DEVELOPMENT OF A RAPID METHOD FOR THE ESTIMATION OF CALCIUM AND PHOSPHORUS IN MILK

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF
MASTER OF SCIENCE
IN
DAIRYING
(DAIRY CHEMISTRY)

BY
ASHU BHATIA

DIVISION OF DAIRY CHEMISTRY
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R.)
KARNAL-132001 (Haryana), INDIA
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Regd. No. 89-M-DC-56

DEDICATED TO MY

PARENTS AND

BROTHER

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This is to certify that the thesis entitled "DEVELOPMENT OF A RAPID METHOD FOR THE ESTIMATION OF CALCIUM AND PHOSPHORUS IN MILK" submitted by MISS ASHU BHATIA in partial fulfilment of the requirement for the award of MASTER OF SCIENCE in Dairying (Dairy Chemistry) of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other Degree or Diploma.

Dated : 15.7.92

(Dr. DES RAJ)

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Analytical methods are the builders of the scientific data. Rapid and simple methods, though a little less accurate, have been more frequently used than the sophisticated instrumental and time consuming classical methods. The rapid methods become more important for the analysis of perishable products like milk and some of the milk products during standardization and processing.

Among the other constituents of milk, minerals especially calcium and phosphorus are equally important as nutrients in the human diet. They also influence some of the Physico- chemical properties of milk, concentrated milk and some milk products. Sindhu (1985) reported considerable increase in heat stability of concentrated buffalo milk with the addition of optimum quantity of monobasic sodium phosphate solution in milk, whereas some of the unconcentrated buffalo milk samples were destabilized. Sindhu (1989) further reported that a required quality of Channa for Rasogolla making can be prepared from buffalo milk by modifying the salt balance of the milk. This indicates that it is essential to know the concentration of major minerals, especially of calcium and phosphorus already present in the milk before doing such modifications.

The estimation of calcium and phosphorus in milk and milk products is very frequent

during research and teaching. It may gain importance during processing of milk, because for better quality and/or heat stability of milk and some of milk products, not only fat and SNF of milk are required to be standardized, but calcium and phosphorus may also, if rapid methods of their determination are available.

The existing methods of calcium and phosphorus determination, either are laborious and time consuming or they require costlier instruments and chemicals. Majority of the instrumental methods are based upon the techniques such as molecular absorption spectrometry, flame photometry,atomic absorption spectrometry. However, these two elements can also be determined by classical chemical methods. But none is used as platform test for the determination of calcium and phosphorus.

In order to develop a simple and rapid method for the determination of calcium and phosphorus in milk, a chemical property, oxalate effect on milk, has been used. Des Raj (1991) observed oxalate effect which states that " a phenomenon of decrease in titratable acidity of milk is observed when neutralized potassium oxalate is added to it. The decrease in the acidity is different for milks of different animals and propotional to the concentration of $Ca_3(PO_4)_2$ content of milk at the neutral pH".

Using this property a simple titrimetric method has been standardized for simultaneously determination of calcuim and phosphorus. The estimation is carried out in conjunction with acidity test of milk. It needs double titration for acidity determination, one before and one after the addition of potassium oxalate solution to the test portion of the milk sample.

CHAPTER 2-

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

A great variety of analytical methods are available for the determination of calcium and phosphorus in milk. Most of them are based on the techniques such as molecular absorption spectrometry. flame photometry and atomic absorption spectrometry. Many chemical methods are also available for the determination of calcium and phosphorus. The various analytical methods available in literature have been reviewed in the following paragraphs.

2.1. METHODS OF CALCIUM DETERMINATION :

2.1.1 EDTA- Method :

The method developed by Schwarzenbach (1946) was based on the complex formation of alkaline earth metals with ethylene- diamine tetra acetate (EDTA). This principle has been used by many workers for blood serum and other biological materials (~~Holfz 1951~~, Cheng and Bray 1951, ~~Gastler 1949~~). However the method was applied to milk by Jenness (1953) and Van Der Have (1954). Jenness (1953) titrated calcium with complexone in a solution of milk ash and in milk serum. Complexone is the disodium salt of the amino polycarbonic acid ethylene -diamine tetra acetic acid. With compound forms with calcium stable and colourless complexes which are water soluble.

Under the conditions of his experiments the phosphate ion interfered with the titration and had first to be eliminated with the aid of an ion exchanger. Van der Have (1954) estimated calcium by titration with a solution of "Complexone 3" using Eriochrome black T as

an indicator without the necessity of eliminating the phosphate. This complexone 3 is also a salt of ethylene diamine tetra acetic acid.

Borus - Boszormenyi and Kovacs (1976) determined calcium in the ash of the samples of both vegetable and animal origin. The ash content was dissolved and precipitated as oxalate in the conventional way. The precipitate was dissolved in cold nitric acid without washing and the solution was then titrated with an EDTA solution using phthalein purple as indicator in presence of masking agents. Generally it was 0.4 to 2.0 mg or even 5 ml of acid solution. The calcium contents of milk and bones could be determined by direct complexometric titration without precipitating calcium as oxalate.

Kindstidt and Kosikowski (1985) improved upon the above method, in which 2-3 gm cheese was ashed, dissolved in dilute acid and the calcium chloride was back titrated with ethylene diamine tetracetic acid using hydroxy naphthol blue as indicator. The samples remained free from turbidity and the titration end point was recognised easily. However, a portion of magnesium of the sample was also measured. Kamal (1960) eliminated the interference caused by the phosphate ions in this complexometric titration by adding the disodium salt of ethylene dinitrilo tetra acetic acid to the neutral system and back titrating the excess with calcium standard solution.

Bird et al (1961) combined different EDTA- methods for determination of calcium. They used the Ling's method for the removal of proteins and

phosphates by the potassuim metastannate. Flashka's indicator Erichrome black S.E. was used for the determination of calcium. Although recoveries were 100% the procedure was not convenient. Before carrying out the actual EDTA titration, Sirnik and Zagozen (1973) diluted 10ml milk with twice the amount of distilled water to 100 ml and added 3ml buffer (5 N KOH in 6.6% Potassuim cyanide solution pH 13 was used for the purpose). Then 10ml of this diluted milk was brought to 50ml with double distilled water. A small quantity of indicator which consisted of .1 gm calcium, 1 gm charcoal and 10gm of potassium chloride was added. Grillo and Munao (1989) used calcon instead of the above indicator in the EDTA titration.

Pearce (1977) observed that when Eriochrome black T or calcon were used as indicators results were higher than when Pattons and Reeder's indicators were used. Boszormenyi and Ternero (1977) carried out back titrations with excess of EDTA to estimate calcium. However, Pearche (1977) showed that direct titration gave more reliable results and that erroneous values for calcium may be obtained unless pH carefully adjusted. Boszormenyi (1977) modified the conventional titration with EDTA solution in that the possibility of the precipitation of calcium as calcium hydrogen phosphate ($CaHPO_4$) was avoided by adjusting the calcium concentration of the titrated solution to <0.3 mM and precipitation of $Mg(OH)_2$ was avoided by adjusting the pH to 10.3. Phthalein purple was used as indicator.

Chaplin (1984) developed a pH stat-method (used to assess end point). The method was a modified one being used at pH 5.5. replacing EDTA by CDTA (trans - 1,2 - diamino cyclo hexane - N,N,N', N', tetra acetic acid) or BAPTA [1,2-bis (2-aminophenoxy) ethane- N,N,N', N', tetra acetic acid]. The complexing agent was added continuously from a syringe pump working at 60 ml/hr and flow rate measured on a chart working at a slow speed of 1cm/min. Thus distance on the chart paper was converted into volume and calculations made. Although the method gave accurate results it involved the used of expensive instruments and chemicals.

Cardwell et al (1991) described a flow based analysis method, discontinuous flow analysis was used for the determination of total calcium in drinking water, milk and wine by titration with ethylene glycol tetra acetic acid. The titration can be cycled continuously with a cycle time of about 1 min. This can be carried out with a single sample or with different samples using an autosampler. The method for water and wine was simple highly reproducible but for milk a back titration method was used because of the complex matrix of the sample. Alvarez Jimenez et al (1988) proposed that without ashing of milk titration could be carried out after precipitating out the protein by salicylic acid. Kondrat'ev (1975) suggested the use of Trilon B (EDTA tetra sodium salt) as titrant.

2.1.2. Spectrophotometric Method:

The spectrophotometric method was developed long back. It gives accurate results but involves the use of expensive instruments. In this

method sodium metavanadate and sodium molybdate are added to nitric acid solutions of the ashed samples. Added molybdate and vanadate reduce interference of phosphate in calcium analysis and calcium is determined at 422.7nm.

Lovachev et al (1973) used the method for calcium estimation in butter. Dry ashing of the nitric acid extract of butter was carried out and colour developed in the usual manner. Ramirez - Munoz (1975) used Beckman Autolam BurnerII and Beckman Model 495 Spectrophotometer. However there was interference due to phosphoric acid. This interference was reduced by selection of a suitable burner position and addition of 500ppm strontium as suggested by Ono, and Odagiri et al. (1976). They also found that removal of proteins with trichloroacetic acid was adequate and that ashing was not necessary. King (1977) used rapid quantitative x-ray spectrometric method. However, they concluded that it was unsuitable for moist foods and foods with high sugar content.

Flame emission spectrometry was carried out by Noller, and Bloom et al. (1978) for milk and milk products. But emphasis was placed with regard to preparation and handling of apparatus.

Basson et al. (1980) used a carle microvolume sampling valve with two identical sample loops. Analysis was based on the reaction between calcium and cresolphthalein complexone in a 2- amino -2- methyl propan - 1- ol basic medium. The colour developed was measured at 580 nm.

