THE CITRIC ACID CONTENT OF MILK

by

Hans Ulrich Siegenthaler

A THESIS

Submitted to the Faculty of Graduate Studies
and Research of

The University of Manitoba

in Partial Fulfilment of the Requirements for
the Degree of Master of Science.

October 1954.

TABLE OF CONTENTS

TABLE INDEX

TABLE INDEX (Continued)

INTRODUCTION

Citric acid is present in milk primarily in the form of its salts, the citrates. Although present in only very small quantities it is a very important constituent of milk. In conjunction with the phosphate salts it plays an important part in stabilizing the colloidal system of milk, especially the protein fractions. The citrates and the phosphates form most of the anions of the system while calcium contributes the majority of the cations.

During certain seasons of the year and especially in the spring when the cows are changed from dry feed to pasture the salt balance of milk changes and considerable difficulty is encountered in certain phases of the Dairy Industry, especially concentrated milk and cottage cheese manufacturing, due to protein instability. The citrates are also important in the development of flavor in cultured dairy products. Citric acid is converted by the flavor producing bacteria to the compounds which contribute the delicate flavor desired in these products. The presence of citric acid in milk is essential to the production of the proper flavor.

For the above reasons it is very important to have a knowledge of the citric acid content of milk under various

conditions. However, there is no well established and generally accepted method available for its determination. The method as presented by the Association of Official Agricultural Chemists has been criticized as to its accuracy by some noted scientists (1). But even if the accuracy of the official method were beyond doubt there would still remain serious objections to its use since four days are required for the analysis. Obviously such a method is not suitable for the determination of a series of samples.

The objects of the present investigation are:

1. To study the methods of determination of citric acid in milk. Two newly developed colorimetric methods are investigated and compared with the official method of the Association of Official Agricultural Chemists.
2. To study the factors correlated with the citric acid content of milk or influencing the citric acid content of milk. For this purpose the colorimetric method of Rose is utilized. Since a review of literature disclosed an extremely wide range of variation of citric acid content under apparently normal conditions, further revelations of the factors influencing its content in milk are of scientific and commercial interest.

REVIEW OF LITERATURE

I. The Origin of Citric Acid in Milk.

II. The Distribution of Citric Acid in Milk.

(a) The Average Content and the Range of Variation.

(b) The Causes of Variation of Citric Acid in Milk.

III. The Methods for the Determination of the Citric Acid Content of Milk.

led them to conclude that to account for all the citric acid found in milk by uptake from the blood would practically exhaust the latter of this substance in its course through the gland since it is several hundred times as concentrated in the milk as in blood. This fact suggested that citric acid was synthesized in the mammary gland and gave rise to speculation as to its blood precursors and the mode of synthesis. They have shown that the mammary gland can synthesize citric acid from substances in the tissue and that the addition of glucose, lactic acid, pyruvic acid, maltose or glycogen increased the citric acid formation in mammary slices.

Considering the observations of the above authors, citric acid could be synthesized in the mammary gland in the following manner:

The reaction might start out with the processes by which glucose is oxidized to CO_2 and H_2O in the muscle metabolism of carbohydrates. Fructose and galactose are converted into glucose. As the normal level of glucose in the blood is reached, it is then converted to glycogen and stored in the liver or in muscle tissues. The glycogen is then, if needed, broken down according to the Emden-Meyerhof cycle to form pyruvic acid and adenosine triphosphate. The pyruvic acid enters the Krebs cycle and reacts with

oxalacetic acid which is another reaction product of this cycle. Then cisaconitic acid, in equilibrium with isocitric acid and citric acid, is formed. The isocitric acid takes part in further reactions of the Krebs cycle. The citric acid again enters the mammary gland and occurred as the citric acid content of the milk.

II. THE CITRIC ACID CONTENT OF MILK

A search of the literature concerning the content of citric acid in milk reveals rather contradictory results, especially regarding the possible causes of variations.

(a) The Average Content and the Range of Variations.

According to the literature the citric acid content of cows' milk varies considerably.

Twenty-five samples of fresh retail milk taken over a year's time were analyzed by Arup (2). The values ranged from 0.150 to 0.206 per cent.

Two other investigators, Supplee and Bellis (31), stated that there was 0.1 to0.2 per cent of citric acid in milk.

Heinemann (16) established the citric acid content

of twenty-seven samples of defatted milk solids taken through the year 1943. These samples varied from 0.188 to 0.209 per cent and averaged 0.1995 per cent.

The variation of citric acid content of three hundred and thirty-five individual animals, as determined by Sherwood and Hammer (29), was from 0.07 to 0.33 per cent and averaged 0.18 per cent.

Richmond (25) stated that the citric acid content of milk ranged from 0.15 to 0.20 per cent.

Comparing the citric acid content of milk from individual quarters of the udders of thirty-six cows, Kieferle et al. (19) found, with one exception, values ranging from 0.20 to 0.40 per cent. There appeared to be less variation between samples from different quarters of the same cow than between samples from different cows.

Very little reference is made to the citric acid content of colostrum. A search of the literature for such data was without success.

(b) The Causes of the Variations of the Citric Acid Content of Milk.

The extremely high range of variations of the citric acid content, exhibited in the figures for analysis under apparently normal conditions, gives rise to several theories.

Some authors believed that factors like breed of cows, type of feed, season of the year or geographical location caused these variations, while other investigators emphasized that these variations represent chiefly a variation between individuals.

Testing the milk of three hundred and thirty-five individual cows, Sherwood and Hammer (29) found that the breed of animal, the time of milking, the stage of lactation and the season of the year had no significant effect on the citric acid content of milk.

Opposed to these results are the conclusions of Heinemann (16). He stated that in a series of samples of defatted milk small but definite seasonal variations were found. The highest value occurred in March and April and the lowest in November. Samples of defatted milk solids taken from eleven different states indicated that there was a slight variation in the citric acid content of milk from different localities.

Arup (2) again stated that his investigations about citric acid did not indicate significant seasonal variation. The same author also determined the citric acid content of eleven samples of defatted milk solids of English, Irish and American origin. There was no significant geographical variation, his results ranging from 0.151

to 0.184 per cent on the reconstituted basis.

Rumments, as mentioned in Hammer and Babel (13), established by his tests an average citric acid content of 0.213 per cent; milk from the same cows toward the end of the lactation averaged 0.134 per cent, thus indicating a definite decrease.

Hunziker (18) pointed out that green feeds increased the citric acid content of milk and that dry winter feeds decreased it.

III. METHODS FOR THE DETERMINATION OF CITRIC ACID IN MILK

An examination of the literature showed very clearly that a detailed study of the citric acid distribution has been seriously circumscribed by the lack of accurate methods to determine this component in the small quantities present in milk.

Apparently, the most satisfactory results for the quantitative determination of citric acid have been obtained by methods based on the pentabromacetone reaction. As early as 1847 Cahours (5) prepared a bromination product from several citrates. According to the reported melting point and solubility data the product which he called "bromoxaform" represented without doubt pentabromacetone. These investigations

were generally overlooked and only since Stahre (30) published a paper on the detection of citric acid through the formation of a bromine derivative of a product of its oxidation with potassium permanganate, the so-called Stahre reaction, has this procedure been utilized. It comprises the oxidation of citric acid into acetone and the substitution of bromine by means of bromine water to form pentabromacetone.

In 1902, however, Woehlk (34) concluded that the reaction was not quantitative since the dilution, the temperature and the quantity of bromine were determining factors for quantitative recoveries of pentabromacetone.

Kunz (22) was the first to utilize the Stahre reaction to determine the citric acid content of milk. He demonstrated that by substituting potassium bromide for bromine water, the reaction could be used for the quantitative determination of the acid.

Dunbar and Lepper (8) made a collaborative study of this method improved by Kunz. They found that with large quantities of acid the results were acceptable.

Exhaustive studies, made by Hartmann and Hillig (14) about the sensitivity of the Stahre reaction led them to conclude that if this provided means for (1) preventing the formation of pentabrom-acetone as the oil, by chilling of the oxidation mixture, (2) suppressing the solubility of

pentabromacetone to a minimum by employing chilled solutions for washing and (3) drying the precipitate for weighing, the method would give results of considerable accuracy. They concluded, further, that the modified Stahre reaction for the conversion of citric acid into pentabromacetone is not only quantitative, but it furnishes a splendid means for accurately determining the acid.

