ANALYSIS FOR CITRIC ACID IN PRESENCE OF CASEIN

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SUMMARY

Factors affecting citric acid determination in the presence of casein were studied with the pyridine–acetic anhydride method. Although the opalescence in skimmilk tests affected results by only 2%, the turbidity introduced by solutions of isolated casein could cause gross overestimation. A pH above 12.3 in the test aliquot assured a clear reaction mixture with both skimmilk and casein solutions. It is, therefore, recommended that test aliquots containing casein should comprise sufficient alkali to insure a clear reaction.

Acid casein required seven resuspensions in fresh washing solution at pH 4.5 for complete removal of occluded citrate, lactose, and calcium. Adsorption of citric acid by casein precipitated at the iso-electric point, or with trichloroacetic acid, was demonstrated and appears to account for the lower citrate concentration in acid sera, as compared to skimmilk. These results extend the conclusions of a previous paper.

In a previous publication (7) the authors recommended determination of citric acid in milk without prior separation of the casein. Results of direct analysis were 5 to 10% higher than values obtained with either trichloroacetic or hydrochloric acid sera. This discrepancy was attributed to adsorption of citric acid by the precipitated protein, a suggestion which had been made by Pucher *et al.* (12), who had observed a similar result (with a different citrate method) when albumin was precipitated from urine samples. The present authors' original conclusion (7) was that: "Attempts to detect interference of proteins have indicated that they do not interfere significantly. . . . We are therefore convinced that the higher values obtained by direct analysis of milk represent its true citric acid content within 2.5%."

In a private communication, Davies (1) reported that most samples of skimmilk gave a slight opalescence when analyzed by the direct method (7), and wondered whether this did not produce significantly high results; also, he doubted that acid-precipitated casein could bind citrate electrostatically. In a recent publication, Evenhuis (2) concluded that the direct method (7) gives erroneously high results, and that adsorption of citric acid by precipitated protein is unlikely.

The present paper shows that the slight opalescence obtained in direct analysis of skimmilk does not cause significant error but can, nevertheless, be completely eliminated. It is also shown that re-solubilized casein does not interfere with the determination of citric acid, provided sufficient alkali is added to prevent opalescence. However, casein preparations can introduce traces of citrate because of incomplete washing. Adsorption of citric acid by casein is demonstrated, extending the results of a study by Van Slyke (16, 17).

MATERIALS AND METHODS

Bulk skimmilk and skimmilk from individual cows were used in the study. Casein was prepared in bulk according to Van Slyke and Baker (15), or by

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adjusting 100-ml. aliquots of skimmilk to pH 4.5–4.6 with hydrochloric acid, then filtering through Whatman No. 2 paper. Casein was also prepared by adding 25 ml. of 60% w/v trichloroacetic acid (12% final concentration) to 100 ml. of milk (14).

Calcium was estimated turbidimetrically (6), lactose was determined colorimetrically (8), and citric acid was analyzed by the pyridine–acetic anhydride method (7, 9). The pH was measured with a Beckman Model G pH meter.

EXPERIMENTAL PROCEDURE AND RESULTS

Prevention of opalescence in the presence of casein. Opalescence was observed in some samples after direct analysis of citric acid in aqueous dilutions of skimmilk. Since this phenomenon had not been observed in previous studies with 1% solutions of casein in 0.1 *N* sodium hydroxide, it appeared probable that opalescence could be prevented by introducing alkali with the test solution. In a preliminary study with the citrate test at 32° C. (7) it was found that up to 0.5 meq. of NaOH could be tolerated per tube;[1] higher concentrations resulted in a marked reduction of color.

The possible effect of added alkali on the determination of citric acid in the presence of casein was studied with 1 and 3% w/v solutions of casein [Van Slyke and Baker (15)]. The casein was dissolved in various concentrations of sodium hydroxide up to 0.40 *N*, and 1.0-ml. aliquots were analyzed. Consistent results and clear reaction mixtures were obtained when the test aliquot was above pH 12.3 (Table 1); a lower pH in the aliquot led to increased turbidity in the test solution, with citrate recoveries as high at 175%. The yellow color of the reaction developed, even though citrate was not added to the casein solutions, indicating that the casein was contaminated with citric acid (see later).