El- Shaarawy and Reith (1982) gave an atomic absorption spectrophotometric method sutiable for large number of samples for calcium estimation. Samples were dry ashed, silicates removed by hydrochloric acid and effect of interfering ions like phosphate etc. were minimised by additon of lanthanum. Perkia- Elmer and varian Techtron machines were used. Sarudi and Varga (1983) further elaborated the procedure, the solution of ashed samples were vaporised in air or acetylene flame and calcium determined at 422.7mm. Gorbunov et al. (1985) used discharge voltage of 6-7 kv in a box at low pressure for the purpose.

Rapid and direct determination of major minerals in raw cow milk by multi-channel inductively coupled plasma emission spectrometry was applied by Takahashi and Sutoh (1990) . Pneumatic atomization of aqueous milk solution was carried out. However, in the subsequent year (1991) they observed that atomization resulted in poor or no absorption of sample solution. To avoid this liquid detergent was inserted after every two milk samples. Fagioli et al. (1991) used plasma atomic emission spectrometry in carbonaceous slurries of samples. They obtained good results.

2.1.3. Ion Exchange Chromatography :

The method was developed by Christianson et al. (1923). In this Method total calcium was extracted from milk as EDTA complex. The extract was injected in the column. Shim pack IC-CI cation exhange column was used with 4 mM tartaric and 2 mM EDTA (pH 3.41) as mobile phase which disrupted the EDTA - Ca

Complex. Muldoon and Liska et al. (1968) analysed raw, pasteurised and sterilized skim milk by the cation exchange resin method developed by Christianson et al. (1923). Later Yagi et al (1989) carried out simultaneous quantitation of both calcium and phosphorus in the cation exchanger.

2.1.4. Flame Photometry :

Ammonium molybdate and an ammonium buffer are added to a solution of milk salt. The photometric determination is then carried out at 554 nm. Sarudi (1973) used the same method but with a new clarification agent. Milk was first deprotenized by $Zn[Hg(SCN)_4]$, the clear serum containing calcium was then treated with ammonium thiocyanate and mercuric chloride. Then flame photometric determination was carried out.

2.1.5 Potassium Permanganate Titration Method.

In this method milk is first treated with trichloroacetic acid to remove proteins and fat. Then calcium is precipitated in the form of calcium oxalate with the addition of ammonium oxalate. This calcium is then dissolved in sulphuric acid and titrated against standard $KMnO_4$ solution. Various workers precipitated the calcium from milk serum as calcium oxalate under various conditions.

Ling et al. (1936) removed proteins and fat from milk with 10% TCA. The filtrate was neutralised with ammonia and reacidified with acetic acid. Ammonium Chloride was added and the calcium precipitated from hot solution with solid ammonium oxalate. After allowing it to stand overnight the precipitate was filtered, washed

with hot water and finally titrated with N/20 $KMnO_4$ after dissolving the precipitate in hot sulphuric acid.

Verma and Sommer (1957) carried out analysis and undertook to study the amounts of calcium and phosphorus in samples of commercial milks. Distribution of constituents into soluble and insoluble frations was also studied. The same study was conducted by Ananta Krishan (1941) in Ass's Milk. Van Slyke and Bos Worth (1916) studied $Ca_3(PO_4)_2$ in milk using the Pasteur Chamberlain filter. Kay (1952) precipitated the proteins and fat by means of colloidal ferric hydroxide. Lanstrip (1936) used picric acid precipitation whereas Sanders (1937) used TCA. Ling and Anantakrishnan (1940) and Verma and Anantakrishnan (1946) used rennet coagulation.

Davies and White (1962) concluded that calcium can be determined primarily on strict adherance to prescribed conditions for precipitation of calcium. Calcium oxalate precipitates at p^H of about 4 and the ratio of $C_2O_4^{--}$ to Ca^{++} in the precipitate should be 1:1 according to Kolthoff and Sandell (1946), that this ratio should be 1:1 is important as the permanganate titration measures the oxalic acid derived from the calcium oxalate. Kramer - Tisdall (1950) modified the method and calcium oxalate precipitated from neutral solution but this resulted in low values for calcium to be derived from the permanganate titration.

Separation of calcium from phosphate ions by cation exchange resins and determination of calcium in the eluent were attempted in milk by the method of

Malkki et al. (1953). The application of ion exchange was however time consuming especially in milk samples where preremoval of proteins is necessary to avoid precipitation in the exchange column.

It was stated by Fresenius (1868) that precipitaion of calcium oxalate was always contaminated by magnesium oxalate. It was therefore, necessary to dissolve the precipitate in hydrochloric acid reprecipitate calcium oxalate with ammonia. But Richards (1931) showed that under certain conditions a single precipitation of calcium was sufficient. Fisher (1928) showed that magnesium oxalate was not precipitated from supersaturated solutions, if a slight excess of ammonium oxalate was used.

2.1.6. Miscellaneous Methods :

Khramov et al. (1983) described the procedure for the application of the Bio - test - Calcium Kit involving formation of red orange colour on reaction of calcium with 2- hydroxyaniline. The method was used for milk diluted with water in 1:10 ratio.

Keogh and Kennedy (1983) measured calcium content using a Radiometer F2112 electrode in different types of milk.

Joe et al. (1968) described a method using Technicon auto-analyser. Samples analysed for calcium determination included whole milk, skim milk and chocolate milk.

Mutzelburg et al. (1980) described a fast method of estimation of calcium, in which the Pierce calcium rapid stat Kit was used. They compared results

of this method with the oxalate precipitation method.The mean values were significantly higher from the oxalate method.

In Russia GajduseK (1975) gave a formal titration method. The content of calcium in milk was calculated according to a formula 59.85 + 17.44 x where x is the difference between milk acidity and acidity after adding oxalate. In comparison to chelometric analysis the maximum error was ± 10%. It was thus concluded that the method could usefully be applied for determining approximate calcium levels in milk for cheese making only.

2.2. Methods of Phosphorus Determination :

2.2.1. Fiske and Subbarow Method :

Phosphorus is estimated till today by the method of Fiske and Subbarow (1925). The method is based upon the colour reaction of phosphorus with ammonium molybdate and measurment of the blue colour Spectrophotometrically at 660 nm or using a red filter in colorimeter. In a modification of this Pena (1931) also utilized ammonium molybdate as a colour developer, interference were precipitated with trichloro acetic acid. Caimi (1959) adapted the Fiske and Subbarow procedure to milk by warming the sample after colour development and before developing colour intensity.

International Dairy Federation (1967) described the Fiske and Subbarow method for phosphorus determination in milk. 10 gm milk ashed, dissolved in hydrochloric acid suitably diluted and treated with perchloric acid. Ammonium molybdate was used to

develop colour. The optical density of the coloured solution was measured using red filter. Bedessem et al. (1970) used the same method but carried out dry ashing of the sample. Pien (1969) discussed that dry ashing was better than wet mineralisation.BIS (1981) described a similar method in which to the ashed sample solution HNO_3 and $NaNO_3$ are added and percipitates filtered. The precipitate was dissolved in standard alkali and titrated with standard acid solution.

2.2.2. Spectrophotometric Method :

The principle of the spectrophotometric method is almost similar to that of the Fiske and Subbarow method except the use of different chemicals and determination of optical density at wavelength other than 660 nm. Jager (1970) carried out photometric determination of phosphorus. Samples were digested with the help of perchloric acid. Ammonium molybdate and ammonium vanadate were used to develop colour which was measured at 660 nm. Linden et al. (1971) slightly modified the method of phosphorus determination. Ammonium molybdate was added in excess in the reaction and the phosphomolybdic acid formed in acid medium was quantitatively extracted with methyl iso-butyl ketone and determined at 313.26 nm. This gave results which agreed well with the Fiske and Subbarow method.

The International Organisation for Standardisation (1974) prescribed the use of Sodium - molybdate - hydrazine sulphate reagent and the molybdenum blue was measured at 700 nm.

King (1977) determined phosphorus by x-ray fluorescent spectrometry but the method was considered unsuitable for moist foods and foods with high sugar content. Oparina and Ustimenko (1979) used amidol for colour development. In 1987 a new standard which supercedes the IDF standard (1967) was given wherein milk after dry ashing or wet digestion was treated with molybdate - ascorbic acid solution. The molybdenum blue was measured at 820 nm.

Takahashi and Sutoh (1990) gave a new rapid and direct method of determination of major minerals in raw milk by multichannel inductively coupled plasma emission spectrometry. In this method approximately 2 gm of milk was made upto 50 ml with water and atomized, but this resulted in poor or no absorption of sample due to contamination of atomizer with milk. Hence the ~~same~~ workers ~~(1991)~~ improved the method by passing liquid detergent in between the atomizer after every two samples.

2.2.3. Miscellaneous Methods :

Among the several methods studied Sirnik and Zagozen (1973) described a simplest method in which 2 ml milk was wet ashed with 5-6 ml H_2SO_4 (Selenium Catalyst) and 10 - 15 drops of conc. HNO_3 or 30 % H_2O_2. After cooling the contents were neutralized with standard NaOH and reacidified with HNO_3. 10 ml of a 1:1:1 mixture of vanadate solution, molybdate solution and HNO_3 were added and photometric measurment was carried out at 480 nm. Although the method was simplified but it was time consuming.

Gorbunov et al. (1983) gave a electron discharge emission spectroscopy method which measured phosphorus at p.p.m. levels.

Laskey et al. (1991) analysed milk samples for phosphorus on centrifugal analyser. This was done without ashing or digestion and was a semi automated micromethod for simultaneous analysis of calcium and phosphorus.

