sources of organic chemicals like oil is reproducible annually, will not be realized until the fundamental approaches to the separation, identification, and structure determination of their components have been fully exploited.

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MILK ANALYSIS

Direct Microdetermination of Calcium in Milk

J. R. MARIER and M. A. BOULET

Division of Applied Biology, National Research Laboratories, Ottawa, Canada

A RAPID MICROMETHOD for the determination of calcium was required for studies on ionic equilibria in milk. The method had to be suitable for the analysis of small aliquots of synthetic mixtures resembling milk serum but containing as little as 10 γ of calcium per ml. Gravimetric, colorimetric, and titrimetric methods (*1*) were unsatisfactory because they were time-consuming or lacked sensitivity with the limited aliquots of test solution available.

The method of Saifer and Clark (*2*), which estimates from 40 to 280 γ of calcium in water, was studied in detail with the aim of adapting it to milk and milk serum. A successful modified method, with increased sensitivity, measures from

A turbidimetric potassium oleate method for the direct determination of calcium in milk and milk serum is described. Samples containing from 40 to 100 γ of calcium are conveniently analyzed in the colorimeter tubes, and the turbidity developed can be measured from 20 to 60 minutes after addition of the reagent. The coefficient of variability of single determinations on standard calcium solutions was ±3.2%. Analysis of various milk samples gave values averaging 0.8% above those obtained by a permanganate titration method.

40 to 100 γ of calcium, and results of the study that led to its development are presented here.

Recommended Method

Reagents Glass-distilled water is recommended for the preparation of all reagents. Glass containers should be avoided because they impart a visible turbidity to the solutions. This is seen after a few days' storage in soft glass and after prolonged storage in borosilicate glass. All solutions remained clear after more than 1 year of storage in polyethylene, and this type of container is therefore recommended. Because ammonia slowly escapes from polyethylene bottles, reagents containing this alkali should be replaced every 2 months. Other reagents are stable for at least 2 months.

Potassium Oleate Solution. Mix 6.40 grams of potassium hydroxide, 25 ml. of water, 46 ml. of 95% ethyl alcohol, and 28.20 grams of oleic acid (USP light color) in a 1-liter distilling flask, reflux gently for 1 hour, then immediately dilute to 1 liter (*2*).

Gelatin Solution. Dissolve 20 grams of ash-free gelatin (Eastman Kodak calf or pig skin) in 800 ml. of water at 55° C., add 20 ml. of concentrated ammonium hydroxide, and adjust the volume to 1 liter.

Gelatin-Oleate Reagent. Immediately before using, mix equal volumes of potassium oleate and gelatin solutions.

Citrate-Phosphate Reagent. Dissolve 36.5 grams of trisodium citrate dihydrate and 7.3 grams of dipotassium phosphate trihydrate in water and dilute to 1 liter.

Potassium-Ammonium Hydroxide Solution. Dissolve 3.2 grams of potassium hydroxide in water, add 10 ml. of concentrated ammonium hydroxide, and make up to 1 liter.

Standard Calcium Solution. Suspend 2.4972 grams of calcium carbonate (British Drug Houses, dried at 105° C. for 18 hours) in 200 ml. of water, slowly add 6.0 ml. of concentrated hydrochloric acid, and make to 1 liter. This solution contains 1 mg. of calcium per ml.

Procedure To a sample containing 40 to 100 γ of calcium in a colorimeter tube, add 1 ml. of citrate-phosphate reagent and water to bring the total volume to 5 ml. Then add 5 ml. of gelatin-oleate reagent and swirl the tubes briskly. In the same manner prepare at least four standards containing 40 to 100 γ of calcium, and a "reagent blank." After 20 to 60 minutes at room temperature, determine the extinction coefficient of the standards and samples corrected for the reagent blank at 420 mμ. (An Evelyn colorimeter was used with the reagent blank set at 100% transmittance.) Estimate the calcium content of the samples by reference to the standards. The extinction coefficient per microgram of calcium is constant over the recommended range.

If the solution being analyzed has a significant absorption under the conditions of the test, a correction must be made. To do this, substitute potassium–ammonium hydroxide solution for the gelatin-oleate reagent in a separate aliquot of the test solution and in a reagent blank. Determine the extinction coefficient, corrected for the blank, and subtract it from the net value obtained in normal procedure. The necessity of including these "correction samples" can be assessed preliminary to full scale analysis by treating a sample of the milk or serum in question with the potassium-ammonium hydroxide and reading against a blank similarly prepared. No waiting period is required before these readings are made.

