# DIRECT DETERMINATION OF CITRIC ACID IN MILK WITH AN IMPROVED PYRIDINE-ACETIC ANHYDRIDE METHOD

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## SUMMARY

The determination of citric acid with pyridine and acetic anhydride has been investigated at reaction temperatures from 17 to 60° C. The optimum proportions of pyridine, acetic anhydride, water, and acetic acid for maximum color intensity and stability are given for each temperature. The procedure has been modified to eliminate the violent nature of the reaction, even when the analysis is done at a reaction temperature of 60° C. Details of a method for the determination of 25–200 μg. of citric acid, at a reaction temperature of 32° C., are presented. In comparison with previously published methods based on the reaction, the recommended technique results in improved sensitivity, stability, and reproducibility without requiring careful timing.

The method has been successfully applied to the routine analysis of milk and milk products. Milk and serum can be analyzed directly, after suitable dilution. Corrections for the interference caused by fat in homogenized milk, and by trichloroacetic acid in T.C.A. serum, can be made easily.

Results of direct analysis of milk were from 5 to 15% higher than those for the corresponding sera and are believed to represent the true values for the citric acid content of milk.

Several methods for the estimation of citric acid depend on the Fürth and Herrmann reaction *(4)*, in which color is formed in the presence of pyridine and acetic anhydride. Saffran and Denstedt *(10)* applied this reaction to the colorimetric determination of citric acid in animal fluids and tissues, whereas Babad and Shtrikman *(1)* modified this procedure to estimate citric acid in milk serum. In both procedures, the sample was held for 10 min. at 60° C. in the presence of an excess of acetic anhydride, pyridine was added, and the mixture held a further 40 min. at 60° C., then cooled in an ice-bath. The two heating periods were accurately timed to minimize variability. Reducing the amount of water added with the sample was found to lower the intensity of the yellow color, but enhanced reproducibility *(1)*. Temperatures higher than 60° C. decreased color intensity and stability, while lower temperatures gave greater sensitivity but required longer holding periods for full color development *(10)*. Addition of pyridine to the hot solution caused a vigorous reaction that made it difficult to keep the tubes stoppered during the subsequent holding period *(1)*. Reinart and Nesbitt *(8)* modified the method to reduce the violence of the reaction at 60° C., whereas Murthy and Whitney *(6)* recommended the use of glass stoppers instead of rubber stoppers, to prevent possible contamination of the sample. However, even by rigid adherence to all of these recommendations, reproducible results were difficult to obtain.

The present investigation was undertaken to study factors affecting color development, with emphasis on lower reaction temperatures, and led to a procedure at a reaction temperature of 32° C. for the direct determination of citric acid in milk.

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## DEVELOPMENT OF THE METHOD

A preliminary study of the basic aspects of the reaction was made. It was found that the reaction was strongly exothermic, that a constant reaction temperature was essential to reproducibility, and that rapid color formation at temperatures below 60° C. could be induced by introducing more water with the sample. However, water and acetic anhydride concentrations were difficult to control if they were allowed to interact before the addition of pyridine. On the basis of these observations, pyridine was added to the sample prior to acetic anhydride and, as color development was not affected by the time interval between the two additions, the need for timing the pyridine step was obviated. All reagents were added at room temperature and the tubes placed in a water bath immediately after adding the anhydride. In this manner, the vigorous nature of the reaction was completely eliminated even at a reaction temperature of 60° C., and it was no longer necessary to stopper the tubes. This modified procedure was, therefore, adopted in the following studies.