From the above review of literature it is evident that the available methods for the determination of calcium and phosphorus either are very time consuming or involve costlier sophisticated instruments. If each and every milk sample is to be analysed for calcuim and phosphorous, it is essential to develop a rapid platform test for their estimation. In the present study an attempt has been made to standardize a rapid method for the simultaneously determination of calcuim and phosphorous in milk. The accuracy of the results obtained is sufficient to recommend the method for routine analyses of milk samples for calcuim and phosphorous.

CHAPTER 3 –

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. MATERIALS :

3.1.1. Milk Samples :

The samples of cow milk, buffalo milk and toned milk were collected from the experimental Dairy of the National Dairy Research Institute, Karnal, for analysis.

3.1.2. Reagents :

All the reagents used in the study were of AnalaR or GR grade except, otherwise stated. For the preparation of reagents glass distilled water was used.

(a) Calcium Solution :

Weighed exactly 12.5 gm anhydrous $CaCO_3$ and taken in a 500 ml beaker. To this added about 300 ml glass distilled water and treated with dilute hydrochloric acid. The contents were neutralised and transferred to 500 ml volumetric flask and made the volume with distilled water. This solution contains calcium 10 mg/ml.

(b) Phosphorus Solution :

Weighed exactly 28.7324gm of disodium hydrogen phosphate ($Na_2HPO_4 . 2H_2O$) in a 500ml beaker. To this added about 300ml glass distilled water and neutralised with N/10 NaOH. The contents were transferred to 500 ml volumetric flask and made the volume upto the mark with distilled water. This solution contained phosphorus 10 mg/ml .

(c) Saturated Potassium Oxalate Solution :

The saturated solution of potassium oxalate ($K_2C_2O_4$) was prepared and neutralised with N/10 NaOH.

(d) N/10 Sodium Hydroxide

(e) N/10 Hydrochloric Acid

(f) 15 % Trichloro Acetic Acid Solution

(g) Ammonium Oxalate Solution : Saturated

(h) Ammonium Hydroxide Solution :

The concentrated ammonia solution was diluted with water in 1 : 1 ratio (v/v).

(i) Dilute Hydrochloric Acid Solution :

The concentrated hydrochloric acid was diluted with water in 1 : 9 ratio (v/v).

(j) N/10 Potassium Permanganate Solution :

Prepared stock solution by dissolving 33gm of Potassium permangnate in a litre of water by heating to 70 - 80° C. 110 ml of the stock solution was transferred in one litre graduated flask and made upto the mark with water. The normality of the solution was checked against N/10 oxalic acid.

(k) Dilute Sulphuric Acid :

Concentrated Sulphuric acid was diluted with water in 1 : 4 ratio (v/v).

(l) 5N Sulphuric Acid

(m) Perchloric Acid 60 %

(n) Ammonium Molybdate Solution :

Dissolved 2.5 gm of ammonium molybdate in glass distilled water and volume made upto 100 ml.

(o) 1-Amino-2-Naphthol-4-Sulphonic Acid (ANSA):

29.25gm of anhydrous $NaHSO_3$: 0.5gm of ANSA and 1gm of anhydrous sodium sulphite (Na_2SO_3) was grinded and kept in a cool place. At the time of the experiment 2.5gm of reagent was dissolved in 100ml glass distilled water and used fresh.

(p) <u>10 % Trichloro Acetic Acid Solution (TCA).</u>

3.2. METHODS OF ANALYSIS

3.2.1 Estimation of Calcium

3.2.1.1 Reference Method :

Calcium was determined by the method given by Verma and Sommer (1957) as described below in brief.

10 ml milk was taken in a clean dry beaker and 30ml of 15% trichloro acetic acid solution was added. The contents were stirred and kept for 5 min. Filtered the contents using Whatman No. 42 filter paper. 20 ml of the filtrate was collected and 10 ml saturated ammonium oxalate was added. It was made alkaline using ammonia liquor and few drops of concentrated acetic acid added to neutralise excess ammonia. The contents were kept undisturbed for 3 hrs. and then filtered through Whatman no. 42 filter paper, Washed the precipitate with hot water six times. Discarded all the washings. Checked the washings with calcium solution for absence of ammonium oxalate , if white precipitate appeared then washed again with glass distilled water. Dissolved the precipitate in dilute sulphuric acid and titrated in hot (80 ^{o}C) with N/10 KMnO to a permanent slight pink colour as end point.

Calculations :

Total calcium in mg/100 ml milk was given by V x 40 where V was the volume in ml of N/10 $KMnO_4$ used.

3.2.1.2. **New Rapid Method :**

To 10 ml milk taken in a 100 ml beaker or a china dish, added 1 ml of phosphorus solution and titrated with N/10 NaOH to phenolphthalein end point. To this 0.5 ml of neutralised potassium oxalate solution was added and kept for two minutes. The developed alkalinity was back titrated with N/10 HCl to the same end point. Calcium in milk was calculated by multiplying the volume of N/10 HCl used with a factor 80 and expressed as mg/100 ml milk.

3.2.2. **Estimation of total phosphorus :**

3.2.2.1 **Reference Method :**

Fiske and Subbarow method (1925) was used with slight modifications as follows :

One ml of milk was taken in 100 ml volumetric flask and the volume was made upto the mark with distilled water and mixed well.

1 ml of this solution was pipetted out and poured in 100 ml Kjeldahl flask. 2.5 ml of 5N H_2SO_4 was added. It was slowly heated over a gas burner, to avoid bumping glass beads were also added. When the contents of the flask blackened, 3-5 drops of 60% perchloric acid was added and again heated till fumes of perchloric acid ceased. After cooling 2-3 ml of distilled water was added to the flask and again heated till all residual perchloric acid fumes escaped off.

After cooling, approximately 5 ml glass distilled water was added and shaken. The contents were transferred to 50 ml volumetric flask. Kjeldahl flask was repeatedly washed with glass distilled water and washings collected in volumetric flask. 2 ml of 2.5% solution of ammonium molybdate and 0.1 ml of ANSA reducing reagent were added and volume made upto the mark (50 ml) with glass distilled water. Samples were kept in boiling water bath for about 7 min. to get maximum colour development. A blank determination was also performed with all reagents except milk samples and then cooled. The intensity of the colour was measured at 660 nm in Spectronic-20 (Bausch and Lomb) against blank .

Calculations :

Total phosphorus in mg/100 ml milk is given by P x 10. Where P is the number of µgm of Phosphorus corresponding to the O.D. as determined from the standard curve (Fig. 1).

Preparation of Standard Curve For Total Phosphorus

28.7324 gm of disodium hydrogen phosphate ($Na_2HPO_4 . 2H_2O$ with molecular weight 177.99) was weighed accurately and transferred to 500 ml volumetric flask. It was dissolved in glass distilled water and the volume made upto the mark. 5 ml of this solution was taken in 250 ml volumetric flask and volume made upto the mark with distilled water. This was the stock solution, which contained 200 mg phosphorus/ml. For further dilution 5 ml of the stock solution was further diluted to 250 ml. This solution contained 4µg

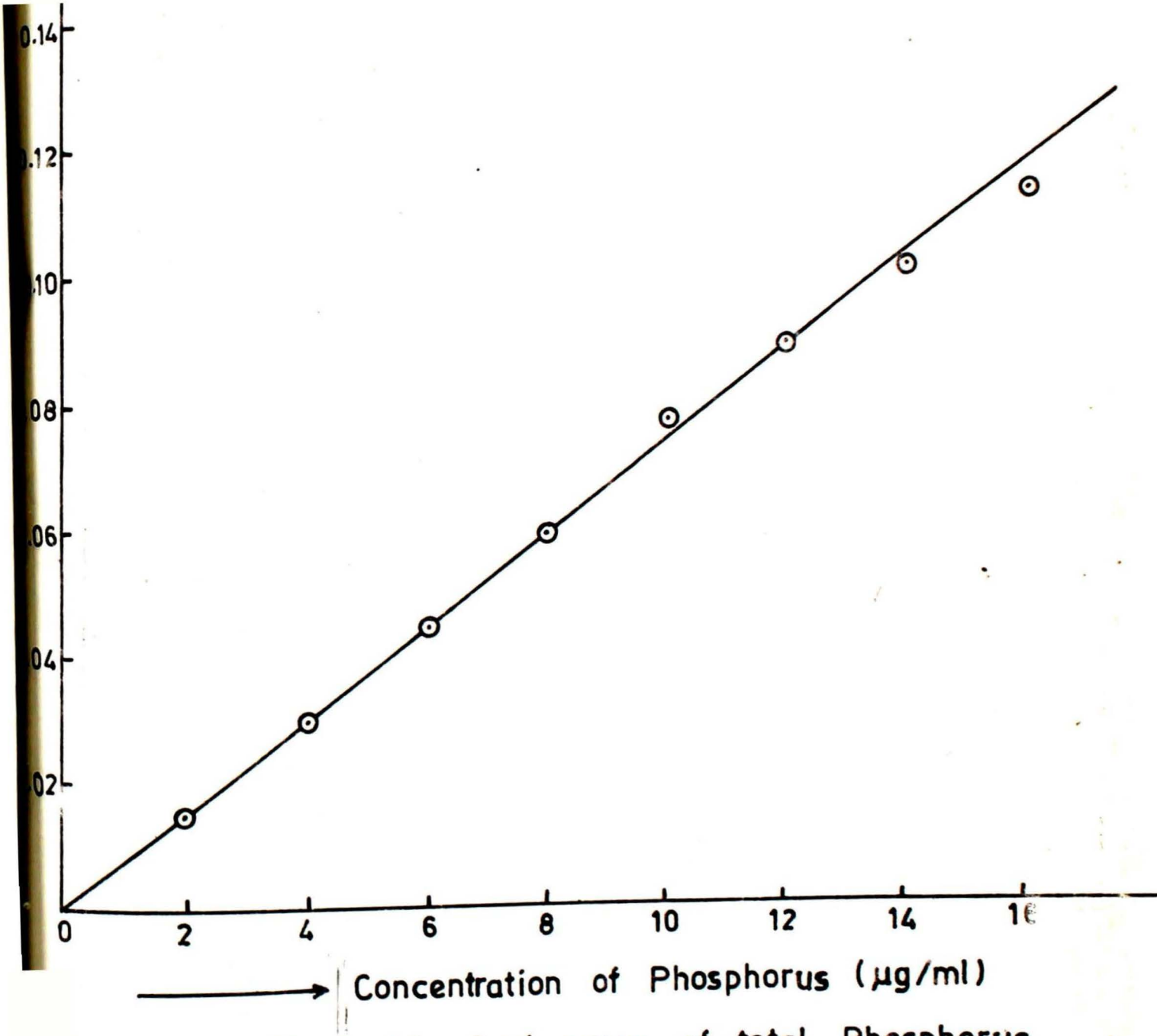

Fig. 1 : Standard curve of total Phosphorus

Phosphorus/ml. This was the working solution.

