STANDARDIZATION OF PYNE CONSTANT AND ITS APPLICATION TO BUFFALO MILK IN THE PRESENCE OF SOME ADDITIVES

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BY

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Abstract
ABSTRACT

STANDARDIZATION OF PYNE CONSTANT AND ITS APPLICATION TO BUFFALO MILK IN THE PRESENCE OF SOME ADDITIVES

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Twenty raw pooled buffalo milk samples each of one litre were collected at intervals from the Hadgud Society of Amul. Fifteen replications were carried out to study the influence on Pyne constant and formal titre values of milk, casein and whey when (i) no calcium ion chelator (T1), (ii) potassium oxalate (T2) and (iii) tetrasodium pyrophosphate (T3) were used. Five replications were separately done to investigate the influence of additives, namely, sodium bicarbonate, sodium hydroxide, formaldehyde and hydrogen peroxide on formal titre values and Pyne constants during storage at ambient temperature (30 ± 2°C). Gross chemical composition of milk was determined in each replication.
Mean values of fat, total protein, total solids, solids-not-fat and ash content (per cent, w/w) of milk were: 8.76, 3.7466, 17.8282, 9.0682 and 0.7018, respectively. Mean values of casein and whey protein contents (per cent, w/w) of milk determined by Kjeldahl method were: 2.8867 and 0.7134, respectively. Mean values of calcium and phosphorus contents (mg/100g) of milk were: 173.5267 and 127.8333, respectively. Specific gravity, titratable acidity (per cent lactic acid) and pH of milk had mean values of 1.0261, 0.1308 and 6.6287, respectively.

For expressing the total protein content of buffalo fresh milk on w/w basis, the Pyne constants for T1, T2 and T3 methods were found to be 1.7620, 1.6488 and 1.7592, respectively, whereas for expressing protein content of milk on w/v basis, the Pyne constants were 1.8079, 1.6918 and 1.6051, respectively. Thus the value of Pyne constant for buffalo milk is lower (1.6488) than the reported literature value for bovine milk (1.70) for expressing total protein content on w/w basis using the oxalate method of formal titration. Similarly, using the same method as of Pyne (1932), the Pyne constant value was found to be lower for buffalo milk (1.6918) than the reported literature value for bovine milk (1.74) for expressing protein content of milk on w/v basis. Statistical analysis showed significant (P<0.05) difference between the Pyne constants in T1 and T2, T2 and T3, while nonsignificant difference between T1 and T3 treatments.
For calculating the casein and whey protein contents of buffalo milk appropriate Pyne constants were also determined. For wider application and flexibility in adoption of either method of choice, Pyne constants were calculated from either formal titre value of milk only or individually from the formal titre values of casein or whey.

For expressing the casein content of milk on w/w per cent basis, the Pyne constants for T₁, T₂ and T₃ methods were: 1.4083, 1.3163 and 1.4070, respectively using the formal titre values of milks in respective method and 1.8490, 1.7045 and 1.8424, respectively using the formal titre values of casein in the respective method. Literature value of Pyne constant derived from the formal titre value of bovine milk for calculating the casein content (w/w per cent basis) using the oxalate method has lower (1.28) value than that found in this study (1.3163) for buffalo milk. Both these values of Pyne constant are, however, lower than the Pyne constant value of 1.38 cited by BIS[SP.IS(Part XI)-1981]. Statistical analysis showed significant difference (P<0.05) in Pyne constants in T₁ and T₂, and T₂ and T₃, while nonsignificant difference between T₁ and T₃ methods when formal titre values of either milk or casein are used for the determination of Pyne constants.

For expressing the whey protein content of milk on w/w per cent basis, the Pyne constants for T₁, T₂ and T₃ methods were 0.3348, 0.3130 and 0.3343, respectively using the formal titre values of milk and 1.4087, 1.3852 and
1.3952, respectively using the formal titre values of wheys in respective methods. Statistical analysis showed significant difference (P<0.05) in Pyne constants in T₁ and T₂, and T₂ and T₃, while nonsignificant difference between T₁ and T₃ treatments, when formal titre values of milk are used to determine the Pyne constants; whereas nonsignificant difference between T₁, T₂ and T₃ methods was obtained, when formal titre values of whey were used to determine the Pyne constants.

Casein content of milk was also calculated on w/v basis using the formal titre values of milk in T₁, T₂ and T₃ methods, the Pyne constants were found to be 1.4460, 1.3507 and 1.4437, respectively but when using the formal titre values of isolated caseins, the Pyne constants were found to be 1.8982, 1.7490 and 1.8905, respectively. These Pyne constants are distinctly different from those used for calculating casein content of milk on w/w basis. Similarly the Pyne constants determined for calculating whey protein content of milk on w/v basis were distinctly different from those required for calculating whey protein content of milk on w/w basis as described earlier. Pyne constants for expressing whey protein content on w/v basis of milk of T¹, T₂ and T₃ methods were: 0.3436, 0.3209 and 0.3430 respectively based on formal titre values of milk and 1.4454, 1.4315 and 1.4315 respectively based on formal titre values of wheys of respective method.
Total protein, casein and whey protein contents of milk in the three methods showed no significant difference between Kjeldahl and formal titration methods when compared by paired t-test. Further suggesting that all three methods of formal titration give protein content estimates which are in close agreement with the Kjeldahl method.

Applicability of the formal titration methods for estimation of total protein of milk was investigated under various manipulations of raw milk such as preservation and souring and neutralization as are commonly encountered in grading and distribution of milk. Effect of such preservatives as formaldehyde, a largely permitted preservative under Prevention of Food Adulteration Act for chemical analysis of milk; hydrogen peroxide, a preservative permitted by FAO/WHO(1973) under restricted conditions and sodium hydroxide and sodium bicarbonate which are unscrupulously used by milk traders for neutralization of sour milk were investigated for their effect on the formal titre values and Pyne constants. Results showed that all the additives excepting hydrogen peroxide lowered the formal titre values suggesting that the same Pyne constant as determined for untreated milk can not be applied for the determination of total protein content of milk in any of the three methods. In order to obtain the correct value of total protein, modified Pyne constants had to be used with the treated milks. These constants for expressing total protein content on w/w basis of milk were: 1.9424, 1.7640 and 1.8248
in the presence of sodium bicarbonate; 1.8466, 1.7082 and 1.8307 in the presence of sodium hydroxide; 3.3018, 3.3040 and 3.2782 in the presence of formaldehyde in T₁, T₂ and T₃ methods, respectively. Whereas, the constants for expressing total protein content on w/v basis of milk were: 1.9929, 1.8100 and 1.9748 in the presence of sodium bicarbonate; 1.8946, 1.7526 and 1.8784 in the presence of sodium hydroxide; 3.3877, 3.3882 and 3.3637 in the presence of formaldehyde in T₁, T₂ and T₃ methods, respectively.

Formal titration methods of total protein determination in milk with and without additives in all the three treatments gave correct estimates of total protein when appropriate Pyne constants were used. This has been concluded from statistical analysis of milk samples by paired t-test.

From the present study it was concluded that formal titration of milk by method 1 (T₁) is the suitable method for determination of total protein, casein and whey protein contents of milk, and hydrogen peroxide as the suitable preservative to preserve the buffalo milk sample for determination of total protein content of milk by formal titration. Fuchsin can be used in place of rosaniline acetate as blank.
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CERTIFICATE

This is to certify that the thesis entitled
"STANDARDIZATION OF PYNE CONSTANT AND ITS APPLICATION
TO BUFFALO MILK IN THE PRESENCE OF SOME ADDITIVES"
submitted by Shri G.V.S. Prasadarao in partial
fulfilment of the requirements for the degree of M.Sc.
(Dairying) in Dairy Chemistry of the Gujarat
Agricultural University is a record of bonafide
research work carried out by him under my guidance and
supervision and the thesis has not previously formed
the basis for the award of any degree, diploma or other
similar title.