The effect of concentration of pyridine and of water on changes in color intensity at 420 mμ with time was studied at fixed concentration of the other reagents. Glacial acetic acid was added as necessary to maintain constant volume and acted only as a diluent. The results obtained at a reaction temperature of 32° C. (Figure 1, A and B) show that increasing concentrations of water or pyridine to an optimum value increased the rate of color formation, but that an

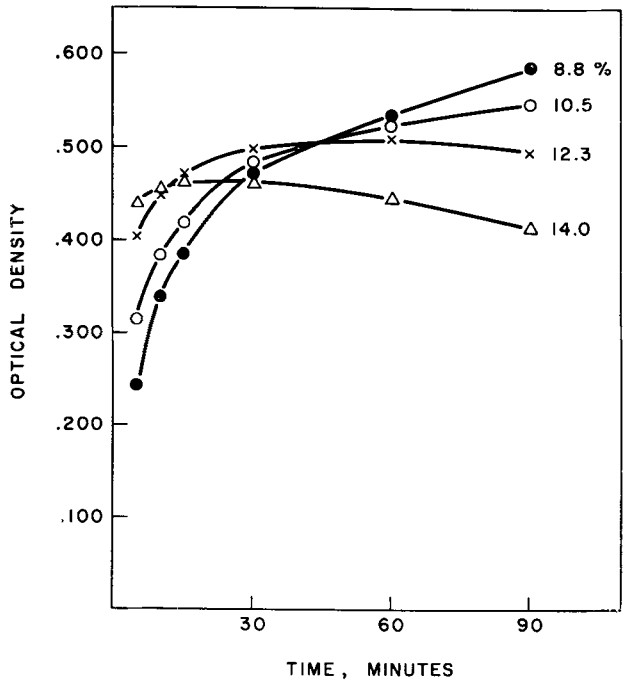


FIG. 1, A: Effect of water on sensitivity and stability. Reaction temp., 32° C.; citric acid, 200 μg/test; total volume, 11.4 ml. Pyridine, 15.8%; acetic anhydride, 70.1%; acetic acid plus water, 14.1%; per cent water is shown by the figures on the curves.

excess of either of these reagents promoted color-fading. Similar effects were observed at other reaction temperaures and with other concentrations of citric acid between 25 and 200 μg. per test. The effect of acetic anhydride could not be illustrated at temperatures below 44° C., because a significant excess could not be added without drastically affecting the pyridine and water concentrations. However, at a reaction temperature of 60° C., the effect of anhydride was similar to that of pyridine and water (Figure 1, C).

Studies were made at several reaction temperatures to determine the optimum proportion of reagents for the development of color of maximum intensity, compatible with a stability of at least 30 min. In this study, glacial acetic acid was not used except at temperatures above 44° C., where it was necessary to substitute it for part of the acetic anhydride, as high concentrations of the latter caused rapid color-fading. The optimum proportions of reagents (% volume / arithmetic total volume) are shown (Figure 2). The amounts of reagents used by Saffran and Denstedt *(10)*, assuming complete removal of water by hydrolysis of acetic anhydride, agreed with our optimum conditions at 60° C., but those used by Babad and Shtrikman *(1)* deviated markedly.

A comparison of results obtained at optimum reagent proportions for various temperatures showed that maximum intensity and stability were obtained at reaction temperatures between 17 and about 35° C. (Figure 3); sensitivity