For the preparation of standard curve, 0, 0.5, 1.0, 2.0, 2.5, 3.0, 3.5 and 4.0ml of the standard solution of phosphorus was taken in different 100ml capacity Kjeldahl flask. These corresponded to 0, 2, 4, 6, 8, 10, 12, 14 and 16 µgm of phosphorus respectively. 2.5 ml of 5N H_2SO_4 was added in each flask. One to two glass beads were also added to avoid bumping and the flasks were heated over the burner till white fumes started coming out. The rest of the procedure followed was same as in 3.2. 2.1. The standard curve between the concentration of phorophorous (µg) against O.D. was drawn (fig.1)

3.2.2.2 New Rapid Method :

To 10ml milk taken in a 100ml beaker or China dish added 1ml of calcium solution and titrated with N/10 NaOH to phenolphthalein end point. To this 0.5 ml of neutralised potassium oxalate solution was added and kept for 2 min. The developed alkalinity was back titrated with N/10 HCl to the same end point. Total Phosphorus in mg/100 ml milk was calculated by multiplying the volume of N/10 HCl used with a factor of 46.5.

3.2.3. Estimation of Inorganic Phosphorus

3.2.3.1 Reference Method :

Fiske and Subbarow (1925) was used with a minor difference.

0.5 ml of milk was taken in a clean dry 100ml beaker. 19.5 ml of 10% TCA solution was added and mixed. The precipitate was filtered and 1ml of the filtrate was taken in duplicate in 50ml volumetric

flask. 2ml of ANSA reducing reagent were added respectively and contents mixed thoroughly. The volume was made upto the mark with glass distilled water and mixed. A blank determination was also done in the same manner using 1ml of 10% TCA instead of the milk filtrate. After 30 ± 2 min the reading of O.D. was taken against blank at 660 nm in spectronic 20. The ugm of inorganic phosphorous was calculated from the standard curve (fig.2)

Calculations :

Inorganic phosphorus in mg/100ml milk is calculated by P x 4 where P is the µgm of phosphorus as noted from the standard curve (fig.2).

Preparation of Standard Curve for Inorganic Phosphorus :

For this purpose 0, 1, 2, 3, 4, 5 and 6 ml of the working solution of phosphorus was taken in different volumetric flask each of capacity 50ml. 1ml of 10% TCA solution was added in each flask. 2.0 ml of 2.5 % ammonium molybdate solution and 0.1ml of ANSA reducing reagent was made upto the mark with glass distilled water, mixed the contents by inversion and kept undisturbed at room temperature for 30 ± 2 min.

3.2.3.2. New Rapid Method :

10ml milk taken in a 100ml beaker or China dish was neutralized with N/10 NaOH to phenolphthalein end point. 0.5ml of neutralized potasium oxalate added and developed alkalinity was back titrated with N/10 HCl to the same end point. The inorganic phosphorus was calculated by multiplying the