Deysher and Holm (7) stated that in addition to losses incurred through incomplete conversion of citric acid to pentabromacetone, losses could occur also because of the volatility of the precipitate under the usual conditions of drying and because of the solubility of the precipitate in the reaction mixture and in the wash water used. They concluded that because of these facts no absolute method could be prescribed for the complete recovery of citric acid as pentabromacetone under all conditions. The method should be standardized with respect to the conditions and products employed and corrections made for the losses incurred in each case.

Allen (1) used the following objections to the method: (1) the formation of pentabromacetone as an oil instead of a crystalline precipitate, (2) the relatively high solubility of the precipitate and (3) the loss due to its volatility when moist. He claimed, furthermore, that the method,

even with the improvements suggested by Hartmann and Hillig (14), was still open to the same objection as the first method, namely, the doubtful quantitative production of acetone dicarboxylic acid.

The official method of the Association of Official Agricultural Chemists for the determination of citric acid in milk is based on the Stahre reaction, modified by Hartmann and Hillig. It is believed that this procedure represents the most accurate method available at the present time.

Many of the citric acid investigations were made using Beau's method (4) based on Deniges' reaction (6). Allen (1) discusses this method as follows:

In the presence of mercuric sulfate citric acid gives, on oxidation with permanganate, a white precipitate of a mercury-acetone-dicarboxylic acid complex of constant composition. This reaction was first observed by Deniges who applied it to the detection of citric acid. The reaction is not given by malic, tartaric, succinic, or lactic acids and though oxalic acid gives a white precipitate with the reagent before the permanganate is added this may be filtered off and does not interfere with the method. It is doubtful, however, whether the reaction is quantitative since it has been shown by Woehlk that acetonedicarboxylic acid breaks

down slowly to acetone and carbon dioxide, even at 0° c.

Pratt (23) oxidized citric acid to acetone, avoiding some danger of decomposition of the dicarboxylic acid by distilling the acetone in a receiver containing Deniges' reagent. Weighing the precipitate formed was claimed to yield satisfactory results. However, Willoman (34) was unable to secure concordant results with this method.

In recent years, many attempts have been made to find more efficient methods of determing the citric acid content of milk. As no assymmetric carbon atoms are present in citric acid, no method based on optical isomers is usable. At the present time there are some methods developed which allow the use of colorimetric or titrimetric procedures.

According to Taussky and Shorr (32) Kometiani (21), in 1931, developed a titrimetric method for the macrodetermination of citric acid (4 to 40 mg). It was based on the reaction of a warm alcoholic solution of organic bromides with sodium or potassium iodide. This reaction was formerly described by Finkelstein (9). The citric acid was first oxidized and brominated to pentabromacetone. Then, the pentabromacetone which precipitated quantitatively, was filtered off and dissolved in alcohol. Acetic acid was added to the alcohol to favor the substitution of bromine by iodine. The above solution was mixed, in a water bath,

with an alcoholic solution of sodium iodide and then heated to just below boiling. The iodine liberated was then titrated with sodium thiosulfate.

A method which included the oxidation of 0.1 to 1.0 mg of citric acid by potassium permanganate in the presence of bromine was developed by Pucher et al (24). After the extraction of the pentabromacetone produced from the oxidation mixture with petroleum ether, it was treated with aqueous sodium sulfide. The colored substance that formed in the aqueous phase was stabilized by the addition of pyridine and the color intensity was measured in a Pulfrich spectrophotometer. Finally, the quantity of citric acid originally present was determined from the calibration curve of the instrument used.

In 1935, Furth and Herrmann (10) developed a reaction, known as the Furth-Herrmann reaction, which consisted in taking up a deproteinized sample in warm acetic anhydride and adding pyridine. This reaction had to be carried out under anhydrous conditions.

Another rapid method which was based on the Furth-Herrmann-reaction was described by Saffran and Denstedt (27). In the presence of trichloracetic acid, acetic anhydride and pyridine a yellow color is developed and read in the Evelyn-colorimeter, with Filter 430 or 400,

aperture of 10 mm.

Babad and Shtrikman (3) adapted the method of Saffran and Denstedt to the determination of citric acid in dairy products. Instead of 1.0 ml, 0.2 ml of citric acid solution was used. This modification was made because water seemingly had a great effect on the uniformity of color development. The authors stated that much better results were obtained this way. They claimed that the accuracy of the method was better than 5 per cent, usually $\pm$ 3 per cent. The most important sources of error were (1) the measurement of the volume of the solution tested, (2) the sensitivity of the colorimeter.

Heinemann (17), since he denied the accuracy of colorimetric methods in which acetic anhydride and pyridine are used to develop color directly from the citrate ion, developed a method based on the oxidation of citric acid by perchlorato-cerate in perchloric acid solution. The results were obtained by titration with sodium oxalate, using nitriferroin as an indicator. The resulting yields were slightly higher than those obtained from the pentabromacetone method and in addition there was considerably less time used for the determination of the acid.

PROCEDURE

I. Plan of Investigation.

II. Methods for the Determination of Citric Acid in Milk.

- A. Official Method of the Association of Official Agricultural Chemists.
- B. Colorimetric Method of Babad and Shtrikman.
- C. Colorimetric Method of Rose.

III. Comparison and Accuracy of Methods.

- A. The Official Method of the AOAC.
- B. The Colorimetric Methods.
- C. Comparison of the Colorimetric Methods with the Official Method of the AOAC.

IV. Determination of Citric Acid Content of Milk.

I. PLAN OF INVESTIGATION

The colorimetric methods for the determination of the citric acid content of milk, one of them developed by Babad and Shtrikman (3) and the second one by Rose (26), were investigated for their accuracy. The investigation was done by comparison of the colorimetric methods with the official method of the Association of Official Agricultural Chemists (33) and by analysing yields of tests executed on solutions of known concentrations.

The citric acid content of a number of milk samples was then determined using the method of Rose. The purpose of these determinations was to resolve the following problems regarding the citric acid content of milk:

1. The variations in citric acid content of milk from individual cows;
2. the influence of lactation period on the citric acid content.
3. the variations of citric acid content of colostrum;
4. the correlation between milk yield and citric acid content;
5. the correlation between total milk solids and citric acid content.

II. METHODS FOR THE DETERMINATION OF CITRIC ACID IN MILK.

A. Official Method of the Association of Official Agricultural Chemists.

The method of the AOAC for the citric acid determination in milk, which is based on the Stahre reaction was carried out according to the instructions given in "Official Methods of Analysis" (33).

B. Colorimetric Method of Babad and Shtrikman.

To an accurately measured quantity of milk, an equal volume of a 10 % trichloracetic acid solution was added. The mixture was then shaken and left for about two hours to ensure a better precipitation of the proteins. It was then filtered through a No. 40 Whatman paper; the first part of the filtrate was discarded.

Exactly 0.2 ml of the filtrate were pipetted into three dry test tubes (150 x 14 mm) and 7 ml of acetic anhydride were added from a burette. The tubes were then firmly closed with rubber stoppers and placed in a constant temperature water bath at $60^{\circ} \pm 1^{\circ}$ C. After ten minutes the tubes were taken out, 1 ml of pyridine (Baker) was added to each, and they were immediately stoppered. Then they were

replaced in the water bath for an additional 40 minutes, during which time they were shaken every 5 to 8 minutes. Afterward the tubes were chilled in an ice water bath for five minutes. Ten to fifteen minutes after their removal a portion of the solution was transferred to an electrophotometric Fischer microcell and the light absorption was read in a Fisher electrophotometer, A C - Model, 115 Volts, 60 cycles, using the "Filter 425".

Two blanks starting with 0.2 ml portion of a 5 per cent solution of trichloracetic acid were run simultaneously in each series of tests, and used to set the colorimeter.

A calibration curve was prepared with solutions of 50 to 150 mg of citric acid in 0.2 ml of 5 per cent trichloracetic acid.