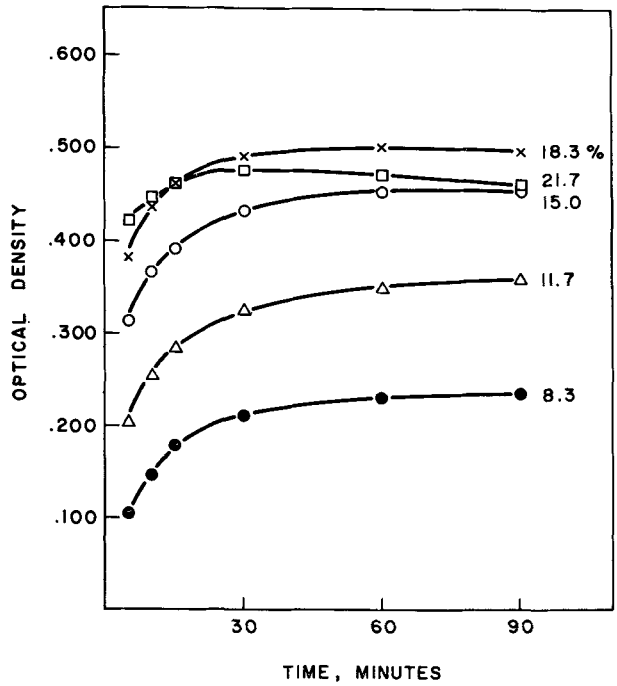


FIG. 1, B: Effect of pyridine on sensitivity and stability. Reaction temp., 32° C.; citric acid, 200 μg/test; total volume, 12.0 ml. Water, 11.7%; acetic anhydride, 66.6%; acetic acid plus pyridine, 21.7%; per cent pyridine is shown by the figures on the curves.

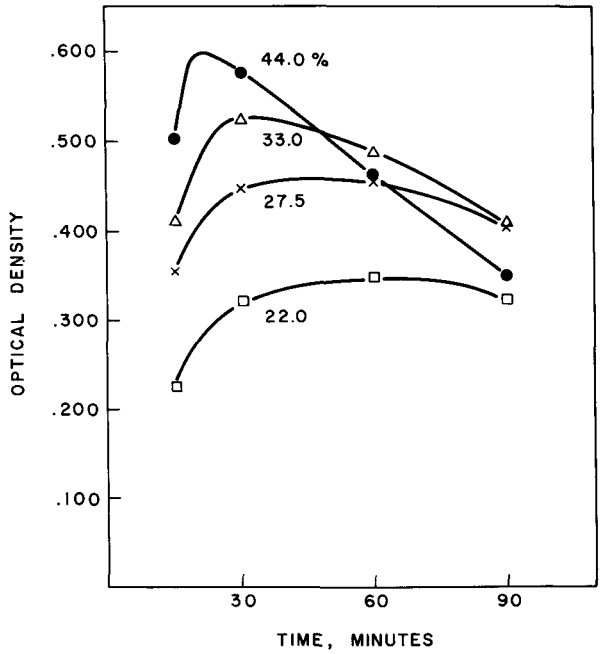


FIG. 1, C: Effect of acetic anhydride on sensitivity and stability. Reaction temp., 60° C.; citric acid, 200 μg/test; total volume, 9.1 ml. Water, 1.1%; pyridine, 11.0%; acetic acid plus acetic anhydride, 87.9%; per cent acetic anhydride is shown by figures on the curves.

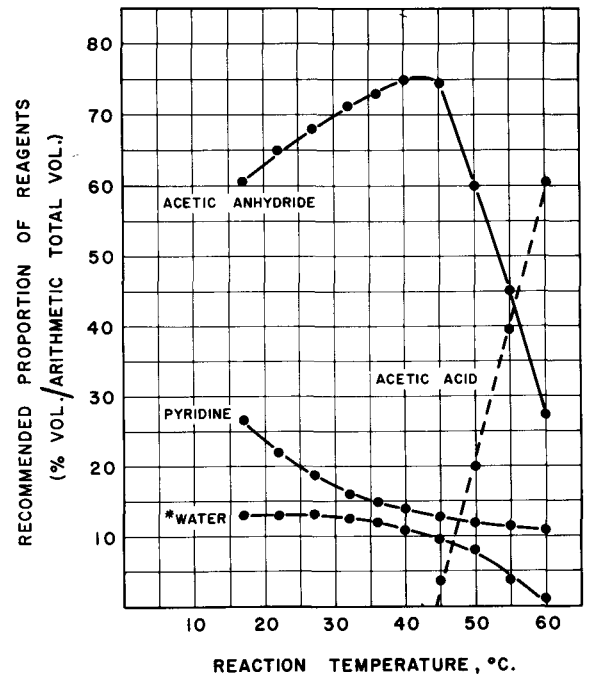


FIG. 2. Optimum proportion of reagents at various reaction tempeatures.
* Includes water of the sample.

and stability decreased slightly at 40° C. and markedly at higher temperatures. The present technique at 32° C. was more sensitive than either the Saffran and Denstedt *(10)* or the Babad and Shtrikman *(1)* procedures (Figure 4). Reproducibility of the method at 32° C. was assessed by analyzing standard solutions of citric acid 14 times during a 3-wk. period; the results showed a coefficient of variability of 0.37%, compared to 1.0% obtained in a parallel study with the Saffran and Denstedt method.