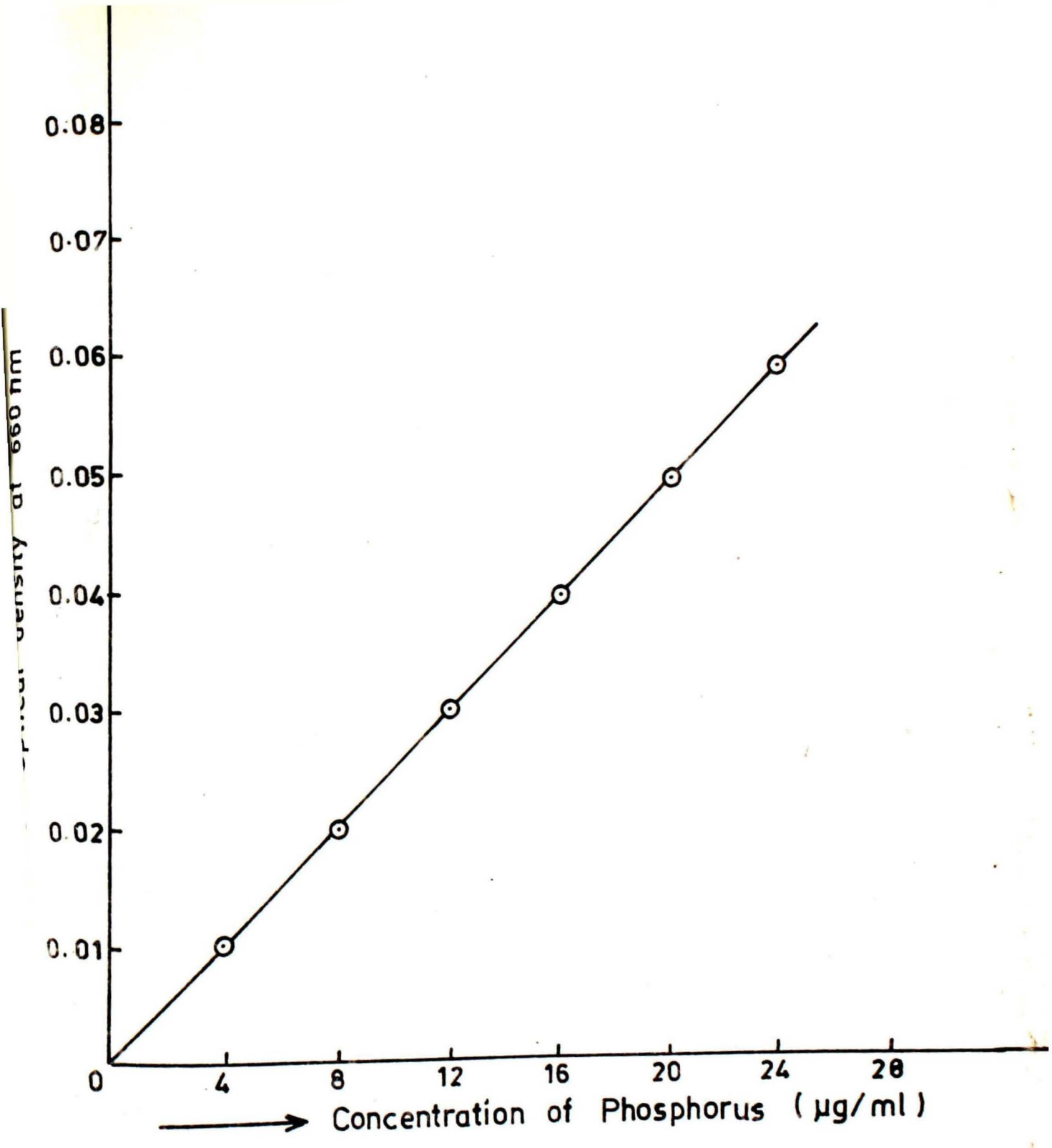

Fig. 2 : Standard curve of Inorganic Phosphorus

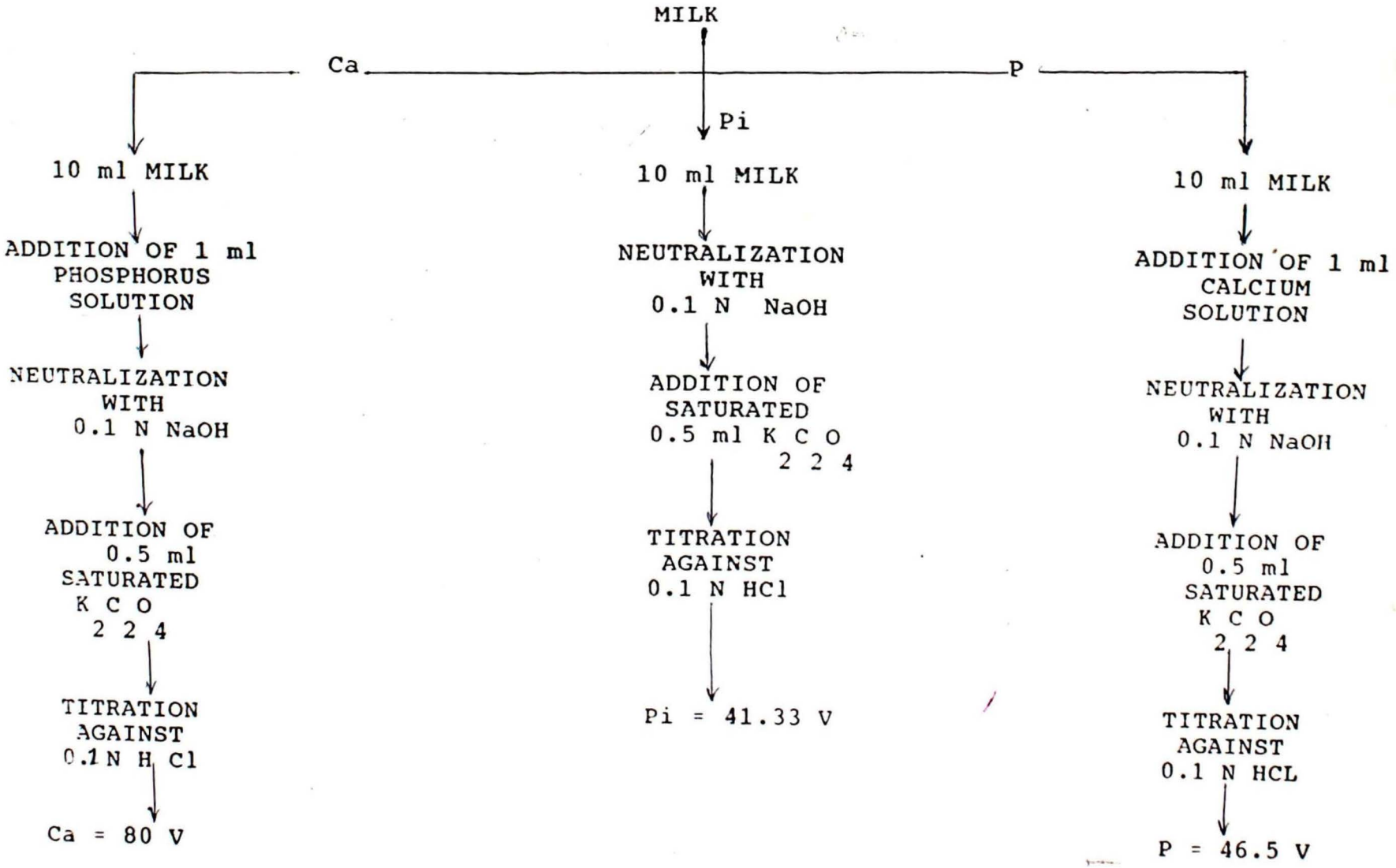

Fig. 3 : Flow diagram of procedure for Calcium and Phosphorus determination

volume of N/10 HCl used with 41.33 and expressed as mg of inorganic phosphorus per 100ml milk.

The new rapid method for the determination of calcium, total phosphorus and inorganic phosphorus in milk are not three different methods. They are only one with a minor difference as explained in the flow diagram (fig.3) and can be followed simulataneously.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 OXALATE EFFECT :

The titratable acidity of fresh milk is due to milk constituents such as milk proteins, calcium, phosphate, dissolved carbon dioxide etc. During titration of milk with N/10 NaOH solution the normal p_H of milk is raised to 8.3, the phenolphathalein end point. At the neutral end point the soluble Ca^{++} and HPO_4^{--} precipitate as depicted in the following reaction:

$$3Ca^{++} + 2HPO_4^{--} \rightleftharpoons Ca_3(PO_4)_2 + 2H^{+} \quad ----(i)$$

If at this stage neutralized potassium oxalate is added to the above neutral contents, the whole material turns alkaline :

$$Ca_3(PO_4)_2 + 3K_2C_2O_4 \longrightarrow 3CaC_2O_4 + 2K_3PO_4 \quad ----*(ii)$$

(neutral) (in excess) (PPt) (alkaline)

The produced alkalinity is due to formation of K_3PO_4. If $K_2C_2O_4$ is added to test portion of milk before titration, the decrease in acidity in reaction (i) was found exactly equal to alkalinity produced in reaction (ii) The alkalinity was determined by titrating the content against N/10 Hydrochloric acid.

In an experiment milk proteins were acid precipitated and the filtrate was neutralized. The same alkalinity was produced in the neutral filtrate when potassium oxalate was added. Similarly in another experiment 10 ml milk was ashed and ash was dispersed in 10 ml water and neutralized . On addition of potassium oxalate same alkalinity was produced as in milk sample. This indicated that the decrease in acidity in milk or

alkalinity produced in neutral milk on addition of potassium oxalate was due to reaction of calcium phosphate with potassium oxalate. To prove this model reactions were carried out using neutral $CaCl_2$ and Na_2HPO_4 solutions.

$$3\,CaCl_2 + 3\,Na_2HPO_4 \longrightarrow Ca_3(PO_4)_2 + 6\,NaCl + H_3PO_4$$

(neu.) (neu.) (neu.) (neu.) (acidic)

The product of the reaction became acidic which was neutralized with N/10 NaOH.

$$H_3PO_4 + 2\,NaOH \longrightarrow Na_2HPO_4 + 2H_2O$$

(neutral)

On addition of neutral potassium oxalate to the neutral content of the above reaction material, the alkalinity was produced .

$$Ca_3(PO_4)_2 + 3K_2C_2O_4 \longrightarrow 3CaC_2O_4 + 2K_3PO_4$$

(alkaline)

The alkalinity as determined by titrating the content with N/10 HCl was found to be propotional to the $Ca_3(PO_4)_2$ content in the reaction mixture.

4.1.1 Factors affecting the oxalate effect

4.1.1.1 Concentration of potassium oxalate :

Neutralized saturated solution of potassium oxalate was added in the neutral reaction mixtures at the rate of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1.0 ml per 10 ml milk . The alkalinity went on increasing upto 0.4 ml level and beyond that it remained constant. It indicated that potassium oxalate present in 0.4 ml solution was sufficient to precipitate whole

of the calcium in the milk samples. However in all the determinations 0.5 ml potassium oxalate was used to keep the oxalate content in excess.

4.1.1.2 Concentration of Calcium and Phosphorus :

The reaction between Ca^{++} and HPO_4^{--} is reversible. Therefore, either calcium or phosphorus is not completely precipitated as $Ca_3(PO_4)_2$. Hence it was essential to add phosphate in excess to the test portion of milk to react whole of the calcium while calcium was to be determined. Similarly calcium ions were added in excess to react the phosphate completely when phosphorus was to be estimated.

Model reactions were conducted between calcium and phosphorus solutions. To 1 ml calcium solution (10 mg / ml) taken in beakers,0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml phosphorus solution (10 mg/ml) were added. After neutralization of the contents 0.5 ml potassium oxalate was added in each beaker. The alkalinity developed was found to increase with the increase of volume of phosphorus solution upto 1.0 ml and after that it remained constant as shown in fig. 1.A. The excess additon of phosphorus beyond 1.0 ml caused the reaction to become very slow and the end point not achieved rapidly and clearly. Under such conditions especially while analysing milk samples less quantity of phosphorus solution (even 0.5ml) was sufficient to react whole calcium content.

In another experiment to 1 ml phosphorus solution (10mg/ml, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 ml

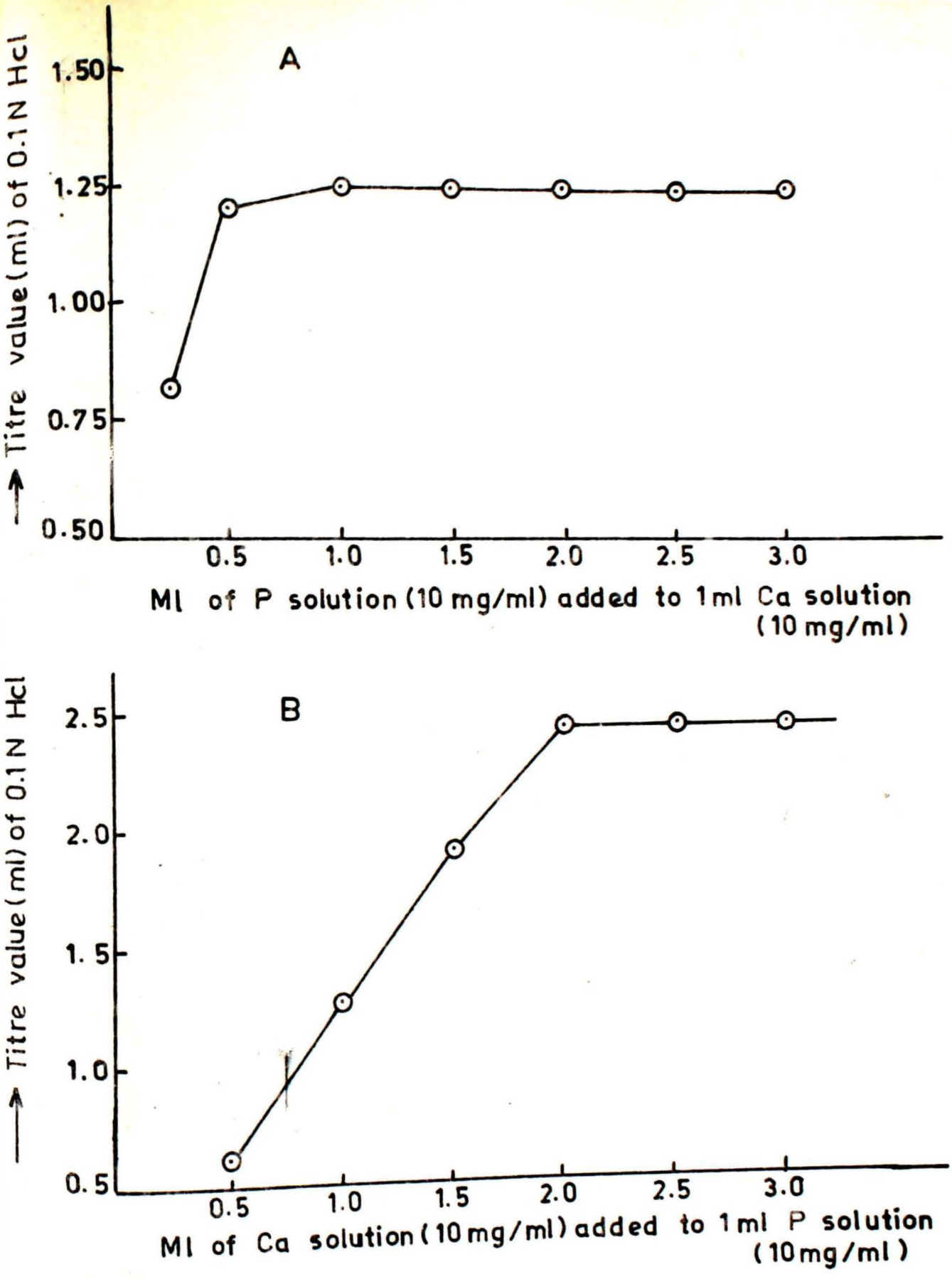

Fig. 4: Effect of various concentrations of Ca and P on the titre value.

