THE DETERMINATION OF THE CALCIUM, MAGNESIUM, AND ACID-SOLUBLE PHOSPHORUS OF MILK BY MEANS OF TRICHLOROACETIC ACID FILTRATES

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INTRODUCTION

The use of a trichloroacetic acid serum filtrate for the quantitative determination of phosphorus in blood, as developed by Bell and Doisy (1) and Benedict and Theis (2), and later introduced by Roe and Kahn (3) for calcium determinations, suggested to the writer the possibility of using a similar procedure in determinations of acid-soluble minerals in cow's milk, in place of the ashing method. The elimination of the ashing method in making such determinations would result in a considerable saving of time and less equipment would be required.

Trichloroacetic acid has been used by Greenwald (4) for the precipitation of blood proteins in order to carry out quantitative determinations of non-protein nitrogenous constituents. Lyman (5) has used trichloroacetic acid as a protein precipitant in both blood and milk for the purpose of preparing filtrates for calcium determinations. Van Slyke and Bosworth (6) have shown that all of the calcium, magnesium, inorganic phosphorus, sodium, potassium, and non-protein nitrogen in milk is found to be in the soluble form in the filtrate when sour milk is filtered.

In the present work the writer has used the trichloroacetic acid filtrate method for calcium, magnesium, and acid-soluble phosphorus determinations in cow's milk, and data are presented to show the accuracy of this method as compared with the ashing method.

Procedure

Trichloroacetic Acid Filtrate Method—20 cc. samples of cow's milk are pipetted into each of two 100 cc. volumetric flasks, the
pipette and flasks having been accurately calibrated. The flasks are filled to the 100 cc. mark with 10 per cent trichloroacetic acid solution, the acid being added slowly and the flask rotated constantly. The contents are thoroughly mixed and allowed to stand stoppered for 30 minutes with frequent stirring. They are then filtered through dry 15 cm. acid-washed filter paper (Schleicher and Schüll No. 589, blue ribbon). The funnel should be covered with a watch-glass during the filtration in order to eliminate, as far as possible, any error due to evaporation.

50 cc. of the filtrate from each sample (corresponding to 10 cc. of milk) are taken for the mineral determination. Then the same procedure is used as in the ashing method after the ash samples have been dissolved and transferred to beakers.

Ashing—10 cc. samples of milk of known specific gravity are pipetted into clean platinum crucibles, dried overnight at 98-100°C to remove moisture, and ashed to a white residue. The ash is taken up with dilute hydrochloric acid and transferred to beakers.

**Calcium**

In certain procedures which have been described for determining blood minerals, notably those of Bell and Doisy (1), Benedict and Theis (2), Roe and Kahn (3), and Greenwald (4), the samples were first diluted with water before the trichloroacetic acid solution was added. In the present work it was felt that by eliminating the addition of water the procedure might be shortened and the degree of accuracy increased. A study was first made of the use of equal parts of milk and acid solution.

**Hydrogen Ion Concentration of Milk and Acid Mixtures**—A number of samples of milk having pH values averaging 6.54 were mixed with equal quantities of trichloroacetic acid solutions; the normalities of the acid solutions were 0.05, 0.06, 0.10, 0.20, 0.40, and 0.609, respectively. When 0.05 N acid solution was used, the pH values of the resulting mixtures were between 4.75 and 4.95. These mixtures could not be effectively filtered. When 0.06 N acid solution was used, the pH values of the resulting mixtures were 4.31 to 4.62. The latter pH value occurred in a mixture in which a sample of rich Jersey milk was used; when the pH value was below 4.6, the approximate isoelectric point of casein, the material filtered readily but the filtrates were slightly
turbid and calcium oxalate crystals precipitated from such filtrates could not be readily filtered and washed. In these cases, calcium recoveries showed an error of −8.9 to −3.8 per cent.

The most accurate calcium recoveries were obtained with the use of the stronger acid solutions. The 0.609 N (approximately 10 per cent) acid solution, when added to an equal volume of fresh milk, resulted in a mixture the pH of which was approximately 0.80. The filtrate was practically clear, with very little or no turbidity; it was entirely free of fat as determined by the Babcock test.