Anand
Date: 12. 01. 1971
(Sukhminder Singh)
Major Advisor
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LIST OF ABBREVIATIONS

°C = Degree Celsius
CV = Coefficient of variation
CD = Critical difference
°F = Degree fahrenheit
g = Gramme(s)
h = Hour(s)
kg = Kilogramme(s)
l = Litre(s)
mg = Milligramme(s)
min = Minute(s)
ml = Millilitre(s)
s = Second(s)
SD = Standard deviation
SEM = Standard error of mean
v/v = Volume by volume
w/v = Weight by volume
w/w = Weight by weight
INTRODUCTION
CHAPTER 1

INTRODUCTION

Milk protein occupies a pivotal position in human nutrition because of its high biological value. The milk protein is a complex and variable mixture of individual proteins. The major milk protein of bovine milk is casein, which exists mainly in the micellar form as calcium caseinate—calcium phosphate complex. Because of this complex nature, it is a carrier of calcium and phosphorus, which are essential for the formation of bones. It has unique properties unmatched by any other protein. It is readily digestible because it is considered to be an open structured and naturally denatured protein. It represents about 80 per cent of the total protein in cow milk but only 40 per cent of the total protein in human milk, rest being whey proteins. Bovine casein has a biological value (BV) of 0.80 and protein efficiency ratio (PER) of 2.5. The whey protein has BV of 0.85 to 0.90 and PER of 3.0. The whole protein has a BV of 0.90 and PER of 3.2 to 3.4. It is because of these nutritional properties that milk proteins are used for the manufacture of infant formulae and other variety of dairy products. Protein content is one of the most important factor affecting the yield of cheese, chhana and paneer. Estimation of protein is also important in the drying of milk.
Protein content along with fat are also correlated for the payment of milk. Therefore, the estimation of milk protein whether in milk or in products is an important aspect from the point of nutrition, yield and in the product manufacture. Milk is a complex mixture of several constituents such as fat, lactose, protein and minerals. The estimation of protein in milk is complicated due to interference by some of these constituents. Not only the interference by these constituents there has also been need for a method which is simple, quick, accurate and cheap. A review on rapid estimation of protein content by Booy et al. (1962) has discussed the problems of several methods of protein estimation. The great diversity of principles have been applied and most of these have also been tried by the investigators with the dairy industry. Booy et al. (1962) have listed some of the requirements that such approximate method should meet. They are:

1. The deviations from the content, as estimated by the Kjeldahl method, must be small for herd samples of milk as well as for milk from individual animals. The standard deviation of these differences should be preferably not higher than 0.06 per cent protein.

2. The method must be applicable to milk during all seasons and from all stages of lactation, except colostrum.
3. Common preservatives should not interfere with the method even with the samples which are one or two weeks old. Some bacterial growth should not cause errors.

4. There must be a simple relationship between the protein content and the measured value.

5. The technique should be so simple, that the estimation can be performed by unskilled operators and does not need complicated apparatus. The price of the determination must be as low as possible.

6. Falsification of milk with nitrogen-containing substances or other interfering compounds must be difficult or not economic.

Out of the several methods published three have found a wider application, namely formal titration, alkaline steam distillation and dye-binding.

The determination of milk proteins by formal titration was first suggested by Steinegger (1905), Richmond and Miller (1906) and Pyne (1932). Later it was adopted suitably for dairy products by Schulz et al. (1956). This method had also been applied in Spain by Aparicio (1966), in Italy by Procoptio and Lorenti (1968), in Brazil by Wolfschoon and Vargas (1977) and in India this method has been recommended by Bureau of Indian Standards(BIS) for determination of casein in milk [IS:1479(Part II)-1961]. It is clear that formal titration has found wide application and there is large literature on the applicability of
formal titration in the dairy industry. The standard deviation of the difference from the Kjeldahl method for milk of individual cows varies from 0.09 to 0.19 per cent and for herd milk from 0.04 to 0.10 per cent. This is rather high but the simplicity of the method has favoured its application which yielded some valuable information (Schulz et al., 1954). This method has an edge over the kjeldahl method, which is considered an accurate and reference method. The Kjeldahl method as applied to milk, includes nonprotein nitrogen, is too complicated to use on a large scale, needs quite complicated apparatus, costly, time consuming and involves the use of highly corrosive digestion mixture which prevents its application as routine method for thousands of samples a day.

Other chemical methods of estimation too suffer from inherent problems. Biuret method is unsuitable for routine analysis as there is need to remove lactose by dialysis which otherwise will interfere with biuret reagent. Folin method is unsuitable for routine analysis since it needs the removal of fat in direct colorimetric methods. Even though Raadsveld (1959) and Posthumus (1960) simplified the dye-binding technique by developing an apparatus, there is drawback with this method, that is, different dye-binding capacities during pasture and stall feeding (Peltola and Mälkki, 1960).
Buffalo milk in contrast to cow milk has different makeup of proteins and minerals. Pyne (1932) reported different values of Pyne constants for bovine and human milk because the latter had higher whey protein and lower casein content. Ling (1963) had given a word of caution that the formal titration method must not be used indiscriminately since there are abnormalities in nitrogen balance in normal milk. Buffalo milk contains 3.2, 0.6 and 0.8 and cow milk contains 2.8, 0.6 and 0.7 per cent respectively of casein, whey protein and ash. Buffalo milk has higher content of calcium and phosphorus, larger diameter of casein micelle and higher ratio of micellar to soluble casein than in cow milk. Voluminosity of the casein micelles from buffalo milk is lower than that of cow milk. The relative proportion of $\alpha_s1$, $\beta$, K— and $\alpha_s2$—caseins in buffalo milk is also different from cow milk. However, no report on the determination of Pyne constant for buffalo milk is available in the literature although plenty of data is now available on distinct differences in the buffalo and cow milks and their proteins. Even for the cow milk there is no unanimity in the methods of formal titration. These variabilities relates to quantity of milk, amount and concentration of indicator, use of calcium ion chelator, normality and type of alkali used for titration and the methods followed for the judgment of
end point of titration. Despite of existence of such variability, the formal titration has been used to determine the protein content in milk, cream and cheese, to control the suppliers milk, to select and standardize the milk for cheese making and preparation of yoghurt and other cultured milk products, to control butter washings, to determine the degree of condensation of evaporated milk, to control the purification of lactose, to determine the degree of protein breakdown in cultures and peptone preparation, to determine the degree of proteolysis in milk, to determine milk-solids-not-fat in fluid milk, dried milk and ice-cream, to determine the quality of industrial casein and to detect adulterants in milk. It is clear that formal titration has been favoured in several determinations in dairy processing without much basic understanding about the application of Pyne constant and factors influencing such determination in milk of buffalo. Wolfschoon and Vargas (1978) have reviewed the reaction mechanism of milk protein determination by formal titration and have suggested the possibility of several products being formed (Schiff's bases, N-hydroxy methyl adducts, thio—alcohols, methylene bridges) for the reaction between formaldehyde and a few milk amino acids. They have also expressed the need to elucidate the complex reaction of oxalate with milk proteins and to demonstrate the exact mechanism of the
reaction of milk proteins with formaldehyde and the nature of the derivatives formed. It was with this basic understanding that the need for the evaluation of Pyne constant for buffalo milk was felt and the present study was undertaken to standardize the formal titration method of protein estimation in buffalo milk and to determine the applicability of this method on preserved and neutralized sour milks under specific conditions.
REVIEW OF LITERATURE
CHAPTER 2

REVIEW OF LITERATURE

The pertinent literature on formal titration was classified into following sub-heads.

2.1 Reaction mechanisms of milk protein determination by formal titration
2.2 Methods of formal titration
2.3 Conversion factors for formal titration
2.4 Status of formal titration among other methods
2.5 Factors influencing formal titration
2.6 Other applications of formal titration

2.1 Reaction mechanisms of milk protein determination by formal titration

2.1.1 A general overview of chemical reactions of formaldehyde and formal titration

Technical formalin contains about 37 per cent formaldehyde and varying amounts of methanol in water. However, for accurate studies solutions of purified formaldehyde are preferable to those containing methanol. Formaldehyde in aqueous solutions acts as a very weak base, however, a moderately concentrated formaldehyde solution neutralizes significant amount of alkali at pH 9. This is an important step for obtaining accurate results in formal titration (French and Edsall, 1945).