calcium solution (10 mg/ml) were added and neutralised. The effect on the alkalinity produced on addition of potassium oxalate, were shown in fig. 1.B. The alkalinity increased with the increase of calcium solution added upto 2.0 ml and beyond that it remained constant.

From the above discussion it is evident that for complete reaction of calcium almost equal amount of phosphorus should be present in the reaction mixture whereas for complete reaction of phosphorus double the amount of calcium is essential. Therefore, for the determination of calcium or phosphorus in milk in the present study, the above approximate ratios have been maintained. In case of calcium determination 1 ml phosphorus solution (10 mg/ml) was added to 10 ml milk portion whereas, 1 ml calcium solution (10 mg/ml) was added to 10 ml milk while phosphorus was to be determined. Although milk also contains calcium and phophorus but those may not be in the required ratio to keep the contents of one ingredient in excess while determining the other.

4.2 STANDARDIZATION OF RAPID METHODS :

From the above discussion it has been established that the decrease in titratable acidity of milk on addition of potassium oxalate or alkalinity produced in the neutral milk content on addition of potassium oxalate are correlated with calcium and phosphorus contents. In order to find out the correlation factors the following experiments were carried out.

4.2.1 Determination of calcium correlation factor :

Four solutions of $CaCl_2$ were prepared to contain calcium 50,100,150 and 200 mg/ 100 ml. 10 ml of each solution was taken in 4 beakers separately and 0.5, 1.0 1.5 and 2.0 ml of phosphorus solution (10 mg/ml) were added to each beaker respectively, so that each beaker contained calcium and phosphorus in 1:1 ratio. However, maximum quantity (2 ml) of phosphorus could be added in each but reaction rate lowered. The contents of each beaker were neutralized with 0.1 N NaOH and then 0.5 ml of potassium oxalate solution was added to each beaker. The alkalinity of the contents was determined by titration against 0.1 N HCl and expressed as number of ml of 0.1 N HCl and termed as "titre value". The correlation factor was calculated by dividing the mg of calcium / 100 ml of the solution by the titre value. The results are presented in table 1.

The correlation factor for calcium varied slightly from 80.6 to 79.7 corresponding to the concentration of calcium from 50 to 200 mg/100 ml. The average value of the factor was calculated to be 80. Therefore, for the determination of calcium in milk, the titre value of 10 ml milk, sample should be multiplied by the factor `80' and the resultant value will be mg of calcium per 100 ml of milk.

4.2.2 Determination of inorganic phosphorus correlation factor :

Four different solutions of Na_2HPO_4 were prepared to contain phosphorus 50,100, 150 and 200 mg/100 ml. 10 ml from each solution were taken in

TABLE 1

Determination of facttor of titre value for calcium estimation using model solution of $CaCl_2$ and Na_2HPO_4.

Calcuim Content (mg/100 ml) (a)	Titre Value (ml of N/10 HCl) (b)	Factor (a/b)
50	0.62	80.6
100	1.25	80.0
150	1.88	79.8
200	2.51	79.7
	Average Factor =	80.0

* Mean value of 4 replicates.

different beakers and 4 ml calcium solution (10 mg/ml) were added in each beaker. The contents of each beaker were neutralized and 0.5 ml potassium oxalate was added in each beaker. The developed alkalinity (titre value) was determined and correlation factor for phosphorus was calculated in the same way as in 4.2.1. The results have been given in table 2.

The value of the correlation factor for inorganic phosphorus varied slightly from 41.66 to 41.15 as the concentration of phosphorus varied from 50 to 200. The mean value of the factor was calculated to be 41.33. Hence by multiplying the titre value of 10 ml sample by the factor 41.33, the inorganic phosphorus content in mg/ 100 ml of sample can be calculated.

4.2.3 Determination of Total Phosphorus Correlation factor :

In 4.2.2 the inorganic phosphorus has been used in the model reactions, whereas, in milk, total phosphorus includes both inorganic and organic phosphorus. Most of the organic phosphorus is associated with milk proteins and little in phospholipids. To determine the total phosphorus correlation factor, the total phosphorus was estimated in various milk samples of cow, buffalo and toned milk, by Fiske and Subbarow method and the titre value by the new method. In the new method, to 10 ml milk 1 ml calcium solution (10 mg / ml) was added to keep the calcium content in excess. After neutralization of the contents 0.5 ml of potassium oxalate was added and the developed alkalinity (titre value) was determined by

TABLE - 2 : Determination of factor of titre value for inorganic phosphorus estimation using model solution of $CaCl_2$ and Na_2HPO_4

Phosphorus Contents (mg/100 ml) (a)	Titre Value* (ml of N/10 HCl) (b)	Factor (a/b)
50	1.20	41.66
100	2.42	41.32
150	3.64	41.21
200	4.86	41.15
	Average Factor =	41.33

* Mean value of 4 replicates.

titrating against 0.1 N HCl. The correlation factor was calculated by dividing the total phosphorus (mg/100 ml) with titre value.

The results presented in table 3 revealed that the mean values of the correlation factor in all the three types of milk samples were almost same (46.5). Therefore, for the determination of total phosphorus in milk, to 10 ml test portion of milk 1 ml calcium solution (10 mg/ml) was added to keep the concentration of calcium in excess and then neutralized. After addition of 0.5 ml potassium oxalate, the titre value (ml of 0.1 N HCl required to neutralize developed alkanlinity) obtained was multiplied by 46.5 to get the total phosphorus in milk.

4.3 CHEMISTRY OF THE NEW RAPID METHOD :

In milk about one third of the calcium exists in soluble form and the rest of total is associated with globular milk proteins, attached by electrovalent bond. Whereas,a large part of the total phosphorus of milk exists in inorganic form and the rest in organic form. Most of the organic phosphorus is covalently bound to proteins and traces to phospholipids .

4.3.1 Derivation of Calcium Factor :

Calcium and inorganic phosphorus of milk during neutralization of the milk,react to form $Ca_3(PO_4)_2$. When inorganic phosphorus was added to milk in excess, then the calcium electrovalently bound to proteins, may also be depleted and converted to $Ca_3(PO_4)_2$ at the neutral point of phenolphthalein (p^H 8.3). At this

TABLE - 3 : Determination of factor of titre value for the estimation of total phosphorus in milk.

Milk Samples	Total Phosphorus by Fiske and Subbarow method (mg/100 ml milk) (a)	Titre Value by new method (ml of 0.IN HCl) (b)	Factor (a/b)
Buffalo milk	93 - 130 (112)	2.04 - 2.80 (2.408)	46.42 - 46.57 (46.51)
Cow milk	78 - 112 (100)	1.66 - 2.42 (2.151)	46.28 - 46.98 (46.49)
Toned milk	85 - 121 (104)	1.82 - 2.61 (2.236)	46.36 - 46.70 (46.51)
		Average factor =	46.50

Values in parentheses are the mean values of 10 samples in each case.

stage when neutralized potassium oxalate was added, the milk contents became alkaline as depicted by the following reaction :

$$Ca_3(PO_4)_2 + 3K_2C_2O_4 \longrightarrow 3CaC_2O_4 + 2K_3PO_4$$

(neutral) (neutral) (neutral) (alkaline)

or

$$2Ca_3(PO_4)_2 + 6K_2C_2O_4 \longrightarrow 6CaC_2O_4 + 4K_3PO_4$$

It means, per 2 mole of $Ca_3(PO_4)_2$ present at the neutral point, after addition of potassium oxalate, 3 mole of a monobasic acid were required to neutralize tripotassium phosphate to lower the pH to the same phenolphthalein end point, as shown in the following reaction :

$$4K_3PO_4 + 3HCl \longrightarrow 3KCl + 3K_2HPO_4 + K_3PO_4 \quad (pH\ 8.3)$$

Out of 4 moles of K_3PO_4, only 3 are neutralized upto pH 8.3 , the neutral end point of phenolphthalein. To prove it, a buffer mixture consisting of KCl, K_2HPO_4 and K_3PO_4 in 3:3:1 molar ratio was prepared. 0.8% solution, corresponding to nearly 100mg of phosphorus per 100 ml, was prepared. The pH of this solution was found to be exactly 8.3.