The optimum conditions for use with the present technique were determined at constant temperature throughout the holding period. However, for routine

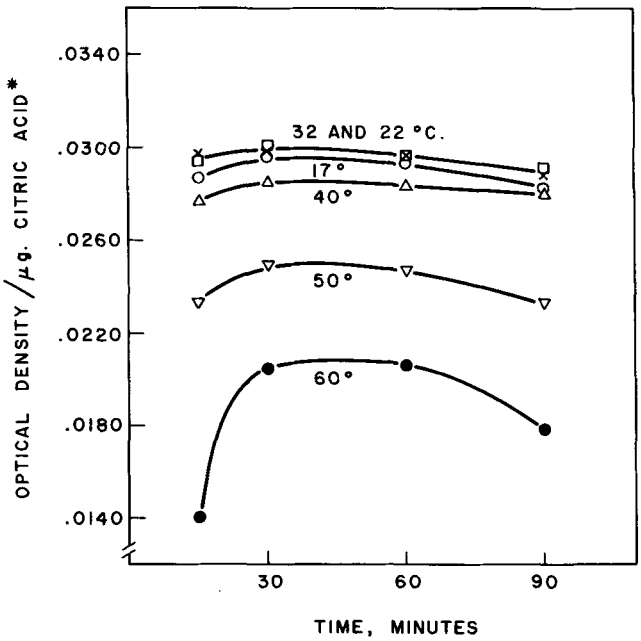


FIG. 3. Effect of various reaction temperatures, using optimum reagent concentrations, on color intensity and stability. (Average of results with 25, 50, 100, and 200 μg. of citric acid per test.)

* *Corrected to a 1.0-ml. final volume for all temperatures. [Dividing the given value by the total volume (ml.) used in the test will give the actual sensitivity coefficient obtainable.]*

analysis, it was convenient to withdraw the samples from the bath as a group after color production was complete (e.g., 30 min.), and the effect of subsequent holding in air at several temperatures was, therefore, investigated. Color stability could be maintained for 30 min. by holding the samples in air at a temperature of 3–10° C. below the bath temperature. If the gradient between bath and room temperatures is not as recommended, readings should be made within 10 min. after removing the tubes from the bath or, if more desirable, another reaction temperature can be selected. Cooling in an ice bath, as previously recommended (*1, 6, 8, 10*), results in a loss of stability and should, therefore, be avoided.

It appears probable that the changes in color intensity with time are the re-

The effect of lactose was studied by introducing up to 50 mg. of the milk sugar with the 1.0-ml. aliquot. Color intensity was reduced by about 2% for every 10 mg. of lactose added, but since 5 mg. or less is present in suitable dilutions of milk, no correction is required.

Values obtained after application of the turbidity correction for fat (described above) agreed within 2.0% with the value for the same milk after fat separation, indicating that milk fat did not contribute to color development.

Possible interference by acids used in the preparation of milk sera was investigated. The effect of hydrochloric and trichloroacetic acids on the intensity and stability of the color was studied by analyzing aliquots of standard citric acid solutions containing 5% T.C.A., or various concentrations of hydrochloric acid up to 1.0 *N*. Hydrochloric acid did not affect the intensity or stability of the color; T.C.A., although it did not affect stability, caused a slight increase in absorption at 420 mμ, but the additional color developed to the same extent in blanks and standards. In a similar manner, the effect of sodium hydroxide was studied. It was found that aliquots of NaOH of up to 0.1 *N* concentration could be tolerated.