C. Colorimetric Method of Rose.

1. Preparation of Serum.

The milk samples, which usually showed a cream layer on top, were put into a warm water bath and well mixed. Then they were cooled to 20° C. and remixed. Following this procedure, 10 ml of milk were transferred into 100 ml volumetric flasks. The flasks were then inverted into a boiling water bath for at least ten minutes. (Colostrum was diluted with 25 ml of distilled water to prevent the formation of a firm coagullum which might prevent an unknown portion of the citric acid from entering the free milk serum. It proved advisable to shake the colostrum samples while inverted in boiling water.) The flasks were cooled and, following the addition of 70 ml of distilled water, they were placed in a water bath at 40° C. As soon as the contents of the flasks were warmed to the temperature of the water bath, 1 ml of 10 per cent acetic acid solution was added while swirling the samples. The samples were removed, held for ten minutes at room temperature and then 1 ml of I N sodium acetate was added. After shaking the flasks, they were cooled to room temperature and the volume was made up to 100 ml by the addition of distilled water at 20° C. The clear filtrate, obtained by

filtering through a dry 11 cm No. 40 Whatman paper, was used for the further analysis.

2. Determination of Citric Acid.

a) Dilution of Sample: From the clear filtrate obtained by the previously described procedure 5 ml were removed and diluted with 5 ml of 0.1 N hydrochloric acid. The resulting sample was thus in 0.05 N hydrochloric acid and formed the basic solution for the determination of citric acid.

b) Preparation of Standards: Analytical grade citric acid monohydrate was dried in an air oven at 72° C. for forty hours and then checked for constant weight by weighing several times after equal intervals. Exactly 20 g of the dried material were dissolved in distilled water and made up to 500 ml of solution. The stock solution thus prepared contained 40 mg of citric acid monohydrate per milliliter. It was stored at 0° C. and renewed every three months.

One milliliter of this stock solution was pipetted into a 25 ml volumetric flask and diluted with 24 ml of distilled water. Then 0, 3, 5 and 10 ml aliquots of the diluted solution were pipetted into 100 ml volumetric flasks. After addition of 10 ml of 0.5 N hydrochloric acid to each flask they were made to volume with distilled water.

Solutions of 0, 48, 80 and 160 micrograms of citric acid in 0.05 N hydrochloric acid were thus obtained. The first of these four solutions (0 ml-concentration) served as a blank to set the electrophotometer at its zero mark.

c) Procedure: All tests were done in duplicate. For reasons which will become obvious later on, only twenty tubes could be included in one batch. They were made up of the following: eight tubes for the four standard solutions in duplicate, twelve tubes for the six samples in duplicate.

To each tube 1 ml of one of the previously described solutions was added. The test tubes were arranged in a manner so that one replicate of the standards was handled before and one after the samples with unknown citric acid content. In intervals of thirty seconds, 8 ml of acetic anhydride (Baker) were added from an automatic burette to each of the test tubes. Immediately after adding the acetic anhydride, the test tubes were loosely stoppered, violently agitated and placed in a constant water bath at $60^{\circ} \pm 1^{\circ}$ C.

At precisely ten minutes after the addition of acetic anhydride to the first sample, the stopper of the mentioned sample was removed and 1 ml of pyridine (Baker) was added. The stopper was then replaced and the tube was again vigorously agitated without being removed from the water bath.

This procedure was continued at thirty second intervals. (Since the acetic anhydride is added to the test tubes in 30 second intervals and the pyridine must be added exactly ten minutes later this limits the number of tubes in a series to twenty.) Thirty minutes after the pyridine was added to the last sample of a series, the first one was removed from the warm-water bath and transferred to an ice-water bath of 5° C. or cooler. The remaining tubes were transferred at thirty second intervals and after the last tube was moved, the cooling was continued for an additional five minutes. Then the batch was removed from the ice-water bath and placed at room temperature for at least five minutes. After a sample was mixed without moistening the upper portion of the test tube, 5 ml were pipetted to the electrophotometric Fischer microcell. The two samples of the standard solution with 0 ml citric acid concentration were used as blank to set the electrophotometer to its zero mark. The light absorption of the remaining samples was read in a Fisher Electrophotometer, A C - Model, 115 Volts, 60 cycles, by using the "Filter 425".

3. Modifications.

The previous described method was originally based on a 0.1 N hydrochloric acid concentration in the samples and

standard solutions used for the citric acid determinations. This concentration, however, seemed to be too high. When the acetic anhydride was added and the tube, containing the samples, was inverted in the warm-water bath, frequently a violent reaction took place. The stopper was thrown out rather violently and part of the solution was ejected from the tube. Various methods of preventing this reaction were tried but the only way which was found satisfactory was to reduce the hydrochloric acid concentration used to dilute the milk serum. A comparison of the color-development by a 0.1 and 0.05 N hydrochloric acid concentration was therefore adopted and used for the citric acid determination of all the milk samples and standard solutions tested in the course of these investigations.

The method of transferring the liquid from the test tube to the electrophotometric microcell should be mentioned. The rubber stopper usually left some black traces on the wall of the test tube. If parts of this deposit were allowed to come in contact with the solution of the tube, either by mixing or when pouring out the sample, a change in color intensity could be observed. To eliminate this error the sample was mixed so as to avoid contact with the upper portion of the tube and was transferred with a pipette to the electrophotometric microcell.

III. COMPARISON AND ACCURACY OF METHODS.

A. The Official Method of the AOAC.

A citric acid stock solution, containing 40 mg of citric acid per milliliter, was used to make up a solution which contained 200 mg/ 100 ml of citric acid. Four analyses were then carried out to determine that citric acid could be recovered from this solution.

B. The Colorimetric Methods.

To establish the accuracy of the two colorimetric methods, three series of samples, containing citric acid in three different concentrations, were analyzed for their contents. The test solutions for the three concentrations, containing 48, 80 and 160 mg of citric acid per 100 ml, were prepared from a citric acid stock solution, containing 40 mg of citric acid per 1 ml. The composition of each series tested is shown in Table 1.

Table 1

Composition of Series.

Concentration (mg of citric acid/ 100 ml)	Number of samples included in one series	Total Number of samples investigated
48	5	15
80	5	15
160	5	15

1. The Babad and Shtrikman Method.

To obtain some idea of the variations occurring with this method, the average deviation and the highest positive and negative deviations from the expected citric acid yield of each concentration group were determined.

2. The Rose Method.

The extent of variations between several series and

between different concentrations, the extent of interaction between series and concentrations and in addition, the standard deviation, the standard error and the coefficient of variation of three different concentration groups, namely 48, 80 and 160 mg of citric acid per 100 ml of liquid, were established.

C. Comparison of the Colorimetric Methods with the Official Method of the AOAC.

1. Colorimetric Methods versus AOAC Method.

To compare the three methods used in this investigation, the following test was done. Using a sample of raw milk, samples were prepared in such a manner that the first one represented ordinary raw milk, the second one raw milk with the addition of 40 mg of citric acid per 100 ml milk, the third one raw milk with 80 mg of citric acid per 100 ml milk and the fourth one raw milk with 160 mg of citric acid.

To determine the citric acid content of these samples the AOAC method was carried out in duplicate; for the colorimetric methods, each determination was done in triplicate.

2. Rose Method versus AOAC method.

The citric acid contents of milk samples of four individual cows were determined by the method of Rose and by the official method of the AOAC. The analyses were carried out in duplicate for both methods.

IV. DETERMINATION OF THE CITRIC ACID CONTENT OF MILK.

The determinations of the citric acid content of milk were done by the colorimetric method of Rose.

All milk samples analysed in the course of this investigation were obtained from the pure breed Holstein herd kept by The University of Manitoba Farm.

For the investigation of the variation of the citric acid content of the milk from individual cows and for the study of daily variations, samples of two following milkings (evening and morning) were taken from twelve cows. Aliquot parts of both milkings, according to the yields of milk, were mixed together and the citric acid content was determined on these mixtures of evening and morning milk.

The above procedure of sampling was also followed for the investigations concerning the correlation between yields of milk and citric acid contents. Twenty-four cows were involved in this analysis.

To investigate variations in colostrum, the citric acid contents of the first ten milkings of ten different cows were determined.

The citric acid content of the milk from six cows on dry feed was established over a four-months' period. This period covered for two cows the third, fourth, fifth and

sixth month and for four cows the first four months of their lactation period. For this investigation the citric acid contents of two following milkings (evening milking and morning milking) were determined.

An investigation of the correlation between the citric acid content and the total solids content of the milk was carried out by milking the four quarters of six cows in separate containers. The total milk solids and the citric acid content of each sample was then established.