A parallel study with bulk skimmilk (diluted 20/100) was also made, in which 0.50-ml. aliquots of the dilution were added per test, along with 0.50 ml. of sodium hydroxide solutions of various normalities up to 0.40 *N*. The desirable amount of alkali was introduced into the colorimeter tube as a separate addition,[2] to avoid loss of citrate by co-precipitation with calcium phosphate (13). The opalescence obtained at low additions of alkali in skimmilk (Table 1) had only a slight effect on determination of citric acid; however, a clear test could be obtained by adding 0.2 meq. of NaOH per test. The effectiveness of NaOH was confirmed with three other samples of skimmilk (diluted 20/100) from different sources. Analysis by the original citrate method (7) gave values (μg/ml) of 1,449, 1,441, and 1,213, compared with (respectively) 1,423, 1,407, and 1,185 in the presence of 0.2 meq. of NaOH/test; the difference was, therefore, 1.8, 2.4, and 2.4%, respectively. However, while the original method re-

[1] With aliquots containing 1% v/v chloroform as a bactericide, a red color can form in the presence of NaOH and pyridine (5) but disappears on adding the acetic anhydride, with no adverse effect on the subsequent citrate reaction.

[2] In adding the NaOH directly into the colorimeter tube, the aqueous phase in the test must be kept constant, since water is one of the reactants (7). The size of the skimmilk aliquot must, therefore, be reduced by an equivalent volume.

TABLE 1
Effect of alkali on citric acid test with casein solutions and diluted skimmilk

Type of aliquot[a]	NaOH added, meq/test	pH of aliquot	After citrate analysis: Citric Acid Measured ($\mu g/ml$)	Citric Acid Recovery (%)[b]	Appearance of test solution
1% Casein	0.015	10.79	17.50	177.5	Turbid
1% Casein	0.025	11.33	12.34	125.2	Turbid
1% Casein	0.050	11.91	11.77	119.4	Opalescent
1% Casein	0.075	12.16	9.73	98.7	Clear
1% Casein	0.10	12.30	9.86	100.0	Clear
1% Casein	0.20	12.40	10.00	101.4	Clear
1% Casein	0.30	12.40	9.59	97.3	Clear
1% Casein	0.40	12.40	9.86	100.0	Clear
3% Casein	0.025	8.38	53.69	175.6	Turbid
3% Casein	0.050	11.10	38.25	125.1	Turbid
3% Casein	0.075	11.93	36.98	121.0	Opalescent
3% Casein	0.10	12.18	32.43	106.1	Opalescent
3% Casein	9.20	12.40	30.57	100.0	Clear
3% Casein	0.30	12.38	30.23	98.9	Clear
3% Casein	0.40	12.39	30.57	100.0	Clear
Skimmilk	0	7.02	1751	101.2	Opalescent
Skimmilk	0.050	12.02	1720	99.4	Opalescent
Skimmilk	0.10	12.28	1694	97.9	Opalescent
Skimmilk	0.20	12.37	1731	100.0	Clear

[a] 1.0 ml. of the alkaline casein solutions was taken for analysis; 0.50 ml. skimmilk (20/100) + 0.50 ml. NaOH were taken for analysis.
[b] Recovery is based on the last sample in each group.

sulted in opalescent solutions after citric acid analysis, the modification with 0.2 meq. of NaOH gave clear reaction mixtures throughout.

Washing of casein. Formation of yellow color with casein solutions, especially in the absence of turbidity (Table 1), suggested that triple-washed casein contained citric acid. A study of the effect of washing on the removal of citric acid from casein was, therefore, undertaken. Acid casein, obtained from 100-ml. portions of skimmilk by adding hydrochloric acid, was dispersed in 100 ml. of approximately 0.1 *M* sodium acetate buffer solution at pH 4.50. The mixture was thoroughly suspended, then refiltered within approximately 10 min.; in this manner, acid caseins resuspended from zero to nine times, along with the corresponding acetate washing solutions, were obtained. Each sample of casein was then dispersed in approximately 50 ml. of water, 3.0 ml. of 10.0 *N* NaOH added, and the volume adjusted to 100 ml. Results of citric acid analysis (Figure 1-A) showed that citrate-free casein was obtained after seven resuspensions in equivalent volumes of acetate buffer; the thoroughly washed casein showed no interference with the citrate method. Throughout, the concentration of citric acid in the casein solution was systematically related to that of the corresponding acetate washing solution, indicating that the resuspension technique was fully adequate for the establishment of equilibrium. Therefore, the concentration found in the acetate buffer reflected the degree of casein purity.