Therefore, on addition of potassium oxalate, per 2 mole of $Ca_3(PO_4)_2$, 3 mole of HCl were required to neutralize the developed alkalinity .

Hence ;

$2\ Ca_3(PO_4)_2 = 3\ HCl$

or , $6\ Ca = 4P = 3\ HCl$

therefore, 3 mole or equivalent of HCl = 6 Ca

1 " " " " " = 2 Ca

= 2 x 40

= 80 g of cal.

Let V ml milk was taken for the titration and v ml of N normal HCl was used to neutralize the developed alkalinity , then

1000 ml of I N HCl = 80 g of calcium

V ml of N normal HCl = (80Nv/1000) g of calcium

Therefore V ml of milk contained calcium = 80Nv/1000 g

and 100 ml " " " = (80Nv/1000) x (100/V) g

= (8 Nv/V) x 1000 mg

= 8000 Nv/V mg

if 0.1 was the normality of the HCl and 10 ml milk was used for test, then

100 ml milk contained calcium = 8000 x 0.1xv/10 mg

= 80v mg.

The formula deduced well resembled with the formula calculated from the experimental data (table.1)

4.3.2 Derivation of Inorganic Phosphorus Factor :

Refer again 4.3.1, that

6 Ca = 4 P = 3 HCl

therefore 3 mole or equivalent of HCl = 4P

1 " " " = 4/3 P

$$= \frac{4 \times 31}{3}$$

= 41.33 g phosphorus.

Similarly proceeding the derivation as in case of calcium (4.3.1) the inorganic phosphorus content per 100 ml milk = 41.33 Nv/V mg. If N/10 HCl was used for 10 ml test portion of milk then inorganic phosphorus = 41.33 v mg / 100 ml milk.

The derived formula was exactly the same as calculated from the experimental data (Table 2) involving the reaction between inorganic phosphorus and calcium.

4.3.3 Derivation of Total Phosphorus Factor :

At the normal pH of milk (6.6 to 6.8), the calcium and phosphorus exist in milk in soluble as well as in colloidal forms. About two third of the calcium in milk is located in the micelle, complexed with HPO_4^{--} and $H_2PO_4^-$ (Rose and Colvin, 1966) . The possible compounds of calcium and phosphorus may be $CaHPO_4$ and $Ca(H_2PO_4)_2$. Both are sparingly soluble in water. When excess of Ca^{2+} is added to milk, $Ca(H_2PO_4)_2$ may also be converted to $CaHPO_4$ as follows :

$$Ca(H_2PO_4)_2 + Ca^{2+} \longrightarrow 2\ CaHPO_4 + 2H^+$$

The liberated H^+ causes the increase in titratable acidity of milk. This really occured in milk when neutralized $CaCl_2$ solution was added to the test portion of milk, the titratable acidity increased tremendously.

$CaHPO_4$ is acidic in nature which gets neutralized during the titration for acidity determination.

$$3CaHPO_4 + 2NaOH \longrightarrow Ca_3(PO_4)_2 + Na_2HPO_4 + 2H_2O$$

(acidic) (netural) (neutral)

The titrable acidity of milk was also found to decrease when neutral potassium oxalate was added :

$$3CaHPO_4 + 3K_2C_2O_4 \longrightarrow 3CaC_2O_4 + 3K_2HPO_4$$

(acidic) (netural) (neutral) (neutral)

It means 3 moles of potassium oxalate were equivalent to 2 moles of NaOH for neutralization of 3 mole of $CaHPO_4$. In other words, when $K_2C_2O_4$ was added after neutralization of milk then 2 moles of HCl were required to neutralize the developed alkalinity.

therfore, $3CaHPO_4 = 2NaOH = 2HCl$

or, $3P = 2HCl$

i.e. 2 mole or equivalent of HCl = 3P

1 " " " " $= \frac{3 \times 31 \text{ gm of phorphorus}}{2}$

= 46.5 g of phosphorus

If v ml of N normal HCl were used for titration of V ml milk, then

100 ml milk contains total phosphorus =

$$= \frac{46.5 \times Nv}{1000\ V} \times 100 \text{ g}$$

$$= \frac{4650\ Nv}{V} \text{ mg}$$

Therefore, when 10ml milk was used for titration and 0.1 is the normality of HCl, then 100 ml milk contains total phosphorous

$$= \frac{4650 \times 0.1 \times v}{10} \text{ mg}$$

$$= 46.5\ v \text{ mg}.$$

The deduced formula was verified experimentally. Samples of cow milk, buffalo milk and toned milk were analysed by Fiske and Subbarow method for total phosphorus determination. The titre value of the same samples (after addition of Ca^{2+} in excess) was also determined. The correlation factor between the total phosphorus and titre value was exactly the same 46.5 (Table 3).

4.4 EVALUATION OF NEW METHODS :

To check the accuracy ofthe new method for the determination of calcium, total phosphorus and inorganic phosphorus in milk, the results of the new method were compared with those of standard reference methods.

4.4.1 Method for Calcium Determination :

Samples of cow milk, buffalo milk and toned milk were analysed for calcium content by the new rapid method as well as $KMnO_4$ titration method. The results are presented in table 4.

The average calcium contents determined by new method in cow, buffalo and toned milk samples were 113.3, 168.9 and 121.9 mg/100 ml milk,respectively which were very similar to those given by $KMnO_4$ titration methods. However the difference in the result of the two methods in individual samples varied from - 6 to + 6, - 12 to + 10 and -10 to + 8 mg/100 ml of cow, buffalo and

TABLE - 4 : Calcium (mg/ 100 ml) determined in milk.

Milk samples*	$KMnO_4$ Method (a)	New Rapid method (b)	Difference (a-b)	Standard error
Cow Milk	104 - 132 (114.5)	100 - 128 (113.3)	-6 to + 6 (1.2)	1.2 ± 3.6
Buffalo Milk	140 - 198 (168.3)	140 - 188 (168.9)	-12 to + 10 (-0.6)	-0.6 ± 6.9
Toned Milk	114 - 132 (121.4)	108 - 135 (121.9)	-10 to+ 8 (- 0.5)	-0.5 ± 5.7

* Samples analysed in each case = 15

Figures given in parentheses are the mean values .

toned milk respectively. The higher difference of results in some milk samples, may be due to a little error in obtaining the correct phenolphthalein end point in the titration. The $KMnO_4$ titration method may also not be free from errors as Sendroy (1944) discussed the conditions in which erroneously high values of calcium were obtained. However, a little practice in visualization of correct end point led to get accurate results in the new method.

4.4.2 Method for total phosphorus determination:

To know the accuracy of the new method, cow, buffalo and toned milk samples were analysed for total phosphorus by Fiske and Subbarow method and new method. The results presented in table 5 reveal that the values of total phosphorus on an average were comparable with those of reference method in cow and buffalo milk samples. Whereas, in toned milk the new method gave a little higher result. Higher difference between the results of two methods in case of individual samples (- 13.7 to 3.6 mg per 100 ml) may not be due to the new method only, but due to reference method also, because Fiske and Subbarow method for total phosphorus is multistep, involving digestion of milk, addition of several chemicals and measurement of colour development spectrophotometrically. Many workers talked about the difficulties. Pena (1931) observed some interferences, and modified the method.

To avoid wet acid digestion, the test portion of milk sample was ashed and the ash was used for total phosphorus determination by the reference method as well

TABLE - 5 : Total Phosphorus (mg/100 ml) determined in milk

Milk samples*	Fiske and Subbarow method (a)	New Rapid method (b)	Difference (a-b)	Standard error
Cow Milk	88.8 - 107.7 (95.7)	86.1- 107.0 (94.3)	-2.6 to 4.7 (1.4)	1.4 ± 2.1
Buffalo Milk	101.0 - 139.3 (120.2)	101.4 - 141.8 (121.3)	-6.9 to 4.3 (-1.1)	-1.1 ± 3.3
Toned Milk	90.2 - 112.0 (98.7)	95.0 - 112.2 (101.1)	-13.7 to 3.6 (-2.4)	-2.4 ± 4.3

* Samples analysed in each case = 15

Figures given in parentheses are the mean values .

as new method. While determining total phosphorus in the ash by new method the factor (41.33) meant for inorganic phosphorus was applied, because whole of the phosphorus in the ash presented in inorganic form. The results are presented in table. 6.

Though the average values of the total phosphorus given bythe two methods were almost same in all the 3 types of milk, yet the difference in results given by two methods in case of individual samples was higher. A little error may be in the new method due to absence of sharp end point, but major difference may be associated with the Fiske and Subbarow method whose values of replicates were not very close. More care and expertise was needed for following the Fiske and Subbarow procedure.

4.4.3 Method for Inorganic Phosphorus determination :

For the determination of inorganic phorphorus in milk by the new method,extra calcium ions were not added before the titration. The native calcium and inorganic phosphorus of milk take part in the reaction during the titrations :

$$3Ca^{2+} + 2HPO_4^{--} \longrightarrow Ca_3(PO_4)_2 + 2H^+$$

The inorganic phosphorus by Fiske and Subbarow method and new method was determined in cow, buffalo and toned milk samples and results given in table 7 reveal that the new method gave on an average 54.6 , 73.7 and 56.1 mg inorganic phosphorus of cow, buffalow and tored milk respectively, where as Fiske and

TABLE - 6 : Total phosphorus (mg/100 ml) determined in milk ash

Milk samples*	Fiske and Subbarow method (a)	New Rapid method (b)	Difference (a-b)	Standard error
Cow Milk	85.6 - 102.9 (96.0)	85.5- 106.4 (95.3)	-8.5 to 7.4 (0.7)	0.7 ± 1.8
Buffalo Milk	100.4 - 135.6 (118.2)	100.8 - 135.0 (118.3)	-14.5 to 6 (-0.2)	-0.2 ± 5.9
Toned Milk	88.9 - 110.6 (99.5)	90.5 - 110.0 (98.3)	- 5.5 to 6.7 (1.2)	1.2 ± 2.8

* Samples analysed in each case = 15

Figures given in parentheses are the mean values .