For all milk samples in the above mentioned investigations, precautions were taken to prevent the break-down of citric acid by bacteria. About one part per thousand of formaldehyde (C.P., Baker's Analyzed) was added to each sample. All tests were done as soon as possible. When samples did have to be stored a storage temperature of 4° C. was used.

R E S U L T S

I. Comparison and Accuracy of Methods.

A. The Official Method of the AOAC.

B. The Colorimetric Methods.

1. The Babad and Shtrikman Method.

2. The Rose Method.

C. Comparison of the Colorimetric Methods with the Official Method of the AOAC.

1. Colorimetric Methods versus AOAC method.

2. Rose method versus AOAC method.

II. Factors Influencing the Citric Acid Content of Milk.

A. The Variations between Individual Cows and the Variations between Daily Milkings.

B. The Influence of the Lactation Period on the Citric Acid Content of Milk.

C. The Variations of the Citric Acid Content of Colostrum.

III. Correlations of the Citric Acid Content with some Related Factors.

A. Correlation of the Milk Yield with the Citric Acid Content.

B. Correlation of the Total Milk Solids with the Citric Acid Content.

I. COMPARISON AND ACCURACY OF METHODS.

A. The Official Method of the AOAC.

A standard solution was added to four samples in order to yield a citric acid content of 200 mg. Table 2 represents the results of this investigation.

Table 2

Recovery of citric acid by the Official method of the AOAC.

Calculated yields of citric acid (mg/100 ml)	Actual yields of citric acid (mg/100 ml)
200	188
200	185
200	185
200	183

B. The Colorimetric Methods.

1. The Babad and Shtrikman Method.

The results of the experiment are shown in Table 3. In Table 4, the average deviation from the expected citric acid yields of the three concentrations as well as the highest positive and negative deviations from these values, expressed in per cent of the expected yields (48, 80 and 160 mg), are listed.

Table 3

Citric acid contents established
by the method of Babad and Shtrikman

Calculated Results	48.0	80.0	160.0
Actual Results	43.5	86.0	155.5
	52.0	72.0	163.0
Series I	52.0	80.0	163.0
	56.0	80.0	155.0
	56.0	80.0	160.0
	41.0	91.0	168.0
	41.0	87.5	170.5
Series II	48.0	78.0	173.0
	48.0	91.0	163.0
	41.0	91.0	168.0
	48.0	81.0	157.0
	48.0	81.0	157.0
Series III	49.5	81.0	150.0
	49.5	74.0	157.0
	48.0	74.0	150.0

Table 4

Deviation in citric acid determined by the Babad and Shtrikman Method

Citric acid content (mg/100 ml)	Average Deviation %	Highest pos. Dev. %	Highest neg. Dev. %
48	10.73	16.67	14.58
80	5.03	13.75	10.00
160	8.07	8.13	6.25

2. The Rose Method.

The results obtained of the analysis by the Rose Method of standard solutions of different concentrations are tabulated in Table 5.

In Table 6, an analysis of variance is represented. Non-significant F-values were obtained for series, concentrations and for the interaction between series and concentrations.

To establish a measure of the variations between the results of each of the three concentration groups, the results of equal concentration groups of the three series were added together and analysed for their mean, the standard deviation, the standard error of the mean and the coefficient of variability. This analysis is shown in Table 7.

Table 5

Yields of citric acid contents established by the method of Rose

Calculated Results	48.0	80.0	160.0
Actual Results	51.0	76.0	153.5
	47.0	79.5	161.0
Series 1	45.0	78.5	156.0
	47.0	78.5	156.0
	47.0	76.0	156.0
	47.0	79.5	158.5
	49.5	79.5	158.5
Series II	49.5	81.5	163.0
	48.5	81.5	163.0
	47.0	83.0	165.5
	49.5	81.5	158.5
	49.5	83.0	158.5
Series III	49.5	80.5	161.0
	48.5	78.5	161.0
	49.5	78.5	161.0

Table 6

The analysis of variance of citric acid content of three concentration groups (48, 80 and 160 mg/100 ml) of three series.

Variance due to	D.F.	Mean Square	F-Value	1% - Point
Series	2	3.835	3.113	3.26
Concentration	2	3.085	2.520	3.26
Series x Conc.	4	0.867	0.709	2.63
Error	36	1.224		
Total	44			

Table 7

Analysis of variations between each concentration group (48, 80 and 160 mg).

Concentration group	Arithm. mean	Standard deviation	Standard Error of mean	Coefficient of variability
48	48.333	± 1.584	± 0.409	3.28
80	79.667	± 2.151	± 0.555	2.70
160	159.867	± 3.350	± 0.865	2.095

C. Comparison of the Colorimetric Methods and the Official Method of the AOAC.

1. Colorimetric methods versus AOAC method.

Table 8 contains the results of the comparison of the comparison of the colorimetric methods of Babad and Shtrikman and of Rose with the official method of the Association of Official Agricultural Chemists.

2. Rose method versus AOAC method.

The citric acid content of four milk samples, established by the Rose method are shown in Table 9.

Table 8

Detection of citric acid content of milk and Milk plus additions of 40, 80 and 160 mg of citric acid per 100 ml by three methods.

Sample	AOAC-Method	Method by Babad and Shtrikman			Method of Rose		
	mg/100 ml	mg/100 ml			mg/100 ml		
Milk without citric acid addition	131	105	130	135	140	140	140
Milk + 40 mg of citric acid	169	135	143	140	174	174	174
Milk + 80 mg of citric acid	209	148	173	173	212	216	222
Milk + 160 mg of citric acid	288	183	233	190	302	302	302

Table 9

Citric acid content of milk(mg/100 ml) determined by the official method of the AOAC and by the method of Rose.

AOAC Method	Rose Method
156	165
182	190
186	190
163	170

II. Factors Influencing the Citric Acid Content of Milk.

A. The Variations between Individual Cows and the Variations between Daily Milkings.

The citric acid contents of the milks from twelve cows over a period of seven days is listed in Table 10.

An analysis of variance was carried out on the results of Table 10 and is presented in Table 11. The F-Value is very high and indicates that there is a significant citric acid content variation between individual animals.

In Table 12 are presented the daily variations of the citric acid content of the milk from twelve cows over a seven-day period.

Table 10

Citric acid contents of milk from twelve cows over a seven day period.

Cow.	Day						
	1	2	3	4	5	6	7
1	200	200	203	205	200	200	195
2	153	158	165	180	165	170	175
3	230	230	220	240	235	240	240
4	185	185	188	185	185	185	185
5	190	188	195	205	200	195	195
6	170	175	190	200	195	190	190
7	200	195	190	175	165	175	175
8	180	165	180	180	180	175	190
9	215	210	203	195	220	210	205
10	210	210	215	215	200	205	205
11	200	190	198	200	200	195	190
12	210	210	215	215	213	210	210

Table 11

Analysis of Variance of the Citric Acid content of milk between cows.

Source of variance	D.F.	Mean Square	F - Value
Between Cows	11	2255	42.51 #
Error	72	53.05	
Total	83		

Exceeds the 5 % point (2.51)

Table 12

The arithmetic means, the standard deviations and the coefficient of variability of the daily citric acid contents of milk from the twelve cows over a seven day period.

Cow	Arithmetic mean	Standard deviation ±	Coeff. of Variability
1	200.428	3.109	1.55
2	166.571	9.363	5.62
3	233.571	7.483	3.20
4.	185.428	1.154	0.62
5	195.428	5.744	2.94
6	187.143	10.747	5.74
7	182.143	13.019	7.15
8	178.571	7.483	4.19
9	208.286	8.195	3.93
10	208.571	5.568	2.67
11	196.143	4.564	1.71
12	211.851	2.415	1.41

B. The Influence of the Lactation Period on the Citric Acid Content of Milk.

The citric acid contents of morning milkings and evening milkings, investigated at monthly intervals for the first six months of the lactation period, are reproduced in Table 13. Six cows were involved in this investigation. The results are also shown graphically in Figure. 1.

Table 13

The citric acid content of the milks (morning milkings and evening milkings) of six cows, covering the first six months of the lactation period.