Formaldehyde in water exists chiefly as methylene glycol \[ \text{\text{(CH}_2 \text{(OH)}_2)\text{]} \], a hydrate of formaldehyde. French and Edsall (1945) reported that formaldehyde reacts with various functional groups


present in amino acids and peptides. It undergoes addition and condensation reactions with amino, imino, amide, guanidino, carboxyl and sulphydryl groups and also with aromatic rings and peptide linkages. These reactions were represented as:

(i) Addition reaction: A compound containing an active hydrogen atom reacts with formaldehyde to form a hydroxymethyl compound as follows:

\[ R - H + CH_2O \rightarrow R - CH_2OH \]

(ii) Condensation reaction: Formaldehyde as methylene glycol undergoes condensation reactions with compounds containing active hydrogen in the following manner:

(a) \[ R - H + CH_2(OH)_2 \rightarrow R - CH_2OH + H_2O \]

(b) \[ R^1 - H + HO CH_2 - R \rightarrow R^1 - CH_2 - R + H_2O \]

The reaction of methylene glycol produced hydroxymethyl compound when condensed with one molecule containing active hydrogen and formed a methylene bridge on reacting further with another. Intramolecular condensation gave cyclic structure whereas intermolecular condensation resulted in the formation of molecular aggregates. Polyoxymethylene chains or bridges were formed with more formaldehyde molecules. Physical-chemical methods of analysis make it clear that each amino group is capable of forming unstable hydroxymethyl derivatives with either one or two moles of formaldehyde:
The oldest formulation of the reaction of formaldehyde with primary amines

\[
R - NH_2 + CH_2O \rightleftharpoons R - NH - CH_2 OH
\]

\[
R - NH_2 + 2 CH_2O \rightleftharpoons R - N (CH_2 OH)
\]

The imino group can react with one molecule of formaldehyde to form a hydroxymethyl compound

\[
R_2 = NH + CH_2O \rightleftharpoons R_2 = N - CH_2.OH
\]

and two imino groups may react to form a methylene compound

\[
2 R_2 = NH + CH_2O = (R_2 = N)_2 CH_2 + H_2O
\]

The amide group can react with formaldehyde to form hydroxymethyl compounds

\[
R - CO NH_2 + CH_2O \rightleftharpoons R - CONH - CH_2OH
\]

in which the hydroxyl group is unusually reactive and readily condenses with another amide molecule to give methylene diamides:

\[
2 R-CO.NH_2 + CH_2O \rightleftharpoons (R-CONH)_2 CH_2 + H_2O
\]

These compounds are usually prepared at elevated temperatures. The hydroxymethyl compounds are somewhat unstable at room temperature and markedly so at higher temperatures, while the methylene diamides are quite stable.

Diketopiperazine reacts with one or two molecules of formaldehyde to form hydroxymethyl groups. Presumably the peptide
linkage is capable of reacting in the same way.

\[ \text{CONH} + \text{CH}_2\text{O} \quad \text{---} \quad \text{CO--N (CH}_2\text{OH)} \text{---} \]

The alcoholic hydroxyl groups react with formaldehyde to form acetals and hemiacetals.

\[
\begin{align*}
R - \text{OH} + \text{CH}_2\text{O} & \quad \text{---} \quad R - \text{O - CH}_2\text{OH} \quad \text{R-OH} + \text{CH}_2\text{O} \quad \text{---} \quad (R-O)_2\text{CH}_2 + \text{H}_2\text{O} \\
2R - \text{OH} + \text{CH}_2\text{O} & \quad \text{---} \quad (R-O)_2\text{CH}_2 + \text{H}_2\text{O} \\
\end{align*}
\]

Hemiacetals are rather unstable while acetals are stable in neutral or alkaline media, unstable in acid. The thioanalogos of acetals and hemiacetals are readily formed by the reaction of sulphydryl group with formaldehyde and are considerably more stable than the oxygen derivatives:

\[
\begin{align*}
R - \text{SH} + \text{CH}_2\text{O} & \quad \text{---} \quad R\text{-S-CH}_2\text{OH} \\
2R - \text{SH} + \text{CH}_2\text{O} & \quad \text{---} \quad (R\text{-S})_2\text{CH}_2 + \text{H}_2\text{O} \\
\end{align*}
\]

The carboxyl group reacts with formaldehyde to form esters of methylene glycol:

\[
\begin{align*}
2R - \text{COOH} + \text{CH}_2\text{O} & \quad \text{---} \quad (R - \text{COO})_2\text{CH}_2 + \text{H}_2\text{O} \\
\end{align*}
\]

In aqueous solutions, however, conditions scarcely favour this reaction and, in general, it appears to be unimportant for amino acids and proteins except when they are in the dry or nearly dry state. Under favourable conditions the active hydrogen atoms of an aromatic ring are capable of reacting with formaldehyde to form hydroxymethyl groups:

\[
\begin{align*}
\equiv \text{C} - \text{H} + \text{CH}_2\text{O} & \quad \text{---} \quad \equiv \text{C} - \text{CH}_2\text{OH} \\
\end{align*}
\]

The C-C bond formed is very stable and so the reaction, though it often proceeds slowly, is practically irreversible. The hydroxyl group formed is frequently followed by condensation with another reactive group resulting in the formation of a methylene bridge. Alternatively condensation may occur between the aromatic
\text{\textup{\textit{\textcolor{red}{CH}}} and a neighbouring hydroxymethyl group attached to another functional group in the same or another molecule.} Such reactions underlie the formation of phenolformaldehyde polymers. In the amino acids these lead to formation of additional rings in tryptophan, tyrosine, phenylalanine and histidine.

French and Edsall (1945) reported that the formal titration of proteins does not differ in principle from that of the amino acids. An alkaline segment of the curve is displaced by formaldehyde (1 to 8 per cent) from a pK value near 10 to one near 7. This very large displacement corresponds to what would be expected for the free \(\varepsilon\)-amino groups of lysine. The best procedure for titrating these groups appear to involve: (1) an initial adjustment of the aqueous protein solution to a pH of approximately 8.5; (2) addition of formaldehyde, which, of course, causes a marked decrease in pH; (3) titration to a final pH of 8.5 in the formaldehyde solution. The alkali consumed should be equivalent to the \(\varepsilon\)-amino groups. If terminal free \(\varepsilon\)-amino groups are present, they should contribute very little to the titration, provided their pK values are similar to those found in peptide (pK values of 8.1 or less). If the initial adjustment of pH in water is made to 6.5 instead of 8.5, the \(\alpha\)-amino groups are presumably included; there is then also a contribution from the imidazole groups of histidine. The \(\varepsilon\)-amino groups, as determined by formal titration, frequently exceed the lysine determined by analysis of the protein hydrolyzate; often this is due to inadequacies in the analytical procedure (Cannan, 1942; French and Edsall, 1945).

2.1.2 Reaction mechanism of formal titration of milk—older concepts

It is well known that milk proteins can be determined
after neutralizing the acidity of an oxalate treated sample and then carrying out a second titration of the same sample following treatment with formaldehyde. The basis of mechanism was established by Richmond and Miller (1906) who suggested that the titratable acidity is produced by the conversion of the amino groups into methylene—imino groups by condensation, with the consequent conversion of the amphoteric nature of the amino acid into acid one.

\[ R-NH_2 + CH_2O \rightleftharpoons R-N = CH_2 + H_2O \]

Ling (1963) suggested that the linkage of formaldehyde with the basic amino groups, which enables the free carboxyl and other acid groups of the protein to exercise their full base-binding capacity, as responsible for production of titratable acidity.