In a duplicate experiment with another lot of milk, the acetate washing

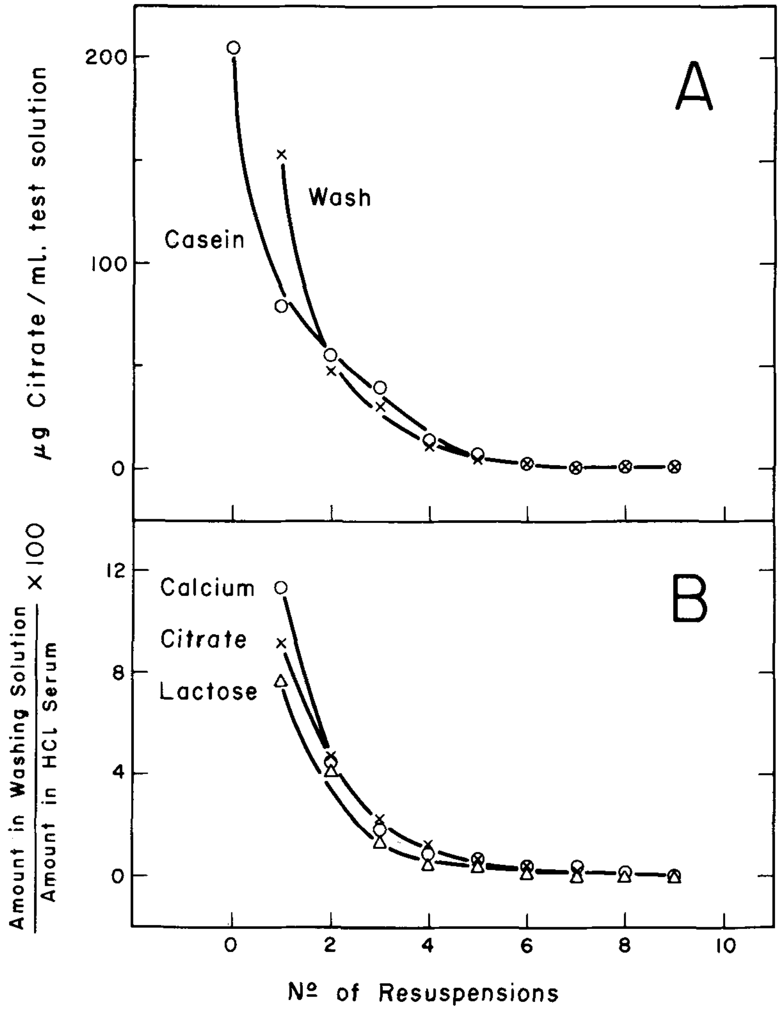

FIG. 1. Washing of HCl casein in 0.1 *M* sodium acetate buffer at pH 4.50 (casein from 100 ml. milk resuspended in 100-ml. volumes of fresh washing solution).

A: Concentration of citric acid in the washing solution, viz., that remaining in the casein (dissolved in an equal volume of 0.3 *N* NaOH).

B: Removal of various milk components from casein, expressed as a percentage of the initial serum concentration.

solutions were analyzed for lactose and calcium, in addition to citric acid (the casein was not analyzed). As in the previous experiment, at least seven resuspensions were required before removal of any of the three components could be considered complete (Figure 1-B). The results, expressed as per cent of the original acid serum concentration, show that retention by (and subsequent removal from) casein is proportional to the initial concentration of the various components.

Adsorption of citric acid by casein at pH 4.5–4.6. Van Slyke and Van Slyke (16) have shown that acid-precipitated casein can retain various acids (i.e., hydrochloric, sulfuric, acetic, and lactic) by means of a Freundlich (charcoal-type) adsorption (17); the authors did not study citric acid. However, Freundlich's data (4) indicated that citric acid retention was to be expected.

To study adsorption, casein was prepared from 100-ml. lots of skimmilk by precipitation with hydrochloric acid or with trichloroacetic acid. The two casein samples were washed nine times by resuspension in 0.1 *M* acetate buffer at pH 4.50, then suspended in 100 ml. of approximately 9.5 m*M*/liter sodium citrate buffer solution at pH 4.58. After 30 min., during which the casein suspensions received periodic agitation, the total volume was noted, the suspensions were filtered, and the filtrates kept for citrate analysis. The caseins were dried in an air oven at 50° C. and their weight was used to correct the total volume.[3]

Suspension of a thoroughly washed casein (whether obtained by HCl or TCA precipitation) in citrate solution at the iso-electric point of casein resulted in a decrease of from 7 to 9% in the citric acid concentration (Table 2).