	Citric acid content											
Month	1		2		3		4		5		6	
Cow	E	M	E	M	E	M	E	M	E	M	E	M
I					189	209	183	188	165	155	158	160
II					272	280	230	258	270	270	233	218
III	279	280	250	270	260	260	218	213				
IV	216	225	200	225	215	183	163	173				
V	225	225	200	225	215	183	163	173				
VI	216	216	183	230	170	213	160	140				

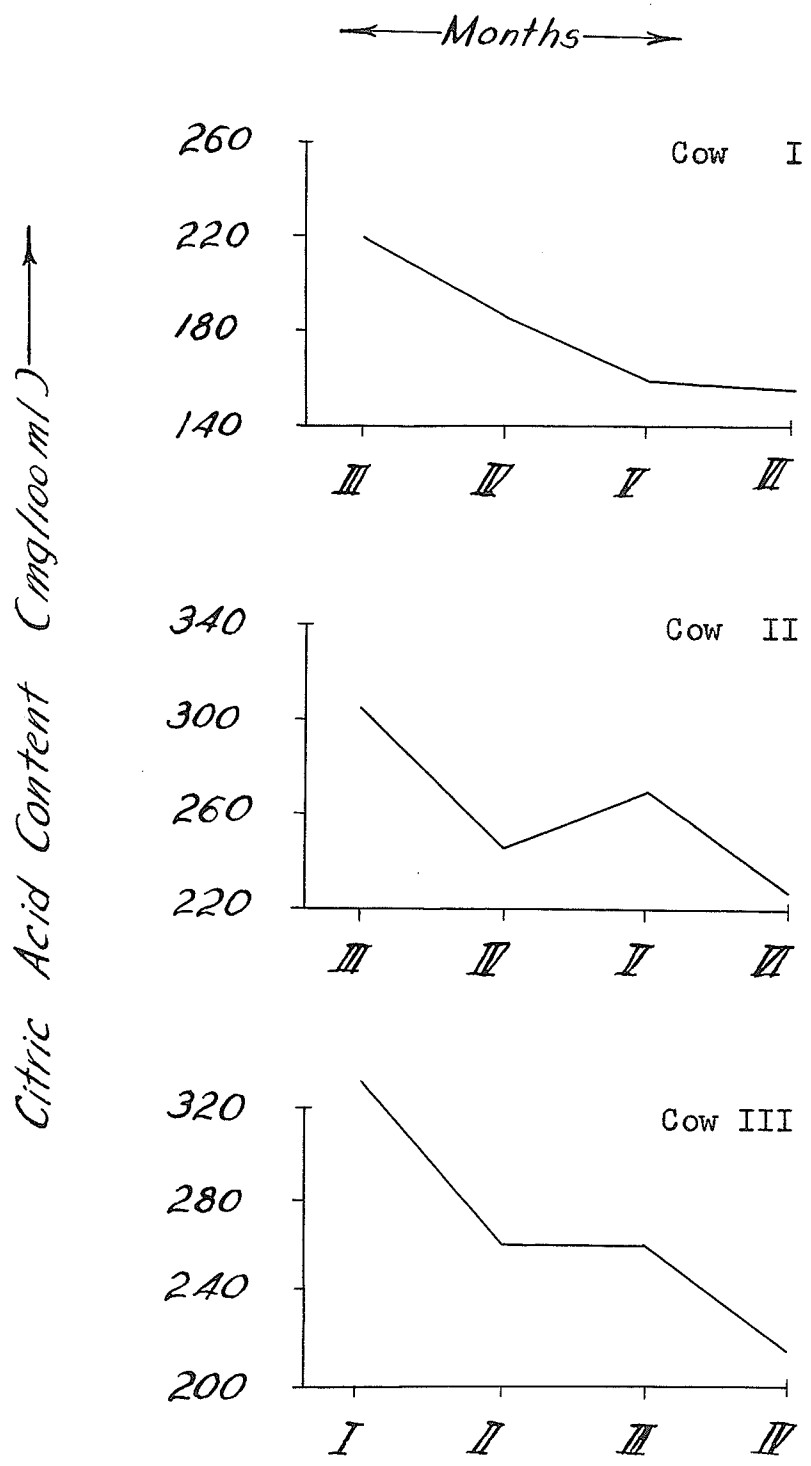

Figure 1 : Citric Acid Content during Lactation Period

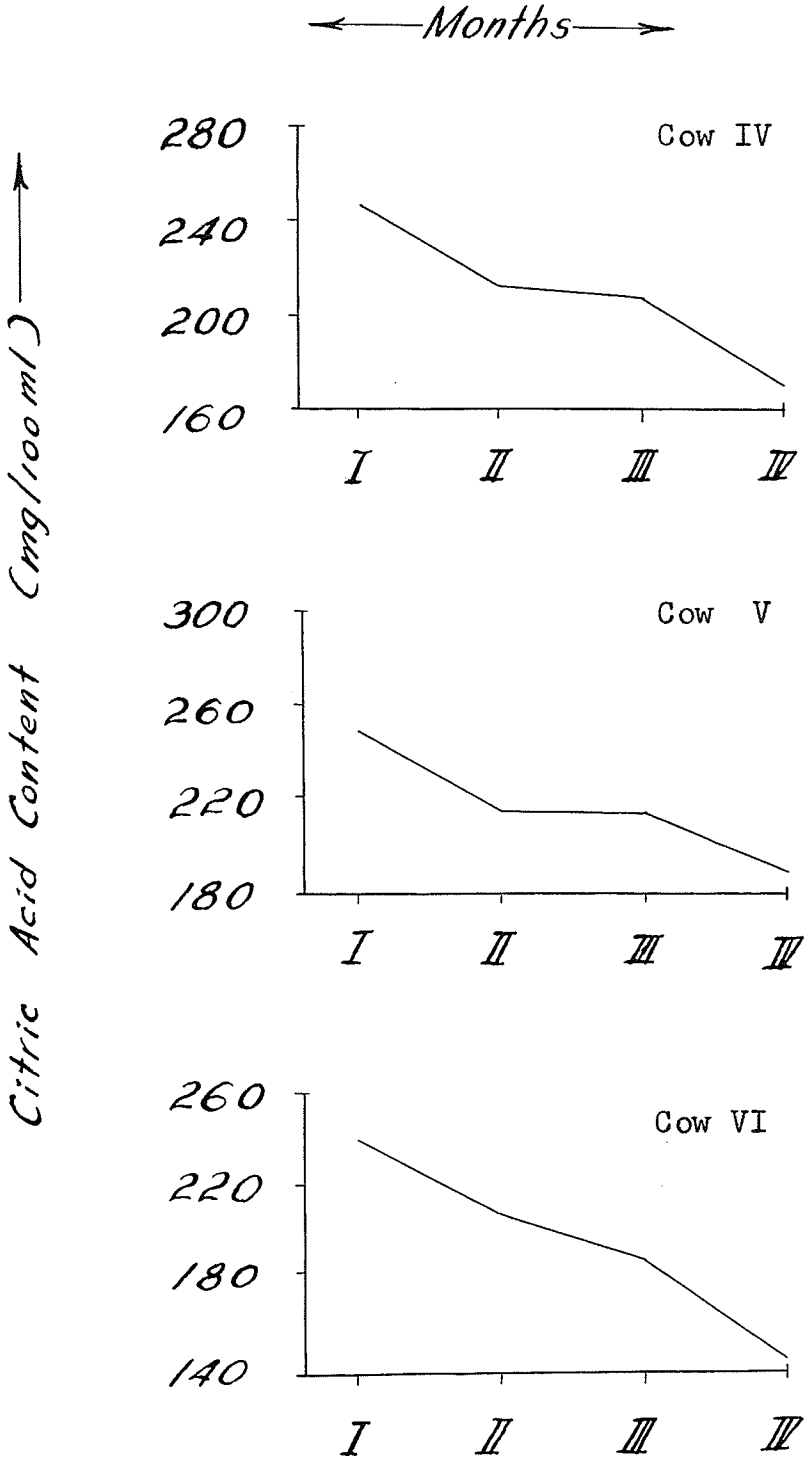

Figure 2: Citric Acid Content during Lactation Period

In Table 14 the results of a Chi square test of the relations between the lactation period and the citric acid content of milk of six cows are given.

Table 14

The ratio of the actual (a) and theoretical (t) citric acid contents of milk of three two-months periods of the first six months of the lactation period.