Aparicio (1966) suggests that the formal mechanism is not only a simple condensation, as previously reported (Richmond and Miller, 1906), but proceed according to the following reaction:

\[ \text{Peptide} - (CH_2)_4NH_2 + CH_2O \rightleftharpoons \text{Peptide} - (CH_2)_4NHzCH_2OH \]

and thus alcoholic group is able to react with an NH group of another neighbouring peptide chain:

\[ RCH - N = O \]

Peptide - (CH_2)_4NHCH_2 - NH - CH-R

\[ O = C - H \]

Jenness and Patton (1969) suggested that formaldehyde reacts with primary amino groups, amide groups, and guanidyl groups and does not react with secondary amide groups (peptide linkage). The initial rapid, reversible reaction seem to be:
It will be remembered that amino groups exist in an equilibrium between charged and uncharged forms.

\[ \text{RNH}_2 + \text{HCHO} \rightleftharpoons \text{RNHCH}_2\text{OH} \]

\[ \text{RNHCH}_2\text{OH} + \text{HCHO} \rightleftharpoons \text{RN(CH}_2\text{OH)}_2 \]

Apparently formaldehyde reacts only with the uncharged form, with the result that the reaction goes completely to the right and the hydrogen liberated is responsible for titratable acidity. In other words, the products of the formaldehyde-amino group reaction are much weaker bases (show less tendency to hold protons) than the original amino groups.

In addition to the rapid primary reactions shown above, further slower reactions occur involving cross linking by means of methylene(-CH₂-) bridges. These may involve two amino groups or an amino group on the one hand and an amide, guanidyl, indole, phenol, or imidazole group on the other. These reactions are essentially irreversible.

2.1.3 Reaction mechanisms of formal titration of milk - newer concepts

2.1.3.1 Reaction mechanisms before addition of formalin

2.1.3.1.1 Reactions of titratable groups

Milk is a complex buffer system consisting mainly of \( \text{CO}_2 \), proteins, phosphates, citrates and lactates (Jenness and Patton, 1969). Citrate which exists mainly in the trivalent form
contributes very little as a buffer during titration of fresh milk within the pH 6.6 to 8.3. But the trivalent citrate species complexes with calcium to form the tricalcium dicitrate:

\[
2 (C_6H_5O_7)^{3-} + 3 Ca^{2+} \rightarrow Ca_3(C_6H_5O_7)_2
\]

The phosphates are the most significant buffers when performing the formal titration as is expected from the pK values of phosphoric acid. Calcium and colloidal calcium phosphate and citrate form linkages between phosphoserine and carboxylic groups of casein. During first titration with the alkali, the carboxylic groups of the amino acid residues present in the polypeptide chain and of the free amino acids, binds sodium. In this step, the carboxyl groups present on micellar side chains, as well as the terminal carboxyl groups and all the ionisable hydrogens within the pH range of 6.62 to 8.39 are titrated.

On the other hand, a release of colloidal phosphate and citrate is possible when alkali is added, as suggested below:

\[
\text{Polypeptide} \quad \text{calcium citrate} \quad \text{NaOH} \quad \text{Polypeptide}
\]

\[
\text{CH}_3 \quad \text{SH} \quad \text{CH}_3
\]

\[
\text{Polypeptide - CHCH}_2\text{PO}_3\text{Ca} \quad \text{Polypeptide - C=CH}_2
\]

\[
\rightarrow \quad \text{Ca}_3(\text{C}_6\text{H}_5\text{O}_7)_2 + \text{PO}_4^{3-} + \text{H}_2\text{O}
\]

Probably, the amount of calcium removed is not enough to stabilize the macromolecule, but is quite enough to expose new titratable groups.

2.1.3.1.2 Reactions of colloidal calcium phosphate

Visser (1962) demonstrated that different types of phosphate can be formed by titrating a solution containing the phosphoric acid in the presence of calcium and casein; he concluded that, between pH 5 and 12, calcium caseinate and
tricalcium phosphate can occur together forming either a soluble chemical complex or a colloidal matrix. He suggested the general formula for this complex as follows:

\[
\text{Colloid} - \text{C} \equiv \text{O} - \text{Ca} - \text{O} \equiv \text{P} \equiv \text{Ca}
\]

It is well known that tricalcium phosphate occurs with milk proteins, although the exact nature of the binding is not yet well defined.

2.1.3.1.3 Reactions taking place in the presence of calcium-ion chelator

Pyne (1932) introduced the use of potassium oxalate to eliminate the disturbing effects of both colloidal and soluble phosphates, but he did not elucidate the reaction mechanism for the oxalate effect. Ling (1963) suggested that the reaction between the oxalate and the colloidal calcium was according to the following reaction:

\[
\text{Ca}_3(\text{PO}_4)_2 + 3(\text{COOK})_2 \rightarrow 3(\text{COO})_2\text{Ca} + 2\text{K}_3\text{PO}_4
\]

and that the alkaline phosphate so formed interferes with subsequent milk titration. Jenness and Patton (1959) stated that the potassium oxalate causes an increase in pH due to the reaction of oxalate with \(\text{Ca}_3(\text{PO}_4)_2\) because of the displacement of colloidal calcium as insoluble oxalate, releasing a phosphate ion which is able to combine with hydrogen ions, of milk thus forming \(\text{H}_2\text{PO}_4^-\) and \(\text{HPO}_4^{2-}\).

On the other hand, Manson (1973) claims that the physico-chemical properties of the caseins are influenced by their content of phosphorus. The phosphate groups occur as O- esters,
formed between hydroxyamino acids of the proteins, serine, threonine, and orthophosphate. He suggested that the elimination of phosphates from these residues occur in the presence of alkali, according to the following reaction:

\[
\text{— HN}
\]
\[
\begin{array}{c}
\text{CHCH}_{2} - \text{OPO}_{3}^{2-} + \text{OH}^{-} \rightarrow \text{C =CH}_{2} + \text{PO}_{4}^{3-} + \text{H}_{2}\text{O}
\end{array}
\]

2.1.3.2 Reaction mechanisms after addition of formalin (Actual formal titration)

2.1.3.2.1 Involvement of lysine

Wolfschoon and Vargas (1977) observed that addition of formaldehyde to milk causes a down in pH to about 7.1 and this occurs in not more than 15 seconds.

Wolfschoon and Vargas (1978) gave the probable reactions of formaldehyde with milk proteins (oxalate treated milk previously neutralized with sodium hydroxide). They proposed that various reaction products, namely Schiff's base, thiomethylol derivatives, hydroxymethyl adducts, dihydroxymethyl adducts and methylldiamines (methylenic bridges) are formed through nucleophilic reactions. When formaldehyde is added to milk it becomes diluted and the following nucleophilic reaction occurs.

\[
\text{H-C-H} + \text{H}_{2}\text{O} \rightleftharpoons \text{H}^{+} \text{H-C-H}
\]

The reaction of the amino group of the lysine is as follows:
The amino group of the lysine acts as nucleophile due to the lone pair of electrons on the nitrogen atom. After reaction with the electrophilic centre (the C atom of formaldehyde) the amine loses a proton forming a methylol intermediate; as the reaction proceeds this intermediate loses water and a methylene-imine (Schiff's base) is formed; the loss of basicity by the methylol intermediate also contributes to the change in pH; this stage should be the rate determining step. The hydroxyl group liberated in II can act as a base attracting the sodium ion thus re-establishing the acidic nature of the carboxyl group (III) therefore permitting a further titration.

With lysine, the N-dihydroxymethyl derivate formation can be written as follows:

\[
\text{Peptide } \overset{+\text{CH}_2\text{(OH)}_2}{\rightarrow} \text{Peptide-}\left(\text{CH}_2\right)_4\text{NHCH}_2\text{OH}+\text{H}_2\text{O}
\]

\[
\text{pH} = 8.4
\]

\[
\text{Peptide } \overset{\text{CH}_2\text{(OH)}_2}{\rightarrow} \text{Peptide-}\left(\text{CH}_2\right)_4\text{NH}_2\text{H}_2\text{O}
\]

\[
\text{pH} = 7.1 < 8.4
\]

Once formed the N-hydroxymethyl (II), the addition of another molecule of formaldehyde to the former (II) is possible,
therefore a N-dihydroxymethyl derivative (III) results.