TABLE 2
Adsorption of citric acid by casein at the iso-electric point

Type of sample	Total volume of suspension	Casein		Net volume	Citric Acid, μg/ml		
		Wt.	Volume		Determined	Corrected for volume	Recovery (%)
	(ml.)	(g.)	(ml.)	(ml.)			
HCl casein filtrate	114.0	3.15	2.10	111.90	1,444	1,616	90.9
TCA casein filtrate	116.0	3.23	2.15	113.85	1,448	1,649	92.8
Citrate solution	....	......	......	100.0	1,777	....	100.0
Milk I HCl serum	....	......	......	....	1,579	1,576[a]	94.3
Milk I Skimmilk[b]	....	......	......	....	1,671	....	100.0
Milk II HCl serum	....	......	......	....	1,623	1,620[a]	95.2
Milk II Skimmilk[b]	....	......	......	....	1,702	....	100.0

[a] Includes corrections (× 1.018) for 3.0 *N* HCl addition and (× 0.98) for approximate volume occupied by casein.
[b] 0.2 meq. of NaOH were added per test.

For purposes of comparison, two samples of skimmilk and their hydrochloric acid sera also were analyzed; the results with hydrochloric acid sera were approximately 5% lower than those obtained with their respective skimmilks (Table 2).

DISCUSSION

The results show that a turbid or opalescent reaction mixture can occur in the citrate test if the aliquot of test solution is below pH 12.3. Waugh and von Hippel (18) have demonstrated that the dispersibility of a re-solubilized acid casein was improved by an alkaline treatment at pH 12.0. Our results indicate that opalescence does not introduce a large error in the analysis of diluted skimmilk (i.e., < 2.5%); however, it can cause overestimation at the

[3] The volume occupied by casein was estimated by suspending 3.0 g. of Van Slyke and Baker casein (15) in 20 ml. of the 9.5 m*M*/liter citrate solution; the total volume obtained was 22 ml., giving an apparent specific volume of 0.67, which approximates the values reported by Ford *et al.* (3).

higher casein levels introduced by aliquots of 1 and 3% solutions of re-solubilized casein (Table 1). It appears probable that lack of gross interference with skimmilk is due—at least in part—to improved dispersibility of the natural casein aggregate under conditions of the citrate test. With normal skimmilk, a clear test reaction is obtainable by addition of 0.2 meq. of NaOH/test; however, with abnormal and modified milks, the required amount of alkali should be determined by a preliminary test. With skimmilk, the desired amount of alkali must be added as a separate addition to the colorimeter tube, and the volume of aqueous phase kept constant by a corresponding decrease in the size of the skimmilk aliquot. Recently, Evenhuis (2) used sodium caseinate solutions mixed in various proportions with a known sodium citrate solution (pH. 6.8) in a study of the pyridine–acetic anhydride method. He also fermented the citrate from milk and found that the casein had to be redissolved by addition of alkali to pH 6.9. In both types of experiment, the pH of the test aliquots was obviously below the value of 12.3 required to assure a clear reaction mixture and, therefore, his conclusion that the effect observed with these re-solubilized casein solutions would apply to analysis of normal skimmilk is erroneous.

Complete removal of citric acid (and other milk components) from acid-precipitated casein requires very thorough washing (Figure 1-A and 1-B); this is particularly important where the effect of casein on a citrate method is being studied. The gradual removal of milk components by successive washing treatments of acid casein has also been noted by Muller (10), who performed lactose determinations on the casein as a means of assessing the efficiency of washing. Patten and Hergstrom (11) recommend agitation or compression of the curd in the presence of the acid whey to extrude calcium, after which the casein is triple-washed. Thoroughly washed casein (resuspension 7, 8, and 9 in Figure 1-A) showed no interference with the citrate method, even though 1.0 ml. of approximately 3% solution (in 0.3 N NaOH) was used in the citrate test. In contrast, the triple-washed Van Slyke and Baker casein (15) used in the casein:alkali study (Table 1) contained approximately 1.0 μg. of citric acid per milligram of casein. It is concluded that the substance identified as chromogenic material in casein in a previous publication (7) was, in fact, residual citric acid.

Citric acid is adsorbed at the iso-electric point by casein obtained either by HCl or by TCA precipitation (Table 2). Retention of citrate by casein is lower in the presence of milk serum than in solutions containing citrate only, probably because other negatively charged components of the serum are adsorbed and displace some citrate (16, 17). A previous study (7) had shown that the citrate content of HCl serum was identical to that of TCA serum. This indicated that the degree of citrate adsorption was similar in both systems, even though the over-all positive charge of the casein particles was much greater in the TCA-treated milk (pH approximately 1.0). Because of this adsorption phenomenon, analysis of HCl or TCA acid serum does not provide a true value for total citrate in skimmilk, but yields a result which is from 4 to 10% low.

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