precipitate *(10 ml milk)* Vol.[b]	Ca and Mg content *(Cal)*[c]	Ca and Mg content of precipitate and occluded serum *(10 ml milk)*	Ca and Mg content by Jenness' method *(2nd cor.)*[d]	Ca and Mg content by proposed method *(avg of 4 replicates)*
	(meq/liter)		*(ml)*	*(ml)*	*(meq)*	*(meq)*	*(meq/liter)*	*(meq/liter)*
1	78.67	78.45	95.2	4.52	0.0356	0.0406	78.95	
2	78.63	78.41	95.0	4.72	0.0371	0.0408	78.78	79.70
3	78.91	78.69	95.1	4.62	0.0365	0.0419	79.23	
4	78.59	78.37	94.8	4.92	0.0387	0.0414	78.64	

[a] Corrected for the estimated volume of precipitate (0.280 ml) calculated assuming 2.8% casein in the milk sample, 80% of which is precipitated under the conditions of the test, with a specific volume of 0.75 ml/g and a hydration of 0.5 g H_2O/g protein.

[b] Volume of occluded serum = 100 ml − vol collected − est. vol of ppt.

[c] Ca and Mg content = obs. Ca and Mg content (meq/liter of milk) × vol of occluded serum (ml)/10,000.

[d] Corrected not only for the volume of ppt but also for the Ca and Mg content of the ppt.

on the assumption that the volume of the occluded serum was equal to the volume of the precipitate and the occluded serum, the calcium and magnesium content of the precipitate and the occluded serum would be significantly higher. Therefore, the precipitate must contain some calcium and magnesium. The observation that the analytical results by Jenness' method even after the correction for the calcium and magnesium in the precipitate are significantly lower than those obtained by the proposed method may be due to small amounts of calcium and magnesium retained by the filter paper during the dispersion and transference of the precipitate and the occluded serum.

When the proposed method was compared with that of Bird et al. (3) (Table 4), the

TABLE 4

Comparison of the proposed method with the method of Bird et al. for determination of total calcium and magnesium in milk

Milk Sample No.	Calcium and magnesium content (Average of four replicates) 1	2
	(meq/liter)	
Proposed method	75.81	72.49
Bird's method	76.17	72.94

Analysis of variance

Source of variation	Degrees of freedom	Mean square	Significance [a]
Method (M)	1	0.667	5%
Milk (m)	1	42.940	V.S.
M × m	1	0.008	N.S.
Error	12	0.117	

[a] V.S. = Very significant; N.S. = not significant.

method of Bird et al. yielded slightly higher values (5% significance level). The difference is small (0.48-0.62%) and can be explained as due to a possible bias introduced into the results of Bird's method by the use of a mean correction factor for the precipitate volume. If one considers the range of values obtained by employing the individual correction factors (Sample 1, 75.59-76.95; Sample 2, 72.64-73.28), it can be seen that results obtained by the proposed method fall either within the range or very close to it.

Recovery of the added calcium and influence of the pH. The recovery of known amounts of added calcium provides a way of testing whether the method measures all of the calcium present. Also, it is known that the pH influences results of the titration. Therefore, the three systems indicated in Table 5 were prepared and their pH adjusted to the values indicated by adding 1 ml of the borate buffer (without NaOH) and appropriate amounts of 0.1 N NaOH or 0.1 N HCl. Titration of the systems according to the proposed procedure yielded complete recoveries of added calcium within the pH range of 8.5 to 10.5. However, at pH 8.0 it is impossible to determine the end point and at pH 10.5 significantly higher values for total calcium and magnesium are obtained. From pH 8.5 to 10.0 no significant difference was obtained in the titration values; however, the end point was much more satisfactory at pH 9.5-10.0.

Effect of other variables. Since the concentration of the indicator, the dilution of the milk sample, and the strength of the buffer may affect the determination of calcium and magnesium with the new method, it is important to know the extent of their influence and the range throughout which the determination is free of these influences. Basically, when one of the variables was investigated, all of the other variables were kept constant.

a. Influence of the concentration of the indicator was investigated with freshly prepared Eriochromeblack T solution. Amounts used were 0.025 ml, 0.05 ml (which represents about three drops), and 0.075 ml.

b. Influence of the dilution of the sample with water was investigated at three levels: 1:10, 1:20, and 1:30. Amounts of indicator and borate buffer and the pH were adjusted accordingly.

c. To investigate the influence of the strength of the borate buffer, it was prepared without sodium hydroxide and 0.5 ml, 1.0 ml, and 1.5 ml were added to the diluted milk. The pH was kept constant in all samples by adding the proper amount of 0.1 N NaOH.