If the tetrahedral intermediate formed does not undergo either intramolecular dehydration, or react with another formaldehyde molecule but reacts with another amino group, it is possible to form methyl diamines (methylene bridges):

\[
\text{Peptide-(CH}_2\text{)}_4\text{NH-CH}_2 + H_2\text{N} \rightarrow \text{Peptide-(CH}_2\text{)}_4\text{NHCH}_2\text{N-Peptide} + \text{H}_2\text{O}
\]

the hydroxyl group formed (II) is usually reactive and may condense with another nitrogen atom containing an active hydrogen atom to form a methylene bridge. The nitrogen atom of the peptide linkage is probably capable of reacting in the same way although steric hindrance is to be expected.

Finally, the titration of the formaldehyde treated milk with alkali proceeds as a normal acid-base neutralization reaction, involving all the released or re-established carboxylic groups of the proteins; the amount of alkali consumed should be equivalent to the amino groups present.

2:1.3.2.2 Amino acid residues other than lysine participating in reactions with formaldehyde but not involved in titration

Amino acid residues other than lysine, which are capable of reacting with formaldehyde are arginine, cysteine and histidine. The reaction occurs with arginine with the involvement of guanidyl group, and with cysteine with involvement of sulphydryl groups and forms thiomethylol.
With histidine the reaction probably proceeds as follows:

\[
\text{Peptide-SH} + \text{H}_2\text{C-H} \quad \text{Peptide} \rightarrow \text{SCH}_2\text{OH} + \text{H}_2\text{O}
\]

One N atom of the imidazole ring of histidine reacts with one molecule of formaldehyde forming the N-hydroxymethyl derivative.

### 2.2 Methods of formal titration

The various methods of formal titration with some modifications existing in the literature were: Steinegger's (1905), Richmond and Miller's (1906), Pyne (1932), Thome (1946), Schulz (1953), Richmond's (1905), Oregon's (Harvey et al., 1954), Karunina and Shilovich's (1953), Richardson's (Everson et al., 1959), Bakalor's (automatic formal titration) (1965), Walker's (1914) and BIS [SP.IS(Part XI)-1981] method.

Some details of the above methods are given in Table 2.1.

### 2.3 Conversion factors for formal titration

Conversion factors obtained by various workers, by taking Kjeldahl method as reference method, were classified according to species.

#### 2.3.1 Cow milk

Pyne (1932) obtained factors of 1.70 (w/w) and 1.74 (w/v) for total protein estimation and 1.28 (w/w) for casein estimation in cow milk.

Everson et al. (1959) obtained a factor of 1.83, after
Table 2.1. Methods of formal titration of milk

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of the method</th>
<th>Quantity of milk</th>
<th>Strength of alkali</th>
<th>Expression of formal titre value</th>
<th>Conversion factor</th>
<th>total protein casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Steinegger</td>
<td>100 ml</td>
<td>0.25 N NaOH</td>
<td>aldehyde figure in 'SH</td>
<td>0.475</td>
<td>--</td>
</tr>
<tr>
<td>2.</td>
<td>Richmond and Miller</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3.</td>
<td>Pyne</td>
<td>10 ml</td>
<td>0.1N NaOH</td>
<td>amount of alkali used in ml</td>
<td>C M</td>
<td>1.7(0/w/v)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.26</td>
</tr>
<tr>
<td>4.</td>
<td>Thome</td>
<td>--</td>
<td>--</td>
<td>aldehyde figure in 'SH</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>5.</td>
<td>Schulz++</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>6.</td>
<td>Karunina and Shilovich</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7.</td>
<td>Richmond</td>
<td>10 or 11 ml Strontia solution</td>
<td>0.1N NaOH</td>
<td>aldehyde figure in 'SH</td>
<td>0.17</td>
<td>--</td>
</tr>
<tr>
<td>8.</td>
<td>Oregon</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>9.</td>
<td>Richardson</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>10.</td>
<td>Bakalor</td>
<td>20 ml</td>
<td>0.1N NaOH</td>
<td>amount of alkali used in ml (T)</td>
<td>0.8428</td>
<td>+ 0.2814</td>
</tr>
<tr>
<td></td>
<td>(Automatic formal titration)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Walker</td>
<td>10 ml</td>
<td>N/9 NaOH</td>
<td>amount of alkali used in ml</td>
<td>--</td>
<td>1.63</td>
</tr>
<tr>
<td>12.</td>
<td>BIS</td>
<td>10 gm</td>
<td>0.1N NaOH</td>
<td>amount of alkali used in ml</td>
<td>--</td>
<td>1.38</td>
</tr>
</tbody>
</table>

+ In these methods addition of neutralized saturated potassium oxalate is followed after phenolphthalein addition.
++ 0.4 ml of 28% K₂C₂O₄ is added before phenolphthalein addition.
CM Cow milk
HM Human milk
+++ the number of ml of 1N strontia to neutralize 1000 ml of milk
following a modified Richardson's method and Vermesanu et al. (1961) gave a factor of 0.480 (when aldehyde figure is in ScSH) by following Schulz method for total protein determination in cow milk.

El-Sokkary and Hassan (1953) obtained a factor of 0.2118 for determination of casein nitrogen content of cow milk.

2.3.2 Buffalo milk

El-Sokkary and Hassan (1953) gave a factor of 0.2166 for determination of casein nitrogen content of buffalo milk.

2.3.3 Ewe's milk

Montemurro (1963) obtained a factor of 0.512 (when aldehyde figure is in ScSH) by following Steinegger's method for protein estimation in ewe's milk.

2.3.4 Goat milk

Montemurro (1963) reported a factor of 0.452 (when aldehyde figure is in ScSH) by following Steinegger's method for protein estimation in goat milk.

2.3.5 Human milk

Pyne (1932) reported a conversion factor of 1.34 for determining the protein content in human milk.

Abou Dawood et al. (1977) obtained conversion factors of 2.09 and 1.29 for protein and casein estimations, respectively, in human milk.

2.3.6 Milk (Species not specified)

Russo (1958) obtained a factor of 0.486 (when aldehyde figure is in ScSH), Castillo (1961) gave a factor of 1.77, Wolfschoon and Vargas (1977) derived a factor of 1.747 for protein estimation.
Castillo et al. (1962) derived a factor of 1.35 and Laskowski et al. (1978) gave a factor of 1.57 (using Walker's method) for determination of casein content from formal titre values of milk.

2.4 Status of formal titration among other methods

Status of formal titration method among other methods of protein estimation had been evaluated by several workers as described in the following review:

2.4.1 Steinegger's method

Lucchetti and Maffei (1942) found good results for total protein but poor results for the fractions (casein, albumin, globulin and amino-acid fractions) with this method.

Tentoni et al. (1959) have shown that the above method is simple, cheap, rapid and giving results in agreement with Kjeldahl results, provided that the factor is established experimentally for the particular supply of milk.

Cusmano and Russo (1960) found an agreement between the protein values obtained by methods of Steinegger, Schulz and Kjeldahl.

Armandola and Brezzi (1961) found an average deviation of ± 1.68 per cent between the protein values determined by Steinegger's method and that obtained by the Kjeldahl method.

Vermesanu et al. (1961) found a correlation coefficient of 0.68 for the above method when Kjeldahl was taken as standard.

Montemurro (1963) found that Steinegger's method is suitable only for the analysis of mixed milk and also indicated that this method is less reliable for the analysis of individual milk samples, particularly those with high and low protein contents.
2.4.2 Richardson's method

Everson et al. (1959) found a correlation of 0.83 between this method and Kjeldahl method.

2.4.3 Pyne's method

DeVleeschauwer and Heyndrickx (1947) found a rectilinear relationship between N values of modified method of Rowland and formal titre values with the above method.

Ilstad (1954) showed the above method as more accurate for the determination of proteins in cheese milk than the ordinary formal titration.

Solberg et al. (1957) found no significant difference between Thome (1947) and Pyne (1932) methods and concluded that the addition of potassium oxalate was unnecessary.

Drux and Bauer (1964) showed that formal titration methods of Pyne and Schulz were suitable and sufficiently accurate for rapid routine determination of the protein content of milk.

Cerna (1968) found that amido-black method with Foss Pro-Milk apparatus as reliable method if instructions are rigidly followed, than Schulz and Pyne's methods of formal titration after doing comparision of these methods with Kjeldahl method.