Months of Lactation Period	Citric acid content (mg/100 ml) 130-179 a	t	180-229 a	t	230-279 a	t	$\bar{X}^2$	D.F.	P-Value
1 and 2	0	3.000	11	8.000	5	5.000			
3 and 4	5	4.500	12	12.000	7	7.500	11.85	4	0.02
5 and 6	4	1.333	1	4.000	3	2.500			

The probability of obtaining such an association between the lactation period and the citric acid content by chance is less than two in a hundred.

C. The Variations of the Citric Acid Content in Colostrum.

In Table 15 the citric acid contents of the ten first milkings of ten individual cows are shown. Also, the arithmetic mean of the values of all ten cows for corresponding days are noted. The results are also represented graphically in Figure 2.

Table 16 represents the values of a Chi square analysis. The P - Value lies between 0.01 and 0.02 which indicates that the probability of obtaining a distribution of the citric acid content as indicated in Figure 2 by pure chance lies between one and ten in a hundred.

Since the Chi square test cannot be conveniently run on these samples the citric acid yields of the first milking were not included in this analysis.

Table 15

Citric acid contents of the ten first milkings of ten cows

Cow	Milking									
	1	2	3	4	5	6	7	8	9	10
1	151	170	189	242	354	252	238	242	232	238
2	171	210	250	225	315	320	275	275	270	250
3	200	194	277	265	307	273	239	211	230	214
4	222	258	270	276	268	276	271	248	221	208
5	234	237	273	309	360	294	294	296	291	291
6	248	275	251	248	243	243	266	229	244	239
7	85	85	150	140	180	175	180	185	165	140
8	100	130	150	187	210	215	205	205	190	200
9	70	60	245	255	275	273	230	225	228	180
10	190	240	235	235	210	180	165	145	145	155

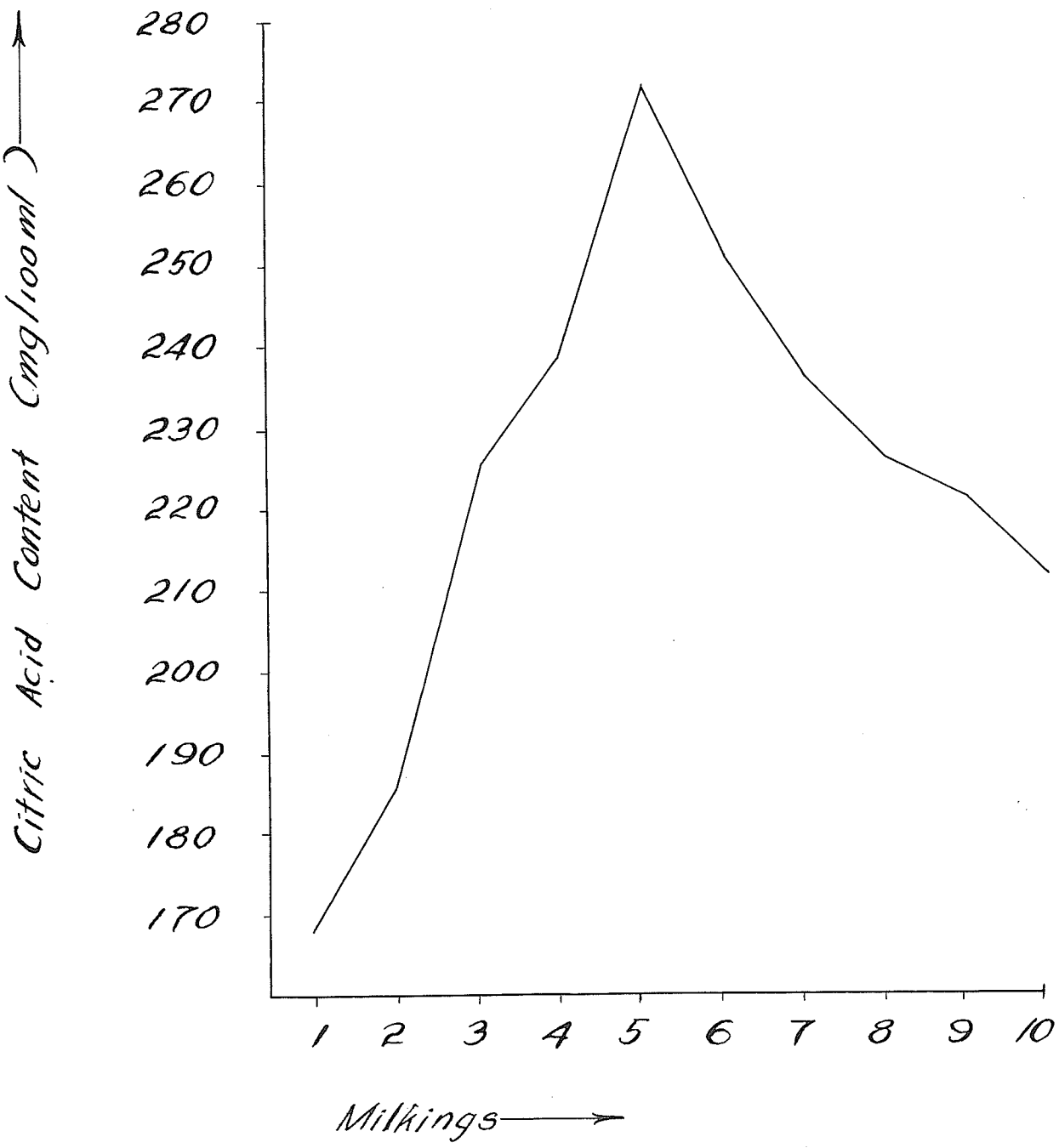

Figure 2: Variation of Citric Acid Content in Colostrum

Table 16

Chi Square test of the Citric Acid content of Colostrum

Milkings	Citric Acid Content (mg/100 ml) 60 - 160		161 - 261		262 - 362		X^{-2}	D.F.	P-Value
	a	t	a	t	a	t			
2, 3, 4	6	3.333	17	17.666	7	9.000			
5, 6, 7	0	3.333	15	17.666	15	9.000	11.85	4	0.02
8, 9, 10	4	3.333	21	17.666	5	9.000			

III. Correlation of the citric acid content with some related factors

A. Correlation of Milk Yield with Citric Acid Content

The yields of milk in pounds and the corresponding citric acid contents in mg per 100 ml milk of twenty-four cows are presented in Table 17.

Table 18 contains the values of a Chi square analysis which indicates a probability-value of 0.50.

Table 17

Yields and citric acid content of milk

Cow	Pounds of Milk	Mg/100 ml of citric acid	Cow	Pounds of Milk	Mg/100 ml of citric acid
1	23.0	195	13	34.5	170
2	25.0	188	14	34.5	210
3	26.0	200	15	35.5	190
4	28.0	220	16	36.0	185
5	28.0	230	17	38.0	200
6	29.0	245	18	38.5	150
7	29.5	185	19	40.0	200
8	31.0	180	20	43.0	210
9	31.0	220	21	44.0	155
10	31.5	190	22	52.5	180
11	31.5	210	23	53.0	180
12	32.0	200	24	55.0	155

Table 18

Chi square test of the correlation between yield of milk and citric acid content

Pounds of Milk	150 - 174		175 - 199		200 - 224		225 - 250		X^{-2}	D.F.	P Value
	a	t	a	t	a	t	a	t			
23.0 - 31.9	0	1,375	5	4.125	4	4,583	2	0.917			
32.0 - 40.9	1	1.000	3	3.000	4	3.333	0	0.167	8.27	9	0.50
41.0 - 49.9	1	0.250	0	0.750	1	0.833	0	0.167			
50.0 - 57.9	1	0.374	1	1.125	1	1.250	0	0.250			

B. Correlation of the Total Milk Solids to the Citric Acid Content of Milk.

The results of this investigation are shown in Table 19.

Table 20 represents the ratio between actual and the theoretical numbers of a Chi square analysis and includes as well the resulting values. The probability of obtaining such an association between citric acid content and total milk solids as obtained in this investigation, by chance, is less than one in a thousand.