Results (Table 6) indicate that none of these variables significantly affects the titration throughout the ranges investigated.

Influence of phosphates and protein. The effect of phosphate concentration upon the test was determined by adding to the diluted milk varying amounts of a solution of Na_2HPO_4 containing 0.950 g P/liter, as indicated in Table 7. The analysis of variance of these results indicates that concentration of phosphate four times the total phosphates present in the

TABLE 5

Recovery of added calcium and influence of the pH upon total calcium and magnesium determination by the proposed method

pH	Calcium and magnesium content (Average of four replicates)			Calcium recovered	
	Milk sample [a]	Standard $CaCl_2$ solution [b]	Milk sample and standard $CaCl_2$ solution [c]		
	(meq)			*(meq)*	*(%)*
	(1)	(2)	(3)	(4)	(5)
10.5	0.2016	0.1947	0.3962	0.1946	99.94
10.0	0.1999	0.1944	0.3941	0.1942	99.94
9.5	0.1997	0.1944	0.3941	0.1944	100.02
9.0	0.1999	0.1944	0.3942	0.1943	99.94
8.5	0.2001	0.1946	0.3944	0.1943	99.86
8.0	End point indistinct				

Analysis of variance

Source of variance	Degrees of freedom	Mean square	Significance [d]
		($\times 10^5$)	
1. Influence of the pH (Columns 1, 2, and 3 above)			
System (S)	2	25,952.304	V.S.
pH	(4)	0.436	V.S.
10.5 vs. others	1	1.698	V.S.
10.0 vs. 9.5, 9.0, and 8.5	1	0.007	N.S.
9.5 vs. 9.0 and 8.5	1	0.027	N.S.
9.0 vs. 8.5	1	0.012	N.S.
S × pH	(8)	0.069	1-0.1%
(M [a] and M + Ca [c] vs. Ca [b]) × (pH 10.5 vs. others)	1	0.534	V.S.
(M [a] and M + Ca [c] vs. Ca [b]) × (pH 10.0 vs. 9.5, 9.0, and 8.5)	1	0.0001	N.S.
(M [a] and M + Ca [c] vs. Ca [b]) × (pH 9.5 vs. 9.0 and 8.5)	1	0.002	N.S.
(M [a] and M + Ca [c] vs. Ca [b]) × (pH 9.0 vs. 8.5)	1	0.001	N.S.
(M vs. M + Ca) × (pH 10.5 vs. others)	1	0.011	N.S.
(M vs. M + Ca) × (pH 10.0 vs. 9.5, 9.0, and 8.5)	1	0.002	N.S.
(M vs. M + Ca) × (pH 9.5 vs. 9.0 and 8.5)	1	0.002	N.S.
(M vs. M + Ca) × (pH 9.0 vs. 8.5)	1	0.0004	N.S.
Error	45	0.017	
2. Recovery of added Ca (Columns 2 and 4 above)			
Added vs. recovered Ca (R)	1	0.014	N.S.
pH	4	0.014	N.S.
R × pH	4	0.002	N.S.
Error	30	0.012	

[a] 25 ml diluted (1:10) milk + 25 ml water (M).
[b] 15 ml $CaCl_2$ solution (0.01296 meq Ca/ml) + 35 ml water (Ca).
[c] 25 ml diluted milk + 15 ml $CaCl_2$ solution + 10 ml water (M + Ca).
[d] V.S. = Very significant; N.S. = not significant.

average milk does not have any significant effect upon the results.