2.4.4 Oregon's method

Harvey et al. (1954) followed Oregon method of formal titration for total protein, casein, lactalbumin and Hart method for Casein determination and found no significant difference between formal titration method and Kjeldahl method.

2.4.5 Thome method

Solberg et al. (1957) found a closer correlation
between formal titration values and total protein than between formal titration values and casein.

2.4.6 Schulz method

Cusmano and Russo (1960) found an agreement between the protein values obtained by methods of Steinegger, Schulz and Kjeldahl.

Vermesanu et al. (1961) found a mean deviation of ±0.07 per cent protein for this method when Kjeldahl method was taken as reference method.

Drux and Bauer (1964) have shown this method as suitable and sufficiently accurate for rapid and routine determination of the protein content of milk.

Orlova et al. (1965) found a percentage of error of < 3% for 90% of samples for casein determination with this method when compared with Kjeldahl method.

Cerna (1968) found that the amido black method with the Foss Pro—Milk apparatus as reliable method than this method, if instructions are rigidly followed.

Bellea (1980) compared dye-binding methods with orange G in crude and purified form; dye-binding with Amido black, using the Pro-Milk II; and this method with Kjeldahl method and found a SD of differences from Kjeldahl method as 0.065% protein for the purified Orange G, 0.078% for the Pro-Milk, 0.0837% for the crude Orange G and 0.089% for this method.

2.4.7 Bakalor’s method

Bakalor (1965) found a standard deviation of differences for his method with Kjeldahl method as 0.038% protein.

2.4.8 Karunina and Shilovich method

Yaganshin and Zueva (1989) found a correlation
coefficients of 0.806, 0.784 and 0.727 for this method, Orange G and a blue sulfo-dye method respectively with Kjeldahl method.

2.4.9 Walker's method

Laskowski et al. (1978) found a standard deviation of 0.32 and 0.10 % for casein determination by this method with 1.47 as factor and 1.57 as factor respectively, when compared to Pro-Milk II colorimetric method.

2.4.10 Formal titration (Method not specified)

Formal titration has been shown to be rapid and having closest agreement with Kjeldahl when compared with Kofranyi method and colorimetric methods of Steinsholt and Schober and Hetzel (Kiermeier and Renner, 1960), whereas, Solberg (1962) found amido-black method as more accurate than formal titration and Nesen (1962) found Kofranyi method as giving better agreement with Kjeldahl, than formal titration or dye binding with Orange G or amido-black.

Correlation coefficients of 0.9349, 0.9333, 0.8390, 0.7084 and 0.5384 0.8290 were obtained with formal titration by Anagama and Kami (1960), Vanderzant and Miah (1961), Castillo (1961), Ol’shevskii (1966) (two values for milks of two individual farms) and Steen (1979) respectively, whereas, 0.9720 and 0.9920 were obtained with steam distillation and amido-black (Vanderzant and Miah, 1961), 0.9253 and 0.9493 (two values for milks of two individual farms) were obtained with Orange G method (Ol’shevskii, 1966), and 0.923 and 0.972 were obtained with Orange G and amido black methods (Steen, 1979) when Kjeldahl was taken as reference.

Standard deviation of 0.06, 0.06, 0.05 and 0.198% protein with formal titration were reported by Renner and Omeroglu
(1972b), Roeper (1974), Wolfschoon and Ferreira (1978) and Steen (1979) which were more than that for Kofranyi, Infra Red Milk Analyser (Renner and Omeroglu, 1972b) and amido black method (Steen, 1978) when compared to Kjeldahl method.

The percentage of error was more in formal titration than direct alkali method (Antila, 1956), and, Kofranyi and refractometric methods (Renner and Omeroglu, 1972a) when compared with Kjeldahl method.

2.5 Factors influencing formal titration

2.5.1 Breed

Harvey et al. (1954) found a greater degree of variability within cows of Jersey than Holstein cows with formal titration.

2.5.2 Stage of lactation

Bannenberg and Hoek (1949) showed that colostrum gave erroneous results their observations that milk from the beginning and end of lactation also gave large deviations, were confirmed by Bosticco (1954) and Schober and Fricker (1954).

2.5.3 Feeding

As Schober and Fricker (1954) showed the errors are larger during stall-feeding than during pasture feeding.

2.5.4 Season

Schulz et al. (1954) found a average lowest values in April and June-July, and higest in May and October, whereas, Roeper (1974) found that formal titration method giving constant casein/true protein ratio and lower results from April to the late May.
2.5.5 Acidity of milk

Schober and Fricker (1954) found that development of acidity as not having effect on formal titre values, whereas, finding of Kiermeier and Renner (1960) and Cerna (1968) shows that higher acidity will lead to give higher results.

2.5.6 Protein content of milk

Yaganshin and Zueva (1969) found that formal titration was in good agreement with Kjeldahl for samples of 3 groups of protein content (3.0, 3.5 and 4.1 per cent) but this method gave higher values for the highest protein level (4.35 ± 0.14 per cent for 17 samples versus 4.06 ± 0.06 per cent by Kjeldahl method).

2.5.7 Cold storage

Cerna (1968) found that formal titration giving high results for cold stored milks and concluded that this method will be suitable for pasteurized milk.

2.5.8 Additives

Schulz et al. (1953) advised the use of mercuric chloride as a preservative, with guaiacol as a warning indicator and also found that potassium dichromate has influence on this method. It was also found that addition of ammonia, even at levels that could not be detected organoleptically, will cause high results (Schulz et al., 1953).

Mulay and Ladkani (1973) found that addition of
sodium bicarbonate, and Singhal and Raj (1989) found that addition of solid adulterants, namely, sugar, starch, sodium chloride and urea will lower the formal titre values. However, cereal flours, gelatin, or other substances normally used in ice-cream mixes do not interfere in the determination of milk solids-not-fat in ice-cream by formal titration method (Crowhurst, 1956).

2.6 Other applications of formal titration

In addition to determination of protein and its fractions in milk, formal titration has also been used (i) for determination of protein in cheese (Lanna and Laurenza, 1960; Armandola, 1962 and 1971), cream (Drux and Bauer, 1964), ice-milk and ice-cream (Hill and Stone, 1964), whey (Wolfschoon and Leite, 1977), butter, crude lactose and commercial peptone (Schulz et al., 1954); (ii) for determination of milk-solids-not-fat in fluid milk, sour milk, dried milk (Verma and Garg, 1966) and ice-cream (Crowhurst, 1956; Verma and Garg, 1966; Jain, 1971); (iii) for assessing the proteolysis in the milk (Knaut, 1958; Pijanowski, 1969; Vujicic, 1973 and Juffs, 1975); (iv) to study the rate of ripening in cheese (Mogensen, 1947; Vakaleris and Price, 1956; Szonntag, 1958 and Balotoni and Bakos, 1958) and (v) to evaluate the quality of industrial casein (Pijanowski and Jakubowski, 1958).
MATERIALS
AND
METHODS
CHAPTER 3
MATERIALS AND METHODS

This chapter covers details of the methods of milk collection and sampling, chemicals, fractionation of milk, compositional analysis of milk, formal titration of milk and its fractions, formal titration of stored samples of milk, determination of Pyne constants and statistical analysis of data.

3.1 Milk collection and sampling

Pooled fresh samples of buffalo milk were collected directly from the milk cooperative society, Hadgud. Milk samples were drawn from milk cans (40 l capacity) which contained pooled milk of about 20 buffaloes. Milk from about 120 buffaloes was randomly poured in the aluminium cans. One litre of well mixed pooled sample of milk was drawn at random from the cans and brought in stainless steel container (1.5 l capacity) to the laboratory within 10 min. Milk was warmed to 40°C and then cooled to the required temperature before taking lactometer reading, pH measurement and sampling for compositional analysis. In all twenty samples of milk were collected on different days and at regular intervals. Fifteen replications were carried out for the determination of Pyne constants for unpreserved raw milk and five replications were carried out separately to investigate the influence of storage on Pyne constants of preserved milk and those of neutralized sour milk.
3.2 The chemicals

Sodium hydroxide pellets (GR) rosaline acetate (LR) and tetrasodium pyrophosphate (GR) supplied by Loba Chemie Indoaustranal Co., Bombay; phenolphthalein (LR) and fuchsin (basic) supplied by BDH Laboratory Chemicals Division, Glaxo Laboratories (India) Ltd., Bombay; absolute alcohol (IP) supplied by Alembic Chemical Works Co. Ltd., Baroda; formaldehyde (37 to 41 per cent, w/v) (AR) supplied by Qualigens Fine Chemicals, Division of Glindia Ltd., Bombay; potassium oxalate (LR) supplied by s.d. Fine Chem. Pvt. Ltd., Boiser and hydrochloric acid (AR) supplied by Samir Tech-Chem Industry, Baroda were used for formal titration of milk.