Table 19

The citric acid content and the total solids content of twenty-four quarter milkings

Cow	Quarters							
	Left Front		Left Rear		Right Front		Right Rear	
	C.A. Mg/100ml	% T.S.	C.A. Mg/100 ml	% T.S.	C.A. Mg/100 ml	% T.S.	C.A. Mg/100 ml	% T.S.
I	178	11.94	195	11.54	183	11.83	185	11.71
II	188	11.13	200	11.00	195	11.19	200	11.71
III	155	9.72	150	9.30	155	9.83	150	10.16
IV	185	11.97	185	11.83	193	11.82	190	11.73
V	175	13.13	175	13.00	175	13.00	175	12.94
VI	170	12.36	160	11.71	160	11.70	163	11.1

Table 20

Chi square test of the correlation between total milk solids and citric acid content.

Total Milk Solids	Citric acid content (mg/100 ml) 150 - 166		167 - 183		184 - 200		X^{-2}	D.F.	P-Value
	a	t	a	t	a	t			
9.00-10.49	4	1.166	0	1.166	0	1.666			
10.50-11.99	3	4.375	2	4.375	10	6.250	23.77	4	<0.001
12.00-13.50	0	1.458	5	1.458	0	2.083			

D I S C U S S I O N

The results of a study of methods of determining the citric acid content of milk as well as the results of an investigation of factors correlated with or influencing the citric acid content of milk have been presented. Any conclusions can be tentative only. The number of animals included in the investigations or, in other cases, the length of time investigations were continued were insufficient to permit the drawing of final conclusions.

This discussion will deal with the results in the order already given, namely (1) methods, including the official method of the Association of Official Agricultural Chemists and the colorimetric methods by Babad and Shtrikman and by Rose, (2) investigation of factors influencing the citric acid content of milk, as the variation between individual cows and the daily variations in colostrum, (3) investigation of the correlation of the citric acid content to the yield of milk and the total milk solids.

I. INVESTIGATION OF METHODS.

A. The official method of the AOAC.

The official method of the Association of Official Agricultural Chemists was not investigated with the intention of checking its accuracy, but for reasons of comparison only. It was intended to use this method as a basis for determining the accuracy of the two colorimetric methods. A trial was first made to determine the recoverability of citric acid in a stock solution of known strength.

While the official method of the AOAC was satisfactory for determining the citric acid content of milk, it proved to be rather troublesome for determining the concentration of a citric acid stock solution. The first complication occurred when the lead salt was separated from the suspending solution. After the required centrifuging period, the liquid portion in the centrifuge bottle remained cloudy. However, by chilling the contents in the centrifuge bottles it was possible to overcome this difficulty.

A more serious error occurred in filtering the hydrogen-sulfide saturated aqueous lead salt solution. While observing all precautions described in the method it was impossible to get a clear filtrate. The precipitate in the solution was

apparently too fine to be retained by the filter paper.

The results listed in Table 1 disclose a relatively high variation and vary from the expected yields. They are consistently lower than expected. The variations may be due to the experimental difficulties mentioned above.

B. The colorimetric methods.

The colorimetric methods were checked to determine which, if any, was the more accurate.

1. Results of the method of Babad and Shtrikman.

The large variations of this method are shown in Table 4. The authors (3) claim an accuracy better than $\pm$ 5 per cent, usually $\pm$ 3 per cent. In the course of this investigation, however, deviations representing as much as 16.67 per cent of the expected yield were observed.

There are several factors which might be responsible for these variations. First of all, the main source might be in the extremely small amount of solution which is tested. Only 0.2 ml of a filtrate of a 1 : 1 solution of milk and trichloracetic acid (10 %) are used for a determination. It is obvious that even a small variation in the measuring of this amount will greatly affect the results. Furthermore, timing

is not uniform in this method. The acetic anhydride is first added to all the samples of a series, and after holding them for ten minutes in a water bath, they are removed and pyridine is added. They are then replaced in the water bath at the same temperature for an additional forty minutes. This procedure does not allow all samples in a series a similar time for color-development. The first tube of a series will have several minutes more time than the last one. Also, the time allowed for color-development will vary from series to series, depending on the time required for pyridine addition. The introduction of a strict time table which would grant to each sample an exactly equal period of time for reactions would probably improve the accuracy of the method.

2. Results by the method of Rose.

The results of the investigation of this method appear in Table 5.

Results obtained by a colorimetric method are considered to depend on factors which are not easy to control. It is recommended that a test should be run on standard solutions with each series of determinations, and calibration curve should be plotted from the results. The fact that the accuracy of colorimetric methods varies at different ranges of

concentrations is also stressed. For these reasons an analysis of variance was made.

a) Analysis of variance of citric acid contents of three concentration groups of three different series.

The data in Table 6 gives the following information: (1) the variations between yields of different series are not significant. The F-Value is below the one per cent level; (2) the F-Value for the variations between yields of the three concentration groups (48, 80 and 160 mg/100 ml) is also below the one per cent point; (3) the interaction between series and concentrations lies below the one per cent point and is also not significant. The fact that the three F-Values are not significant indicates that there are no signficant sources of error to be expected from the above mentioned causes. An established calibration curve might, therefore, be used for a number of series. It has to be renewed only when one of the integral parts connected with the establishment of the calibration curve (chemicals, parts of the electrophotometer, stock solution) is changed.

b) Analysis of variation between concentration groups.

The variations between the fifteen replicates of each of

the three concentration groups (48, 80 and 160 mg/100 ml) are large. They might be caused (1) by inaccuracy in procedure; (2) by the sensitivity of the electrophotometer. However, colorimetric methods are usually not expected to show absolute accuracy. The coefficient of variability is, especially for the two higher concentration groups (2.1 and 2.7 per cent), in an acceptable range and would, even when doubled (2 x standard deviation), still be near the $\pm$ five per cent limit which is usually assumed for colorimetric determinations. If the coefficient of variability for the lowest concentration group is somewhat larger, there is no reason for alarm because Rose's method uses concentrations which yield results between the 80 and 160 mg/100 ml concentration groups.

C. Comparison of the Colorimetric Methods and the Official Method of the AOAC.

A tabulation of the yields of the three methods reveals a high recovery of citric acid by the official method of the Association of Official Agricultural Chemists and by the method of Rose. However, the yields by Rose's method seem to be approximately five per cent above the results of the official method. The variations between the tests of each concentration do not exceed the five per cent limit.

The results obtained by the method of Babad and Shtrikman are not satisfactory at all. The recovery record and the variations between three parallel tests of each concentration are unduly large.

A comparison of results obtained by the official method and by the method of Rose, listed in Table 9, reveal a similar tendency as observed in Table 8. The results of the colorimetric method are approximately four per cent above the yields obtained by the official method. It is, however, difficult to conclude whether the official method of the AOAC yields too low or if the colorimetric method by Rose yields too high results. In a study of factors influencing the citric acid content of milk as well as in an investigation of correlations between the citric acid content of milk and factors related to it, the use of a method delivering slightly high results will not, as long as the variations are consistent, affect the final conclusions.

II. FACTORS INFLUENCING THE CITRIC ACID CONTENT OF MILK.

A. The Variations between Individual Cows and the Variations between Daily Milkings.

1. Variations between individual cows.

Table 11 shows that there is a very high variation

between the citric acid contents of milk from different animals. Some of these variations might be because the cows were in different stages of lactation. But as the feed and the environment were the same for all of them and as the test was carried out so as to avoid daily variations, most of the variation is due to difference between individuals.

2. Daily variations.

The results listed in Table 12 show the range of daily variations. The feed and the environmental factors were the same for all animals involved in this test. The stage of lactation for the different individuals was left out of consideration. It might, however, be responsible for a portion of the variations observed. The coefficient of variability ranges form 0.62 to 7.15, indicating that a daily variation in the citric acid content of milk exists.

B. The Lactation Period.

Figure 1 represents the citric acid content of the milk of six cows over a six-month portion of the lactation period. The general course of these curves shows a steady decrease in the citric acid content throughout the lactation period. The results of a Chi square analysis (Table 14) indicate that such

a relation between the citric acid content and the lactation period will occur in ninety-eight out of one hundred cases.

As the number of cows involved in this investigation is small and as the time of observation is limited to four months, the obtained results allow only tentative inferences. The period of observation was originally planned to be nine months. It was observed, however, that the change from dry feeding to green feeding affected the citric acid content of milk to a much greater extent than the lactation period. Therefore, only the results established during the period of dry feeding were included in this investigation. Samples of the evening-and-morning-milkings were tested in order to reduce the factors interfering with the problem in question.

C. Colostrum.

The citric acid content of the first milkings of freshening cows, the so-called colostrum, exhibits definite variations. Figure 2 shows the variation in citric acid content of the first ten milkings.