The effect of milk proteins on the Babad and Shtrikman method *(1)* has been studied by Murthy and Whitney *(6)*, who concluded that the presence of residual protein in milk serum resulted in erroneously high citric acid values. In our experiment, the possible interference of casein was investigated with 1% solutions of casein [Van Slyke and Baker *(12)*] in 0.1 *N* NaOH, or anhydrous pyridine. For the test, full aliquots of the pyridine or NaOH solution replaced—respectively—the pyridine or water required in the normal procedure. The test solutions obtained with the NaOH preparation were clear, but a faint turbidity—for which no correction was made—was present in the pyridine samples (this casein turbidity is not encountered in direct analysis of milk). The turbidity correction described could not be applied, because glacial acetic acid dissolves the casein.

The results indicated that solutions of casein in pyridine contained chromogenic material equivalent to 1.0 μg. of citric acid per milligram of casein, whereas the NaOH solution contained 2.0 μg per milligram. Precipitating the casein from the NaOH solution with hydrochloric acid failed to reduce the amount of chromogenic material obtained on re-solution of the casein, while an approximately equivalent amount was found in the mother liquor. These results indicate that isolated casein contains little chromogenic material (even if no allowance is made for the turbidity present in the pyridine test), but that appreciable amounts may be formed during dissolution in alkali. Interference by the casein in milk should, therefore, be less than that observed with isolated casein. However, even if one assumes chromogenic material in casein equivalent to 1.0 μg. of citric acid per milligram, the error involved in the direct analysis of milk (assuming 1,700 μg. of citric acid per milliliter and 3% casein) would be 1.8%.

A study of the combined effect of all milk components, on the reaction involved in the test, was done with solutions containing various amounts of skim-milk at fixed concentrations of citric acid. These solutions, prepared by adding

cooling coil, the bath capacity should be at least 400 ml. of water per sample).

c) Self-filling pipettes for both acetic anhydride and pyridine. Prolonged inhalation of pyridine vapor is injurious to health *(2)*.

Reagents

a) Reagent-grade acetic anhydride and pyridine.

b) Anhydrous citric acid γ obtained by heating the monohydrate at 90° C. to constant weight—about 72 hr.—was used to prepare a stock solution (50 mg/ml) which is stable for at least 1 yr. at 0° C. Standards, prepared from the stock solution, are stable for 1 mo. at 0° C.

Procedure

To a colorimeter tube, add 1.0 ml. of sample (containing from 25 to 200 μg. of citric acid), or citric acid standard, or water (for the reagent blank), followed by 1.30 ml. of pyridine, and swirl the tube briskly. After similar additions have been made to all tubes in a series, add 5.70 ml. of acetic anhydride, swirl the tube again, and immediately place it in the constant-temperature bath.[2] Color development is complete after 30 min., and the color is stable for at least another 30 min., either in the 32° C. bath or in air at a temperature of 22–29° C. Read the color intensity at 420 mμ with the blank set at 100% transmission and estimate the citric acid content of the samples by reference to the standards.

## ANALYSIS OF MILK AND SERUM

*Preparation of samples.* Milk and serum are diluted directly to a citric acid content between 25 and 200 μg. per milliliter; whole milk is centrifuged, or filtered after dilution, to decrease the fat content. If serum contains trichloracetic acid (T.C.A.), prepare an extra blank containing an equivalent amount of T.C.A. and subtract the optical density (2-log G) of this blank from that of the samples; this will correct the samples for the increase in absorption caused by T.C.A. Homogenized milk requires a correction for the turbidity introduced by fat. To determine this correction, the recommended "Procedure" is used with a separate aliquot of the dilute milk, but 5.70 ml. of glacial acetic acid is substituted for the acetic anhydride. This allows estimation of the interfering turbidity in the absence of color. Subtract the optical density of this solution from that obtained in the actual test, and calculate the citric acid content of the test sample from the corrected value.

*Possible interfering substances.* Calcium chloride and monopotassium phosphate were added to standard citric acid solutions so as to introduce various proportions (from 100 to 600 μg.) of calcium and phosphorus with the 1.0-ml. aliquot. The opalescence that formed on addition of pyridine to these solutions disappeared after addition of acetic anhydride, and the intensity and stability of the color were not affected.