Hydrogen peroxide (30 per cent w/v, purified) supplied by E. Merk (India) Ltd., Bombay and formaldehyde (37 to 41 per cent, w/v) (AR) supplied by Qualigens Fine Chemicals, Division of Glindia Ltd., Bombay were used as preservatives for milk. Sodium hydroxide pellets (GR) supplied by Loba Chemie Indoaustranal Co., Bombay and sodium bicarbonate (USP) supplied by Medi Pharma Drug House, Bombay were used as neutralizers for milk.

For the estimation of calcium, phosphorus, nitrogen and other compositional analyses the following chemicals were used: disodium ethylenediaminetetracetate (EDTA) (AR) supplied by Glaxo Laboratories (India) Ltd., Bombay; 1-amino-2-naphthol-4-sulphonic acid (purified) supplied by Loba Chemie Indoaustranal Co., Bombay; sulphuric
acid (AR), ethanolamine (LR), sodium bisulphite (LR), potassium sulphate (LR) and cupric sulphate (LR) supplied by Samir Tech-Chem. Industry, Baroda; sodium acetate anhydrous (GR), ammonium oxalate (GR), eriochrome black-T (LR), magnesium acetate anhydrous (GR), and ammonium molybdate (LR) supplied by Sarabhai M. Chemicals Ltd., Baroda; sulphuric acid (LR) and 2-methoxy ethanol (LR) supplied by S.D. Fine Chem. Pvt. Ltd., Boisar; sodium sulphite anhydrous (LR) and potassium dihydrogen orthophosphate supplied by SD's Lab. Chem. Industry, Bombay; methyl red (LR) supplied by British Drug House Ltd., BDH Laboratory Chemicals Division, Poole, England and trichloroacetic acid (LR) supplied by The Central Drug House, New Delhi.

3.3 Fractionation of milk into casein and whey

Fractionation of milk into casein and whey was done according to BIS method ([SP.IS(Part XI)-1981]) with some modifications. Ten millilitres of sample was taken into a 15 ml centrifuge tube. Sample was centrifuged for 5 min. Then the tube was transferred to an ice-bath to solidify the top fat layer. The fat plug was carefully removed using a spatula. This defatted milk was warmed in a water bath at 45 C for 15 min and to this was added 1 ml of 10 per cent (v/v) acetic acid and the contents were mixed well. After 10 min, 1 ml of 1N sodium acetate solution was added and the contents were mixed again and centrifuged for 10 min. Whey fraction was decanted into a porcelain dish and preserved for formal
titration. Casein which formed the pellet was taken out with the help of a thin spatula in a separate porcelain dish and centrifuge tube washed thrice with 5, 10 and 5 ml of distilled water and wash water transferred into the dish containing the casein. Casein was dispersed with the flat end of a glass rod.

3.4 Compositional analysis of milk

3.4.1 Estimation of total solids in milk

Total solids in milk were determined according to the procedure described in IS : 1479 (Part II)-1961.

In a previously weighed aluminium dish, 10 g of milk was poured and the contents were weighed quickly. The dish containing milk was placed on a boiling water bath until milk had dried and after which it was transferred into well-ventilated oven maintained at 98 ± 2°C. After 4h dish was transferred into a desiccator and allowed to cool for 30 min. After cooling, the dish was removed from the desiccator and weighed. Drying, cooling and weighing were repeated until the loss in weight between two consecutive weighings did not exceed 0.5 mg.

Total solids content was calculated by the following formula:

\[
\text{Total solids (per cent by weight)} = \frac{100 \times w}{W}
\]
where,

\[ w = \text{weight in g of the residue after drying, and} \]

\[ W = \text{weight in g of the sample taken for estimation}. \]

3.4.2 Estimation of fat in milk

Fat content in milk was estimated by the Gerber method described in IS: 1224 (Part I)—1977.

Ten millilitres of sulphuric acid was taken into a butyrometer to which 10.75 ml milk and 1 ml amyl alcohol have been added. Butyrometer was stoppered and shaken until no white particles were seen in the liquid and then it was inverted for few times. Then the butyrometer was placed in a water bath maintained at 65 ± 2°C for 5 min. After tempering, butyrometer was placed in the Gerber centrifuge and centrifuged at 1100 rpm for 4 min. Again the butyrometer was placed in the water bath maintained at 65 ± 2°C for 5 min. Fat column was quickly read and the percentage of fat in milk was recorded.

3.4.3 Determination of titratable acidity of milk

Titratable acidity of milk was determined by following the procedure described in IS : 1479 (Part I) — 1960.

Ten millilitres of thoroughly mixed milk was taken into a porcelain dish and an equal volume of distilled water was added to it. Then 1 ml of phenolphthalein indicator
solution [1g of phenolphthalein powder dissolved in 100 ml of ethyl alcohol (95 per cent, v/v) and 0.1N sodium hydroxide added until 1 drop gave a faint pink colouration. Distilled water was then added to make the volume to 200 ml] was added into the sample. The contents of dish were titrated with 0.1N sodium hydroxide till a light pink tinge of the solution was obtained.

Titratable acidity was calculated using the following formula:

\[
\text{Titratable acidity (as per cent lactic acid)} = \frac{9V_1N}{V_2}
\]

where,

- \(V_1\) = Volume in ml of 0.1N sodium hydroxide required for titration,
- \(V_2\) = Volume in ml of milk taken for titration, and
- \(N\) = Normality of sodium hydroxide

3.4.4 Estimation of ash in milk

Ash content in milk was determined according to the procedure described in IS : 1479 (Part I) - 1960.

A clean and dry silica dish was weighed with 10 g of milk and contents were subjected to heat until evaporated to dryness. The dish was then transferred into a muffle furnace and the temperature of the furnace was raised to 550 to 600°C. Milk was incinerated until free from
carbon. Later on the dish was transferred to a desiccator and cooled. After cooling, the dish was removed from the desiccator and weighed.

Ash content was calculated by the following formula:

\[
\text{Ash (per cent by weight)} = \frac{100 \times w}{W}
\]

where,

- \( w \) = Weight in g of the ash, and
- \( W \) = Weight in g of the sample taken for ashing

3.4.5 Estimation of calcium in milk

Calcium content in milk was estimated by the method described by Davies and White' (1962).

For the estimation of calcium in milk, 5 ml of trichloroacetic acid filtrate [prepared by diluting 20 ml of milk to 50 ml in a volumetric flask with trichloroacetic acid solution (20 per cent, w/v) and shaking the flask vigorously for few seconds, from which filtrate was collected through Whatman No. 40 filter paper after 30 min] was poured into a 50 ml centrifuge tube (round bottom, graduated) and 1 ml of ammonium oxalate solution (4 per cent, w/v) and 1 drop of methyl red solution [0.02 per cent (w/v) in 95 per cent (v/v) ethanol] were added. The tube was rotated to mix its contents and sodium hydroxide solution (20 per cent, w/v) was added dropwise until the colour of the mixture was pale yellow and then few drops of 1N hydrochloric acid were added.
until the colour was very faint pink. After 4 h incubation at room temperature, the contents were diluted with distilled water to 20 ml and centrifuged in a centrifuge (Remi Tg model) for 10 min at 3000 rpm. The precipitate of calcium oxalate was used for the estimation of calcium after discarding the supernatant layer as follows:

Two millilitres of 1N hydrochloric acid was added to the calcium oxalate and mixed to dissolve calcium oxalate. To this was then added 2 ml of 0.05 M EDTA solution, 2 ml of ethanolamine solution (20 per cent, v/v) and a drop of Eriochrome black-T solution (prepared by dissolving 50 mg Eriochrome black-T in 100 ml of 2—methoxy ethanol and filtering contents through Whatman No. 40 filter paper and protecting it from light) and without delay excess of EDTA solution was titrated fairly rapidly with 0.015M magnesium acetate to red colour end point. For a blank determination the whole procedure was repeated with 5 ml of trichloroacetic acid solution (12 per cent, w/v) but before the addition of 2 ml of 1N hydrochloric acid 0.2 ml of ammonium oxalate solution was added.