A number of determinations\(^1\) of the calcium content of normal cow's milk were made by the ashing method and the trichloroacetic acid filtrate method. 1 part of 10 per cent acid solution was added to 1 part of milk. When skim milk was used, the filtration method produced results which were usually slightly high for calcium, not greater than 1.5 per cent; when whole milk was used, the positive error was as great as 4.03 per cent. The results indicated an increased content of calcium in skim milk as compared with the same milk before skimming, this increase corresponding closely with the percentage of fat in the whole milk.

At the time that this work was in progress, a second paper dealing with the determination of blood calcium was published by Roe and Kahn (9). They recommended the use of 4 parts of trichloroacetic acid solution with 1 part of blood serum. The writer made a study of this improved procedure and found that the use of 4 parts of trichloroacetic acid solution with 1 part of milk was more effective in removing the proteins and more accurate for calcium determinations than the use of the 1:1 mixture. Further, in the improved procedure, the calcium oxalate crystals could be digested by heating in order to hasten precipitation, as recommended by McCrudden (8), instead of being allowed to stand overnight. This advantage results from the fact that there is no appreciable amount of heat-coagulable protein present to cause interference in the filtration and washing of calcium oxalate crystals when the 4:1 ratio of acid and milk is used. Typical results are shown in Table I.

\(^1\) Calcium analyses were carried out by the method of Meigs, Blatherwick, and Cary (7), which is a modification of the McCrudden (8) method. Calcium was determined as calcium oxalate, by permanganate titration.
Van Slyke and Bosworth (6) have shown that approximately 33.3 per cent of the calcium and 53.8 per cent of the magnesium in fresh milk occur in a soluble, non-colloidal form. The balance of the calcium, existing principally as colloidal dicalcium phosphate and calcium caseinate (probably Ca₄ caseinate), becomes soluble in the presence of a sufficient amount of acid. It is believed that the colloidal calcium of calcium caseinate is displaced by H⁺ ions of the acid and appears in the form of soluble monocalcium salts.

Rothwell (10) has presented a method of determining calcium in cow's milk by precipitating it directly as calcium oxalate, and centrifuging. In the case of human milk, calcium recoveries were

### TABLE I

Comparison of Filtration Method (1 Part of Milk to 4 Parts of 10 Per Cent Trichloroacetic Acid Solution) with Ashing Method.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fat in milk</th>
<th>Ca per 100 cc. milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>Ashing method</td>
</tr>
<tr>
<td>Mixed whole milk*</td>
<td>4.0</td>
<td>129.2</td>
</tr>
<tr>
<td>&quot; skim &quot;</td>
<td>0.01</td>
<td>134.8</td>
</tr>
<tr>
<td>Holstein whole &quot;</td>
<td>2.95</td>
<td>115.6</td>
</tr>
<tr>
<td>&quot; skim &quot;</td>
<td>0.01</td>
<td>118.2</td>
</tr>
<tr>
<td>Jersey whole &quot;</td>
<td>4.8</td>
<td>136.0</td>
</tr>
<tr>
<td>&quot; skim &quot;</td>
<td>0.01</td>
<td>142.0</td>
</tr>
<tr>
<td>Mixed whole &quot;</td>
<td>3.8</td>
<td>125.2</td>
</tr>
</tbody>
</table>

* The same milk before and after skimming.

(as does the balance of the magnesium) in the presence of a sufficient amount of acid. It is believed that the colloidal calcium of calcium caseinate is displaced by H⁺ ions of the acid and appears in the form of soluble monocalcium salts.
incomplete. Lyman (5) has reported low results in the direct precipitation of calcium in cow's milk. In the present work, calcium recoveries in skim milk, by direct precipitation, were within ±2 per cent of those obtained by ashing; in whole milk, however, calcium recoveries by direct precipitation were incomplete, ranging from 91.2 to 96.2 per cent of the ashing values.

Magnesium

Because of the small amount of magnesium occurring in milk, it is advisable, in gravimetric analyses, to use 30 cc. of milk for each analysis instead of 10 cc.