The contents of the first five milkings, corresponding to the first three days, increase regularly from a subaverage to a peak value. The following milkings exhibit a decreasing citric acid content and tend to approach more nearly normal values. As the results in Table 16 indicate, ninety-five times

out of one hundred the citric acid contents of the first ten milkings will vary in the same way as the one illustrated in Figure 2.

III. CORRELATION OF THE CITRIC ACID CONTENT WITH SOME RELATED FACTORS.

A. Correlation of the Milk Yield with the Citric Acid Content.

According to the results of this investigation (Table 17) there is no correlation between the yield of milk and the citric acid content of milk. The milk of a cow with a high yield might contain a very high or a very low citric acid content. This fact supports the theory that the main reason for the variations of the citric acid content is in the individuals.

B. Correlation of the Total Milk Solids and the Citric Acid Content.

The analysis concerning this correlation yields an unusual high P-Value ($P = <0.001$) and indicates, therefore, the existence of a close correlation between the two factors in question. To obtain the closest possible relation between the citric acid content and the total milk solids, the investigation was done on quarter milkings.

The correlation between the milk solids content and citric acid has been known for a number of years, and there are formulas for the calculation of the approximate content of milk solids in powder milk and in bread which are based on the citric acid content of these products (15). That this practice is sound is supported by the correlation between these two constituents revealed by this investigation.

C O N C L U S I O N S

1. The colorimetric method of Rose appears to be suitable for the determination of the citric acid content of milk. The accuracy is within the ± 5 per cent limit assumed for colorimetric methods.

2. The colorimetric method of Babad and Shtrikman yields variations which may be as large as 16 per cent of the expected content. Therefore, this method does not appear to be suitable for the determination of the citric acid content of milk.

3. There is a definite variation between the citric acid content of milk samples originating from twelve cows.

4. An investigation of the daily variations of the citric acid content of cow's milk yields coefficients of variability ranging from 0.62 to 7.15 and indicates therefore the existence of daily variations.

5. There is a considerable variation in the citric acid content of colostrum. The contents of the first five milkings, corresponding to the first three days of a

lactation period, are characterized by an aphoristic increase from a sub-average value to a peak value. The next five milkings exhibit a decreasing reduction of the citric acid content and lead toward a normal level.

6. A study of the correlation between the milk yield and the citric acid content of twenty-four cows revealed that there is no correlation between the two factors.

7. A very close correlation exists between the content of total solids and the citric acid content of milk. These two factors may therefore be related to each other.

REFERENCES

(1) ALLEN, L.A. The mineral constituents and citric acid content of milk. J. Dairy Res. 3: 1 - 51. 1932.

(2) ARUP, P.S. Citric acid determination in Milk and milk products. Analyst. 63: 635 - 640. 1938.

(3) BABAD, J. and SHTRIKMAN, N. The estimation of citric acid in dairy products. J. Dairy Res. 18: 72. 1951.

(4) BEAU, C. Rev. gen. du Lait, p. 386. 1904. (Original not seen; see reference (1).)

(5) CAHOURS, A. Ann. chim. 19: 484. 1847. (Original not seen; see reference (11).)

(6) DENIGES, G. Comptes Rend. 130: 32. 1900. (Original not seen; see reference (11).)

(7) DEYSHER, E.F. and HOLM, G.E. Determination of citric acid in pure solutions and in milk by the pentabromacetone method. Indus. and Engin. Chem., Analyt. Ed. 14: 4 - 7. 1942.

(8) DUNBAR, P.B. - and LEPPER, H.A. - Methods of Analysis. Association of Official Agricultural Chemists, p. 215. Washington. 1952.

(9) FINKELSTEIN, H. Darstellung organischer jodide aus den entsprechenden Bromiden und Chloriden. Ber. chem. Ges. 43: 1528. 1910.

(10) FURTH, O. and HERRMANN, H. Biochem. Z. 280: 448. 1935. (Original not seen; see reference (27).)

(11) GOLDBERG, A.S. and BERNHEIM, A.R. Citric acid determination. J. Biol. Chem. 156: 33. 1944.

(12) GOLDEN, C.H. Methods of statistical analysis. 2nd ed. p. 47 - 120. New York, J. Wiley & Sons, Inc. 1952.

(13) HAMMER, B.W. and BABEL, F.J. Bacteriology of Butter Cultures: A Review. J. Dairy Sci. 6: 83 - 168. 1943.

(14) HARTMANN, B.G. and HILLIG, F. Application of the Stahre reaction to the accurate determination of citric acid. Assoc. Off. Chem. J. 10: 264 - 272. 1927.

(15) _____ Approximation of the milk solids of a milk product by means of its citric acid content. Assoc. Off. Chem. J. 16: 427. 1933.

(16) HEINEMANN, B. Seasonal and geographical variations in the citric acid content of defatted milk solids. J. Dairy Sci. 27: 773. 1944.

(17) _____ Determination of citric acid in milk products by cerate oxidimetry. J. Dairy Sci.27: 377 - 383. 1944.

(18) HUNZIKER, O.F. Condensed milk and milk powder. 7th ed. p. 38. La Grange, Illinois, published by the author. 1949.

(19) KIEFERLE, F., SCHWAIBOLD, J. and HACKMANN, Chr. Zitronensaeure-Gehalt in Milch. Zeitschr. Physiol. Chem. 145: 18 - 26. 1925.

(20) KNODT, C.B. and PETERSEN, W.E. Studies of the carbohydrate metabolism of mammary gland tissue in vitro; metabolism of citric acid and B - hydroxybutyric acid in tissue slices. J. Dairy Sci. 29: 115 - 120. 1946.

(21) KOMETIANI, P.K. Z. Anal. Chem. 86: 359. 1931. (Original not seen; see Reference (32).)

(22) KUNZ, R. Arch. Chem Mikrosk. 7: 285. 1914 (Original not seen; see reference (14).)

(23) PRATT, D.S. Determination of citric acid. U.S. Dept. Agric. Bur. Chem. Circ. 88. 1912.

(24) PUCHER, G.W., SHERMAN, C.C. and VICKERY, H.B. A method to determine small amounts of citric acid in biological material. J. Biol. Chem. 113: 235. 1936.

(25) RICHMOND, H.D., ELSDON, G.D. and WALKER, G.H. Dairy Chemistry. p. 21. Charles Grifflin & Co. Ltd., London. 1942.

(26) ROSE, D. Food Chemistry Section, Division of Applied Biology, National Research Council, Ottawa. Privat Communication. 1954.

(27) SAFFRAN, M. and DENSTEDT, O.F. A rapid method for the determination of citric acid. J. Biol. Chem. 175: 849. 1948.

(28) SCHEIBE, A. Landw. Vers. Sta. 39: 153 - 170. 1891. (Original not seen; see reference (13).)

(29) SHERWOOD, F.F. and HAMMER, B.W. Citric acid content of milk. Jowa Agr. Expt. Sta. Res. Bul. No. 90. 1926.

(30) STAHRE, L. Nord. Farm. Tids. 2: 141. 1895.
(Original not seen; see reference (16).)

(31) SUPPLEE, G.C. and BELLIS, B. Citric acid content of milk and milk products. J. Biol. Chem. 48: 453. 1921.

(32) TAUSSKY, H.H. and SHORR, E. A microcolorimetric method for the determination of citric acid. J. Biol. Chem. 169: 10s. 1947.

(33) WASHINGTON. Official Methods of Analysts of the Association of Official Agricultural Chemists. p. 227. 1950.

(34) WILLOMAN, J.J. Modification of the Pratt Method for the determination of citric acid. J. Amer. Chem. Soc. 38: 2193. 1916.

(35) WOEHLK, Z. anal. Chem. 41: 77. 1902.
(Original not seen; see reference (14).)

ACKNOWLEDGMENTS

Thanks are extended to Dr. J.M. Nesbitt, Prof. J.J. Ritchie and Mr. A. Reinart, of the Department of Dairy Science, for their guidance and assistance in carrying out this work. The author also wishes to thank Dr. A.D. Robinson, Professor of Biochemistry, for many valuable criticisms and suggestions in writing this thesis and Dr. P.A. Kondra, Department of Poultry Husbandry, for valuable advice with the statistical analysis of the experimental data.