TABLE - 7 : Total Phosphorus (mg/100 ml) determined in milk

Milk samples*	Fiske and Subbarow method (a)	New Rapid method (b)	Difference (a-b)	Standard error
Cow Milk	52.4 - 64.8 (59.8)	50.5- 62.1 (54.6)	1.9 to 8.3 (5.2)	5.2 ± 1.9
Buffalo Milk	64 .1 - 82.3 (73.9)	62.8 - 82.7 (73.7)	- 2.7 to 4.1 (0.2)	0.2 ± 1.5
Toned Milk	55.8 - 64.2 (59.4)	51.7 - 59.9 (56.1)	- 1.4 to 8.4 (3.3)	3.3 ± 2.0

* Samples analysed in each case = 15

Figures given in parentheses are the mean values .

Subbarow method gave 59.8 , 73.9 & 59.4 mg/100 ml in the same milk samples respectively. The results of both the methods were the same in case of buffalo milk, whereas , the new method underestimated the inorganic phosphorus in cow milk and toned milk. This may be due to the reason that buffalo milk contains sufficient calcium (about 180 mg/100ml milk) to react with whole of the inorganic phorsphorus. Whereas, in cow milk and toned milk the calcium contents were less(114 and 121 mg/100 ml) respectively. The ratio of total calcuim & inorganic phosphorus in buffalo milk, cow milk and toned milk were calculated to be 2.28, 1.91 and 2.04 respectiely. Therefore, due to low concentration of calcium in cow and toned milk,whole of the inorganic phosphorus was not utilized in the reaction during titration. The addtion of extra calcium ions to milk is not advisable because the added calcium not only reacts with the inorganic phosphorus but affects the organic phsophorus also. This is a little limitation of the method.

4.4.4 Recovery of added calcuim and phosphorus :

In order to conduct recovery trials, to each 100 ml milk, 1,2,3, and 4 ml of $CaCl_2$ solution (containing 10 mg of calcium/ml) were added and calcium was determined by the new method and compared the results with the calculated values. The results presented in table 8 show that the recovery of the added calcium was satisfactory with minor differences from - 3.7 to 1.8 mg/ 100 ml.

TABLE - 8 : Recovery of added calcium in milk by new rapid method

S.No.	Milk (ml)	$CaCl_2$ Solution added (ml)	Calcium Determined: Titre value (ml)	Calcium Determined: Calcium (mg/100ml) (a)	Calcium Calculaed (mg/ 100ml)	Difference (a-b) (a - b)
1.	100	0	1.65	132.0		
2.	100	1	1.72	137.6	140.7	-3.7
3.	100	2	1.87	149.6	149.4	0.2
4.	100	3	2.00	160.0	158.2	1.8
5.	100	4	2.10	168.0	166.9	1.1
					Mean difference =	0.0

$CaCl_2$ solution contains calcium 10 mg/ml.

For the recovery of total phosphorus, to each 100 ml milk, 1,2,3, and 4 ml Na_2HPO_4 solution (containing 10 mg phosphorus/ml) was added and the total phosphorus was determined. The results obtained are presented in Table 9. The total phosphorus content determined by the method went on increasing marginally above the calculated values as the concentration of added phosphorus increased. Upto addition of 2 ml of Na_2HPO_4 solution, there was no appreciable difference. Beyond that the new method estimated a little higher phosphorus than the actual present.

The reason for this deviation may be that the native phosphorus is distributed in milk as inorganic and organic phosphorus in a particular ratio. If a little inorganic phosphorus is added to milk, it may also be distributed in both the forms. The organic phosphorus may be responsible for the heat stability of the milk, that is why in certain milk samples a little addition of Na_2HPO_4 improves the heat stability of milk. (Sindhu 1985). But there may be a limit to absorb the phosphorus in the casein micelle. Beyond that the added phosphorus may remain as inorganic phosphorus and imbalance in the ratio takes place. In the new method the titre value was multiplied by the factor 46.5 to calculate total phosphorus, which estimated the extra added inorganic phosphorus also,as total phosphorus. However if the difference in titre value beyond 2.41 (in case of pure milk) was multiplied by the factor 41.33 (for inorganic phosphorus) and the resultant value is added to 112.1 (the original value in

TABLE - 9 : Recovery of added Phosphorus in milk by new rapid method

S.No.	Milk (ml)	Na_2EPO_4 Solution added (ml)	Phosphorus Determined: Titre value (ml)	Phosphorus (mg/100ml) (a)	Phosphorus Calculated (mg/ 100ml) (b)	Difference (a-b) (a - b)
1.	100	0	2.41	112.1	-	
2.	100	1	2.60	120.9	120.9	0.0
3.	100	2	2.80	130.2	129.8	0.4
4.	100	3	3.05	141.8	138.7	3.1
5.	100	4	3.30	153.5	147.7	5.8

Na_2HPO_4 solution contains Phosphorus 10 mg/ml.

pure milk) then the recovery will be same as calculated, otherwise results will be slightly higher.

4.4.5 Repeatability of the method :

Any method of analysis is useless if repeatability is not good. To check the repeatability of the proposed new method, calcium and total phosphorus were determined 8 times in the same samples of cow milk, buffalo milk and toned milk. The results of calcium and total phosphorus are presented in table 10 & 11 respectively.

For calcium determination the titre value (Table 10) varied slightly between 1.6 to 1.7 , 2.3 to 2.4 and 1.55 to 1.65 ml in cow, buffalo and toned milk, respectively, and the corresponding calculated calcium varied between 128-136, 184-192 and 124-132 mg/100 ml in the same milk respectively.

Similarly for phosphorus determination the titre values (table 11), were almost same nearer to mean values of 2.113, 2.538 and 2.056 ml in case of cow, buffalo and toned milk samples respectively. The values of the phosphorus calculated were also not appreciably different from the mean values of 98.2 ,118.0 and 95.6 mg/100 ml in cow, buffalo and toned milk samples respectively.

From the foregoing discussion, it is evident that the developed method for the simultaneous determination of calcium, total phosphorus and inorganic phosphorus in milk, is fairly accurate rapid method having good, repeatability. For routine testing of normal milk samples for calcium and phosphorus the

TABLE 10

Repeatability of titre value (ml) and calcium content (mg/100 ml milk) by new method .

Sample	Parameter Studied	No. of Replicates 1	2	3	4	5	6	7	8	Mean
Cow milk	Titre Value	1.6	1.60	1.65	1.60	1.60	1.65	1.7	1.6	1.625
	Calcium	128	128	132	128	128	132	136	128	130.0
Buffalo milk	Titre Value	2.35	2.30	2.30	2.40	2.35	2.35	2.30	2.4[illegible]	2.344
	Calcium	188	184	184	192	188	188	184	192	187.5
Toned milk	Titre Value	1.60	1.55	1.55	1.60	1.60	1.65	1.60	1.55	1.588
	Calcium	128	124	124	128	128	132	128	124	127.0

TABLE 11

Repeatability of titre value (ml) and total Phosphorus content (mg/100 ml milk) by new method .

Sample	Parameter Studied	No. of Replicates								
		1	2	3	4	5	6	7	8	Mean
Cow milk	Titre Value	2.10	2.15	2.15	2.10	2.05	2.10	2.10	2.15	2.113
	Total phosphorus	97.7	100.0	100.0	97.7	95.7	97.7	97.7	100.0	98.2
Buffalo milk	Titre Value	2.50	2.50	2.55	2.60	2.55	2.55	2.50	2.55	2.538
	Total Phosphorus	116.3	116.3	118.6	120.9	118.6	118.6	116.3	118.6	118.0
Toned milk	Titre Value	2.00	2.05	2.05	2.10	2.05	2.00	2.10	2.10	2.056
	Total Phosphorus	93.0	95.3	95.3	97.7	95.3	93.0	97.7	97.7	95.6

method may prove to be the best. However, still more work is needed to be conducted: (i) to study the effect of interfering elements (if any). (ii) to sharpen the end point during titration, (iii) applicability of the new method to adulterated milk samples (iv) to study the effect of neutralizers and preservatives, added to milk, on the new method, etc.

CHAPTER 5

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

A rapid method of the estimation of calcium and phosphorus in milk has been developed based upon the oxalate effect. The factors effecting the oxalate effect have been studied and conditions for the test have been standardized. The decrease in titratable acidity on addition of potassium oxalate to milk or development of alkalinity (titre value) on addition of potassium oxalate to neutralized milk, have been found to be correlated with the concentration of calcium and phosphorus contents of milk.

The chemistry of the rapid method has been discussed and the factors for multiplying the titre values to calculate calcium,, inorganic phosphorus and total phosphorus in milk, have been derived to be 80, 41.33 and 46.5 respectively. The factors were also calculated from the experimental data, which resembled with the derived values.

The accuracy of the developed method was evaluated by analysing 15 samples each of cow milk, buffalo milk and toned milk. The same samples were also analysed by the standard reference methods for calcium, total phosphorus and inorganic phosphorus and the results obtained were very close to those of the rapid method.

Satisfactory recovery trials, good repeatability of the test, easy and simple procedure and no involvement of costlier chemicals and instruments, may tempt the users to adopt the method for rapid analysis of milk for calcium, total phosphorus and inorganic phosphorus, in the near future.

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