[2] Turbidity forms on addition of acetic anhydride to solutions containing protein, but clears during the holding period in the bath.

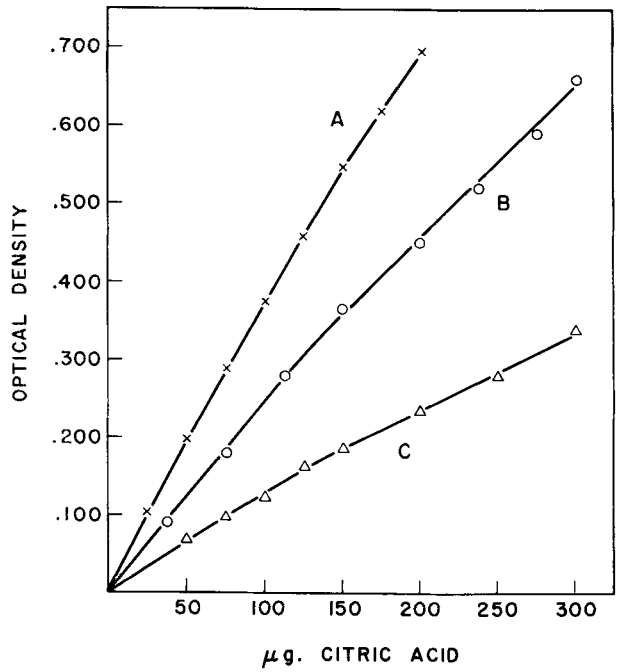


FIG. 4. Comparison of three methods for the estimation of citric acid. A: Present method, 32° C., 8.0 ml. total volume. B: Saffran and Denstedt method *(10)*, 60° C., 10.0 ml. total volume. C: Babad and Shtrikman method *(1)*, 60° C., 8.2 ml. total volume.

sult of three reactions occurring almost simultaneously, i.e., a color-forming reaction, a color-destroying reaction, and hydrolysis of acetic anhydride with water. All three reactions are affected by temperature, and by concentration of pyridine, water, and acetic anhydride. The ingredients responsible for color production are also involved in its destruction (fading) with time, especially when in excess (Figure 1, A, B, and C). Color formation is dominant initially but, as would be expected, soon decreases in rate as citric acid or one of the reagents becomes limiting; meanwhile, the rate of color-destroying reaction increases in proportion to the degree of pigmentation. When the color-forming and color-destroying reactions are occurring at equivalent rates, the color intensity remains constant for a time interval related to the over-all rate of reaction. If—at this stage of the reaction—the rate of both reactions is decreasing, the period of apparent equilibrium will be increased. Hydrolysis probably acts in this manner, reducing the rate of reaction by decreasing the concentration of water and acetic anhydride.

## RECOMMENDED METHOD

### Apparatus

a) A colorimeter or spectrophotometer, equipped to measure color intensity at 420 mμ (an Evelyn colorimeter was used in this work).

b) A water bath at 32 ± 0.25° C., with sufficient capacity to insure dissipation of the heat generated by the reaction. (With moderate stirring, and no

known amounts of citric acid to dilutions of milk to give final concentrations of approximately 80 or 200 μg. per test, were analyzed and the results throughout agreed with the expected total, within 1.5% (Table 1).

TABLE 1

*Analysis of solutions containing various amounts of skimmilk, adjusted to constant levels of citric acid*

| Skimmilk added *(ml. per test)* | *Citric Acid (μg. per test)* | | | |
|---|---|---|---|---|
| | Added | Determined | Expected | % Accuracy |
| 0.120 | 00.0 | 213 | ..... | ..... |
| 0.105 | 25.5 | 213 | 211.9 | 100.5 |
| 0.090 | 51.0 | 213 | 210.8 | 101.0 |
| 0.075 | 75.8 | 212 | 208.9 | 101.5 |
| 0.060 | 101.3 | 208 | 207.8 | 100.1 |
| 0.045 | 126.8 | 206 | 206.7 | 99.7 |
| 0.030 | 152.3 | 205 | 205.6 | 99.7 |
| 0.015 | 177.0 | 205 | 203.6 | 100.7 |
| 0.045 | 00.0 | 80.3 | ..... | ..... |
| 0.030 | 25.5 | 79.5 | 78.8 | 100.9 |
| 0.015 | 50.3 | 76.5 | 76.9 | 99.5 |
| | | | Average | 100.4 |