Experimental

In the original method (*2*), Duponol (sodium lauryl sulfate) was used to stabilize the suspension of calcium oleate and to prevent precipitation of magnesium oleate. In preliminary investigations, it was found that Duponol was not an effective "stabilizer" when phosphate and citrate were present in relatively high concentrations. The addition of citrate gave greater sensitivity than was obtained with Duponol and also permitted determinations in the presence of phosphate. Citrate was therefore substituted for Duponol in the procedure, after its effect had been studied in detail.

Increasing citrate from 0 to 13 mg. per 10 ml. of final solution caused the greatest increase in sensitivity (Figure 1); higher concentrations resulted in a gradual decrease. This decrease appeared to be a nonspecific effect of high salt concentration, as it was practically eliminated when the total salt concentration (per aliquot) was kept constant by adding excess potassium chloride and decreasing potassium chloride as citrate was increased. Although maximum sensitivity at low salt levels was obtained with 13 to 18 mg. of citrate, magnesium interference was serious at this level and 32 mg. of citrate was selected as giving satisfactory sensitivity without danger of magnesium interference in normal samples.

Gelatin was added because it contributed to sensitivity, improved stability, and helped to reduce interference of various ions. Ammonia provided the necessary alkalinity, and its admixture with the gelatin prevented gelation. Variations in ammonia from half to double the recommended concentration (*2*) had no effect. A mixture containing equal volumes of gelatin-ammonia and oleate solutions gave uniform sensitivity over the range of the method.

For reproducible sensitivity, the final pH of the reaction should be 10.5 to 10.7 and the gelatin-oleate reagent introduces sufficient alkalinity to provide this pH with test solutions of pH 6.5 to 10.0. When the pH of the test solution is lower, the amount of alkali required should be determined before analyses are undertaken. The desirable amount of alkali is then introduced into the colorimeter tube after addition of citrate, so as to avoid loss of calcium as precipitated calcium phosphate.

Saifer and Clark (*2*) had recommended a wave length of 420 mμ for the determination and this was confirmed in the course of this investigation. Precipitation of calcium oleate is apparently accompanied by an increase in yellow color, which contributes to the sensitivity at 420 mμ.

Full color and turbidity developed rapidly and readings taken after 10 minutes and 1 hour did not differ.

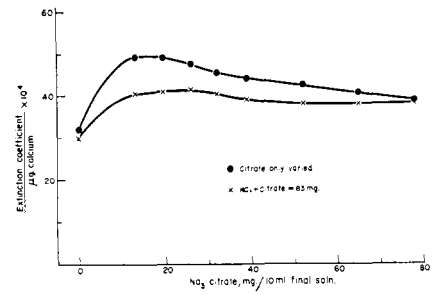

Figure 1. Effect of citrate on sensitivity of turbidimetric method for calcium
Average of quadruplicate determinations at calcium concentrations of 40, 60, 80, and 100 γ per 10 ml. of final solution

Table I. Ranges of Magnesium That Can Be Tolerated

Calcium, γ/10 Ml. Final Soln.	Citrate Added, Mg. 15.5	32	39
	Magnesium, γ/10 Ml. Soln.		
20	Nil	Nil	Nil
40	80–160	0–40	5–10
80	40–160	0–320	5–40

Table II. Effect of Salts on Sensitivity of Method

(Av. of duplicate values obtained with salts added separately. Calcium concentration 50 γ per 10 ml. of final soln.)

Salt Added[a], Mg.	Extinction Coefficient per γ Ca	% Loss in Sensitivity
None	0.00469	...
4	0.00464	1.1
6	0.00460	2.0
10	0.00454	3.2
20	0.00441	6.0
80	0.00371	21.0

[a] Potassium chloride, sodium sulfate, sodium carbonate, or sodium lactate.

In 3 hours, the average extinction coefficient decreased by less than 3%. Analyses performed at 16° and 35° C. gave results which did not differ significantly from those obtained at room temperature.

The effect of the major inorganic constituents of milk was studied. Phosphate did not interfere even when 5 mg. were added in addition to the 1 mg. in the citrate-phosphate reagent, but the high levels caused a slight decrease in the rate at which turbidity developed.