TABLE II
Comparison of Filtration Method with Ashing Method in Determining Magnesium Content of Milk

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fat in milk</th>
<th>Mg per 100 cc. milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>Ashing method</td>
</tr>
<tr>
<td>Jersey</td>
<td>5.2</td>
<td>17.0 mg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.0</td>
</tr>
<tr>
<td>Mixed</td>
<td>4.1</td>
<td>14.2 mg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.4</td>
</tr>
<tr>
<td>Holstein</td>
<td>3.0</td>
<td>13.2 mg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.1</td>
</tr>
</tbody>
</table>

40 cc. samples of milk are pipetted into 200 cc. volumetric flasks and the flasks are filled to the mark with 10 per cent trichloroacetic acid solution. After filtering, 150 cc. aliquot portions, corresponding to 30 cc. of milk, are used. In the ashing method, 30 cc. samples of milk are used. The magnesium determinations were carried out by Method II of the Association of Official Agricultural Chemists (11) p. 29), except that in the analyses of the trichloroacetic acid filtrates, the first addition of alcohol was omitted.

Consistently accurate results were secured by the new method. Representative data are shown in Table II.
Acid-Soluble Phosphorus

Greenwald (12) has shown that the phosphorus compounds of blood may be divided into three classes: (a) lipid, (b) an acid-soluble form, probably inorganic in nature, and (c) phosphorus combined with protein. There is little doubt that the phosphorus in milk exists in these three forms. Van Slyke and Bosworth (6) analyzed both fresh and sour milk, using a porous porcelain filter to separate the soluble from the insoluble constituents, and found approximately 53.6 per cent of the phosphorus in a soluble form in fresh milk and from 76.8 to 79.7 per cent in the inorganic form. They showed that all the inorganic phosphorus becomes soluble in the process of souring. They found that the ratio of organic to insoluble inorganic phosphorus varied from 1:0.83 to 1:2.47. From their data of analyses of sixteen samples of milk, the amounts of soluble phosphorus plus insoluble inorganic phosphorus are calculated as follows: maximum 83.1 per cent, minimum 73.0 per cent, average 78.3 per cent.

Concerning the organic phosphorus compounds, Bosworth and Van Slyke (13) found that purified casein contains about 0.71 per cent phosphorus; Dornic and Daire (14) reported 0.058 per cent lecithin in milk, and they stated that Stoklasa found 0.09 to 0.113 gm. and that Glikin found 0.05 to 0.15 gm. of lecithin per 100 cc. of milk. Koch and Woods (15) found an average of 0.043 per cent lecithin and 0.036 per cent cephalin, a total of 0.079 per cent of both. Lecithin having a formula C₄₄H₉₀N₃O₉ (molecular weight 807.99) contains 3.84+ per cent phosphorus. Cephalin contains about 4 per cent phosphorus.

On the assumption that there is approximately 0.07 gm. of lecithin plus cephalin in milk per 100 cc., the phospholipid phosphorus would amount to approximately 2.75 mg. per 100 cc. of milk. A sample of milk containing 3.0 per cent casein would contain approximately 21.3 mg. of casein phosphorus per 100 cc.; a total of 24.05 mg. of organic phosphorus would be present.

Quantitative determinations of acid-soluble phosphorus were made in a number of samples of cow's milk. The samples were prepared as described for calcium, trichloroacetic acid being used as the protein precipitant and mineral solvent. Phosphorus analyses in the filtrates were carried out by the gravimetric pro-
procedure of the Association of Official Agricultural Chemists ((11) p. 3). Representative data are shown in Table III.

**TABLE III**

*Comparison of Filtration Method with Ashing Method in Determination of Phosphorus in Milk*

<table>
<thead>
<tr>
<th>Casein per cent</th>
<th>Fat per cent</th>
<th>Ashing method mg.</th>
<th>Ashing method Average mg.</th>
<th>Filtration method mg.</th>
<th>Filtration method Average mg.</th>
<th>Per cent total P</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.60</td>
<td>3.65</td>
<td>115.2</td>
<td>92.8</td>
<td>92.4</td>
<td>80.1</td>
<td></td>
</tr>
<tr>
<td>2.64</td>
<td>3.60</td>
<td>112.7</td>
<td>92.0</td>
<td>92.0</td>
<td>81.9</td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>2.90</td>
<td>95.2</td>
<td>74.4</td>
<td>74.5</td>
<td>79.2</td>
<td></td>
</tr>
<tr>
<td>2.42</td>
<td>3.10</td>
<td>93.6</td>
<td>72.0</td>
<td>71.5</td>
<td>77.8</td>
<td></td>
</tr>
<tr>
<td>3.04</td>
<td>4.60</td>
<td>125.9</td>
<td>86.2</td>
<td>86.0</td>
<td>68.4</td>
<td></td>
</tr>
<tr>
<td>2.55</td>
<td>3.00</td>
<td>92.2</td>
<td>64.3</td>
<td>64.8</td>
<td>69.5</td>
<td></td>
</tr>
<tr>
<td>2.60</td>
<td>4.00</td>
<td>105.9</td>
<td>78.6</td>
<td>77.2</td>
<td>72.0</td>
<td></td>
</tr>
</tbody>
</table>