*Application.* A sample of skimmilk was analyzed at six intervals during a three-day period and the results varied from 1,685 to 1,694 μg. per milliliter, with an average of 1,689 ± 4 μg. The milk was stored at 0° C., and a fresh dilution was prepared for each analysis.

The method has been applied to the analysis of skimmilk, heated skimmilk, commercial evaporated milk, instant skimmilk powder, sera prepared from the various milks by precipitation with 12% T.C.A. *(9)*, and sera prepared from skimmilk by precipitation with 6% v/v addition of 1.0 *N* hydrochloric acid, by coagulation with rennet *(5)*, and by ultrafiltration *(11)*. Determinations were made at a reaction temperature of 32° C., also at 17 and 50° C. (with the optimum reagent proportions given in Figure 2 and reading the samples within 10 min. after removal from the bath). Values obtained for the sera were multiplied by 0.97 or 0.94 to correct for the volume change caused by removal of protein, or protein plus fat, respectively.

The results obtained (Table 2) at the three temperatures agreed within 2.5%, attesting to the degree of accuracy obtainable at various reaction temperatures. The citric acid content of each serum was lower than that of the corresponding milk by approximately 10% (15.1% for the ultrafiltrate). The apparent amount of chromogenic material measured in isolated casein would account for only about 2% of this difference, while the agreement between results of sera prepared with T.C.A. and either HCl or rennet indicates that serum proteins do not interfere.

Throughout our investigations, there has been no evidence to suggest that results of direct analysis of milk are erroneously high. Of the milk components, only fat has been found to interfere at levels likely to be encountered in the course of analysis. Attempts to detect interference of proteins have indicated that they do not interfere significantly. Ultrafiltration invariably yields a low

TABLE 2

*Citric Acid content of skimmilk, evaporated milk, and milk powder, and their sera, determined at various reaction temperatures*

| | Citric Acid *(μg/ml)* | | |
|---|---|---|---|
| | 17° C. | 32° C. | 50° C. |
| Skimmilk, untreated | 2,198 | 2,182 | 2,142 |
| Skimmilk, heated[a] | 2,205 | 2,251 | 2,226 |
| Skimmilk, T.C.A. serum | 2,088 | 2,024 | 2,062 |
| Skimmilk, HCl serum | 2,056 | 2,042 | 2,047 |
| Skimmilk, Rennet serum | 2,048 | 1,983 | 2,018 |
| Skimmilk, Ultrafiltrate | 1,882 | 1,853 | 1,943 |
| Evaporated milk[b] | 2,023 | 1,992 | 2,101 |
| Evaporated milk, T.C.A. serum | 1,954 | 1,915 | 1,953 |
| Skimmilk powder[c] | 1,891 | 1,890 | 1,881 |
| Skimmilk powder, T.C.A. serum | 1,695 | 1,687 | 1,700 |

[a] Skimmilk, heated at 180° F. for 15 min.
[b] Commercial evaporated whole milk, reconstituted 1: 2 with water.
[c] Commercial instant skimmilk powder, reconstituted to 9% solids with water.

value for serum citric acid, a result which can be ascribed to casein: citrate association as reported by Eilers and Jense *(3)* and White and Davies *(13)*. The intermediate values obtained with acid and rennet sera can be attributed to adsorption of citric acid by the precipitated protein, as suggested by Pucher *et al. (7)*. We are, therefore, convinced that the higher values obtained by direct analysis of milk represent its true citric acid content within 2.5%.

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