To investigate the influence of the concentration of the protein, calcium- and magnesium-free skimmilk was prepared by shaking 200 ml skimmilk for 1 hr with 250 g cation exchange resin (Amberlite IRC-50 equilibrated with sodium malate buffer $\Gamma/2 = 0.14$, pH 6.7). Varying amounts of this milk were added to the diluted milk sample and the pH adjusted to 9.5 with the borate buffer and additional 0.1 N NaOH. Results are shown in Table 7. Analysis of variance of these values indicates that a fourfold increase in protein content of the sample did not significantly affect the titration, although the performance of the test is easier

TABLE 6

Influence of concentration of indicator, dilution of the sample, and strength of the buffer upon total calcium and magnesium determination by the proposed method

Conc. of indicator		Dilution of sample		Strength of borate buffer	
Volume of indicator sol.	Ca and Mg content [a]	Ratio milk to water	Ca and Mg content [a]	Volume of buffer	Ca and Mg content [a]
(ml)	*(meq/liter)*		*(meq/liter)*	*(ml)*	*(meq/liter)*
0.025	79.32	1:10	79.36	0.5	79.43
0.050	79.35	1:20	79.21	1.0	79.50
0.075	79.32	1:30	79.18	1.5	79.27

Analysis of variance

Source of variance	Degrees of freedom	Mean square	Significance [b]
1. Indicator			
Conc of indicator	2	0.001	N.S.
Error	9	0.013	
2. Dilution			
Ratio of dilution	2	0.038	N.S.
Error	9	0.029	
3. Strength of buffer			
Vol of the buffer	2	0.057	N.S.
Error	9	0.039	

[a] Average of four replicates.
[b] N.S. = Not significant.

at lower concentrations of protein.

Reproducibility. To determine the reproducibility of the proposed method, 20 different milk samples were analyzed in quadruplicate. The

TABLE 7

Influence of concentration of phosphates and proteins upon total calcium and magnesium determination by the proposed method

Phosphates		Proteins	
Added phosphate solution [a]	Ca and Mg content [b]	Added cation exchanged milk [a, c]	Ca and Mg content [b]
(ml)	*(meq/liter)*	*(ml)*	*(meq/liter)*
0.0	79.11	0.0	79.11
2.5	79.07	2.5	79.08
5.0	78.97	5.0	79.34
7.5	78.98	7.5	79.20

Analysis of variance

Source of variance	Degrees of freedom	Mean square	Significance [d]
1. Phosphates			
Conc of phosphates	3	0.018	N.S.
Error	12	0.020	
2. Proteins			
Conc of proteins	3	0.054	N.S.
Error	12	0.058	

[a] Vol added to diluted milk sample containing 2.5 ml of the original milk.
[b] Average of four replicates.
[c] Ca and Mg content of the cation-exchanged milk is zero.
[d] N.S. = Not significant.

standard deviation of a single determination from its mean was observed to be ± 0.20 meq/liter of milk, or ± 0.264% of the total calcium and magnesium content.

DISCUSSION AND CONCLUSIONS

Since the recovery of the added calcium in milk by the proposed method is complete (range of recovery 99.86-100.02%), and the values obtained for total calcium and magnesium are very close to those of other commonly used methods, the method appears to be measuring all the existing calcium and magnesium in the milk. The values are about 1% higher than those obtained by Jenness' method (9). This difference appears to be due, at least in part, to the retention of calcium and magnesium by the precipitated proteins in the Jenness procedure. When compared with the method of Bird et al. (3), the results appear to agree, if one considers the possible variations in the correction factor for the volume of the precipitate employed in the Bird et al. procedure.

The test can be performed over a broad range of pH values, concentration of indicator, dilution of the sample, and strength of the buffer, as indicated in Tables 5 and 6. pH of the sample is controlled by the addition of the borate buffer. The amount of the NaOH added in the buffer has been selected so that when 1 ml of the buffer is added to 50 ml of diluted milk (1:20), the pH is increased to about 9.5, whereas in the 50-ml diluted standard calcium chloride solutions, which have no buffer capacity, the pH is not increased above 10.0. If abnormal milks are to be tested, in which the pH might be not only lower than the optimum range (9.5-10.0) but even lower than the working range (8.5-10.0), then addition of one to two drops of 1 N NaOH before titration should properly adjust the pH.

The presence of phosphates and proteins in concentrations at least four times the normal total phosphates and proteins concentrations of the milk do not cause any interference with the method.

The method is very reproducible, the standard deviation being ± 0.20 meq/liter of milk, or ± 0.264% of the total calcium and magnesium content, which is of the same order of magnitude as those obtained by the Jenness and Bird et al. methods. Kamal reports a standard deviation of ± 1.46% for calcium and ± 3.25% for magnesium.

Because the method is performed by direct titration, it provides a fast, easy, and accurate measurement of the total calcium and magnesium in milk.

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