Average...2.55 3.55 105.7 79.8 75.5

Of the total phosphorus the percentage recovered in the acid filtrates varied between 68.4 and 81.9 per cent; the average was 75.5 per cent. The value 2.55 (average per cent casein) multiplied by 0.0071 equals 0.018 or approximately 17.5 mg. of casein phosphorus per 100 cc. of milk. This figure added to 79.8 mg. (the
average acid-soluble phosphorus) equals 97.3 mg. of acid-soluble plus casein phosphorus. When the above estimated figure for lipid phosphorus is added, the total falls below the figure 105.7 mg. obtained for average total phosphorus.

It is impossible to conclude from the data in Table III whether the figure for acid-soluble phosphorus corresponds exactly with the figure for inorganic phosphorus in milk. Briggs (16) indicated that the acid-soluble phosphorus fraction of blood represents the true value for inorganic phosphorus.

In order to ascertain whether any organic phosphorus was present in the acid-soluble phosphorus fraction, trichloroacetic acid filtrates were prepared in quadruplicate from two different samples of milk. In the case of each, phosphorus was determined in aliquots of two of the filtrates; the other two filtrates were ashed wet in Kjeldahl flasks with sulfuric and nitric acids and the phosphorus was then determined in the ashed samples. The results were identical. There was no appreciable amount of organically combined phosphorus in the trichloroacetic acid filtrates.

A series of trichloroacetic acid filtrates of a sample of milk was prepared in which the milk and acid mixtures were allowed to stand for \(\frac{1}{2}, 24, \text{ and } 72\) hours, respectively, before filtering. The phosphorus contents of these filtrates were all found to be the same. The solution of phosphorus apparently reaches equilibrium within \(\frac{1}{2}\) hour.

It was found impractical to determine acid-soluble phosphorus in Folin and Wu (17) filtrates for the reason that in a solution of milk salts the presence of tungsten interferes in the precipitation and separation of phosphorus by means of molybdate mixture. When the trichloroacetic acid filtrate method is used, there are no interfering materials present in the filtrates.

Protein Precipitation—Efficient protein precipitation is an essential factor in preparing milk filtrates for the determination of minerals or of non-protein nitrogen constituents. In the present work it was found that the completeness of protein precipitation is dependent upon the concentration of trichloroacetic acid in the milk and acid mixtures. Below pH 4.6, protein solubility gradually decreased with decrease in the pH of the mixture. This is contrary to the effect observed when acids such as hydrochloric are used. Protein precipitation was most effective in the case of
milk having a low buffer value; the precipitation was further favored by increasing the dilution of the system. For all samples of cow’s milk, the use of 4 parts of 10 per cent trichloroacetic acid with 1 part of milk produced practically complete protein precipitation.

Trichloroacetic acid solution added to milk produces a much coarser precipitate than does the mixture of sodium tungstate and sulfuric acid used in the Folin and Wu (17) method; the trichloroacetic acid mixture filters much more rapidly and yields a considerably larger amount of filtrate in proportion to the amount of milk used.

**SUMMARY**

A procedure is described for determining calcium, magnesium, and acid-soluble phosphorus in milk without ashing. The procedure is much more rapid than the ashing method. A saving of at least 1 working day in preparing the samples is effected. The use of 4 parts of 10 per cent trichloroacetic acid solution with 1 part of milk was found most effective in preparing protein-free filtrates for mineral determinations. A high degree of accuracy was attained in determinations of calcium and magnesium.

In a number of milk samples, 68.4 to 81.9 per cent of the phosphorus was found to be acid-soluble. There was no definite correlation between the amount of acid-insoluble phosphorus and the amount of casein; in general, however, milk high in casein was high in acid-insoluble phosphorus. The procedure outlined herein should be of value in studying phosphorus combinations in milk, with particular reference to casein and lipid phosphorus.

**BIBLIOGRAPHY**

Ca, Mg, and P Determination in Milk