Magnesium interference was studied by adding from 2.5 to 640 γ of magnesium per 10 ml. of final solution at three calcium and three citrate concentrations. Values lying between 97.5 and 102.5% recovery (on the basis of samples containing no magnesium) were considered acceptable. At the low calcium level—e.g., 20 γ—magnesium interfered at all citrate concentrations by formation of yellow magnesium oleate (*3*). At the higher calcium levels, when magnesium interfered, it gave either high or low recoveries, depending on the citrate concentration. At the low citrate level, coprecipitation of magnesium produced high recoveries. This tendency was progressively offset by increasing citrate until, at the high citrate level, magnesium interference gave low recoveries, probably by altering the dispersion of the precipitated particles. At the intermediate citrate level, the two adverse effects seemed to be compensating and a broad over-all range of magnesium levels could be tolerated for good recovery (Table I). The addition of 32 mg. of citrate per 10 ml. of final solution is therefore recommended and will permit the tolerance of all magnesium concentrations likely to be introduced by milk or serum. In the absence of magnesium, the method can be used for aliquots containing less than 40 γ of calcium. However, in this low range the extinction coefficient per micrograms of calcium is not constant and standards having a calcium content close to that of the unknown must be used.

Interference of some ionic components of milk was studied. Potassium chloride, sodium sulfate, sodium lactate, and sodium carbonate all had similar effects, tending to decrease sensitivity of the method about 3% for every 10 mg. of salt added (Table II). However, the total salt present in a suitably diluted aliquot of normal milk would be approximately 0.35 mg. (assuming 0.7% ash), and even in experiments with sera depleted of calcium, a salt concentration of 4 mg. per aliquot would be exceptional. Interference from these salts is therefore negligible. Satisfactory analyses can be made at high salt concentrations by adding the equivalent amount of salt (within 10 mg.) to the standards and blank. A loss in sensitivity will result, but accuracy will be maintained.

To assess the reproducibility of results by this method, single series of standard solutions containing 20, 40, 60, 80, and 100 γ of calcium per aliquot were analyzed seven times during a 9-week period with one lot of reagents. The extinction coefficients per microgram of calcium, averaged over all five levels, varied nonsystematically from 0.00493 to 0.00516; average was 0.00503 ± 0.00009. Individual values varied from 0.00464 to 0.00546, with a standard error of ±0.00016 (coefficient of variability ±3.2%).

Although the method was designed for the determination of micro amounts of calcium, its accuracy was checked against the AOAC permanganate titration method (*1*). Three different lots of skim milk and one of milk serum were analyzed. To simulate possible variation in milk and serum, samples with added calcium, and others with some of the calcium removed by treatment with Amberlite IRC-50, were prepared from each lot. The turbidimetric analyses of skim milk I (Table III) were done after dry-ashing, while those of skim milks II and III, as well as rennet serum, were done directly. The results (Table III) show that the calcium contents estimated by both methods were in good agreement. The average value by the turbidimetric method was 0.8% higher than by the permanganate method, and the average difference, ignoring direction, was 1.5%.

Table III. Calcium Content of Milk and Serum

(Averages of duplicate determinations. γ per ml.)

	$KMnO_4$ Titration	Turbidimetric Method[a]	Difference, %, Turbidimetric Minus Permanganate
Skim milk I			
Untreated	1151	1144	−0.6
+100 γ Ca	1251	1246	−0.4
+200 γ Ca	1337	1335	−0.1
+300 γ Ca	1440	1430	−0.7
+4% Amberlite	1094	1106	+1.1
+10% Amberlite	909	936	+3.0
Skim milk II			
Untreated	1210	1222	+1.0
+100 γ Ca	1298	1351	+4.0
+200 γ Ca	1394	1412	+1.3
+300 γ Ca	1494	1507	+0.9
+4% Amberlite	1124	1133	+0.8
+10% Amberlite	975	966	−0.9
Skim milk III			
Untreated	1268	1261	−0.6
+200 γ Ca	1477	1463	−1.0
+400 γ Ca	1666	1659	−0.4
+8% Amberlite	1195	1241	+3.8
+15% Amberlite	1178	1215	+3.1
Rennet serum			
Untreated	420	409	−2.7
+100 γ Ca	499	511	+2.4
+200 γ Ca	599	608	+1.5
+4% Amberlite	394	389	−1.3

[a] Samples of skim milk I ashed before analysis; all others analyzed directly.

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