The difference between the blank and sample in the volume of magnesium acetate solution is equivalent to the calcium content in the sample. Calcium content in the milk was calculated applying the following formula:

\[
\text{Calcium (mg/100g)} = \frac{30.06 (V_1 - V_2)}{d}
\]
where,

\[
V_1 = \text{Volume in ml of } 0.015M \text{ magnesium acetate solution required for the blank determination,}
\]

\[
V_2 = \text{Volume in ml of } 0.015M \text{ magnesium acetate solution required for the titration of sample,}
\]

and

\[
d = \text{Specific gravity of milk at 28.9 }^\circ \text{C}.
\]

3.4.6 Estimation of phosphorus in milk

Phosphorus content in milk was estimated by the method of Fiske and Subbarow (1925) using the ash solution [obtained by dissolving the ash in dilute hydrochloric acid (1:4 by volume) and the volume made to 100 ml with distilled water in a volumetric flask].

To 0.1 ml of the ash solution in a 25 ml test tube, 0.4 ml of 5N sulphuric acid, 0.8 ml of ammonium molybdate reagent (2.5 per cent, w/v) and 0.4 ml of 1-amino-2-naphthol-4-sulphonic acid reagent (prepared by dissolving 0.25 g of 1-amino-2-naphthol-4-sulphonic acid, 1.2 g of sodium sulphite and 1.2 g of sodium bisulphite in 10 ml of distilled water) were added. The final volume of the mixture was made to 10 ml with distilled water and the contents were thoroughly mixed. After 10 min incubation at room temperature, the optical densities were measured at 700 nm in Spectronic-20 against reagent blank.

A standard curve was prepared with the phosphorus concentration ranging from 20 to 100 μg/ml using...
potassium dihydrogen phosphate as the standard.

3.4.7 Determination of specific gravity of milk

Specific gravity of the milk was determined according to the IS:1183-1965 using Zeal lactometer calibrated at 84°F (28.9°C). Lactometer reading was taken within 30s after the insertion of lactometer in milk. Specific gravity of milk was calculated using the following formula:

\[
d = 1 + \frac{C L R}{1000}
\]

where,

\[d = \text{Specific gravity of milk at 28.9°C, and}
\]

\[CLR = \text{Corrected lactometer reading at 28.9°C}
\]

3.4.8 Determination of pH of milk

pH of fresh milk was determined at ambient temperature (32°C) using Digital pH meter (Elico, Model LI - 120).

3.4.9 Determination of protein content in milk

3.4.9.1 Determination of total protein content in milk

Total protein in milk was estimated by Kjeldahl method according to the procedure of Davies and Mac Donald (1953).

Ten grammes of milk was weighed and transferred into 300 ml digestion flask. To this 25 ml of concentrated sulphuric acid was added along with 10 g of digestion mixture.
consisting of copper sulphate and potassium sulphate (1:50, w/w) and few glass beads. The material was digested over the flame until it became transparent. After cooling, the mixture was transferred into a distillation flask, rinsing with distilled water. Sodium hydroxide solution (50 per cent, w/v) was poured into the distillation flask to make it alkaline. The mixture was heated and the distillate was collected in a conical flask containing 50 ml of 0.1N sulphuric acid solution and 7 to 8 drops of methyl red indicator. About 150 ml of distillate was collected and was then titrated against 0.1N sodium hydroxide.

A blank was prepared following the above procedure excepting 10 ml of water was used instead of milk. Blank reading was equivalent to about 0.2 ml of 0.1N sulphuric acid.

Total nitrogen was calculated according to the following formula:

\[
\text{Total nitrogen (per cent by weight) = } \frac{(50 - S) - B}{W} \times 0.14
\]

where,

- \( S \) = Volume in ml of 0.1N sodium hydroxide required in the back titration of sample,
- \( B \) = Blank reading (50—volume in ml of 0.1N sodium hydroxide in the back titration of blank), and
- \( W \) = Weight of the sample in g taken for the estimation
Total protein content of the sample was calculated by multiplying the nitrogen content by the factor 6.38.

3.4.9.2 Determination of casein content of milk

Ten grammes of milk was fractionated into casein and whey according to the procedure described in Section 3.3. Twenty millilitres of the casein dispersion in distilled water was transferred quantitatively to the digestion flask and casein nitrogen in the precipitate was determined by the method described in section 3.4.9.1. Casein content of milk was calculated by multiplying the casein nitrogen by the factor 6.38.

3.4.9.3 Determination of whey protein content of milk

Whey protein content of milk was determined by using the whey separated from milk as described earlier (Section 3.3). Whole amount of whey was transferred quantitatively into a digestion flask and nitrogen content was determined by Kjeldahl procedure described in Section 3.4.9.1. Whey protein content of milk was calculated by multiplying the nitrogen content of whey by the factor 6.38.

3.5 Formal titration of milk and its fractions

3.5.1 Treatments of milk and its fractions in formal titration

Formal titration was carried out by three
methods which differed in the treatments to milk so as to obtain better judgement of end point. These treatments were:

(i) Treatment 1 (T-1): In this treatment no Ca ion chelator was added to milk and its fractions as described later in Method 1 (Sections 3.5.2.1, 3.5.2.2 and 3.5.2.3)

(ii) Treatment 2 (T-2): In this treatment 0.4 ml of saturated potassium oxalate was added as Ca ion chelator to 10 ml milk and its fractions obtained from 10 ml milk. This treatment was followed in Method 2 described later (Sections 3.5.2.1, 3.5.2.2 and 3.5.2.3)

(iii) Treatment 3 (T-3): In this treatment 0.4 ml of saturated tetrasodium pyrophosphate was added to 10 ml milk and its fractions obtained from 10 ml milk. This treatment was followed in Method 3 as described later (Sections 3.5.2.1, 3.5.2.2 and 3.5.2.3)

3.5.2 Methods of formal titration

3.5.2.1 Methods of formal titration of milk

Formal titration of milk was carried out by three methods as shown in the Flow diagram (Fig.3.1) and described in details below:

Formal titration of milk was performed mainly in two steps: Step 1, involved titration of milk to the end-point of phenolphthalein without addition of formaldehyde solution (37 to 41 per cent, w/v) and Step 2, involved titration of milk to the end-point of phenolphthalein in the presence of formaldehyde. Titration
Fig. 3.1 Flow diagram for formal titration of milk

\[ T_1 = \text{Treatment 1}, \quad T_2 = \text{Treatment 2}, \quad \text{and} \quad T_3 = \text{Treatment 3} \]
done in Step 2 is the actual formal titration since the titre value obtained in this step was considered for determination of Pyne constant and calculation of protein content. Three methods of formal titration were followed which differed in the use of Ca ion chelator in Step 1.

3.5.2.1.1 Formal titration Step 1

Ten millilitres of thoroughly mixed milk was taken in a porcelain dish. Then 1 ml of phenolphthalein indicator solution (1 g of phenolphthalein powder dissolved in 100 ml ethyl alcohol (95 per cent, v/v) and 0.1N sodium hydroxide added until 1 drop gave a faint pink colouration. Distilled water was then added to make the volume to 200 ml) was added into the sample and titration with 0.1N sodium hydroxide was performed as per the methods. Method 1: No Ca ion chelator was added to milk and milk as such was titrated after 2 min to faint pink colour end point; Method 2: 0.4 ml of saturated potassium oxalate solution was added to milk. This was followed by addition of 0.5 ml 0.1N hydrochloric acid acid and titrated after 2 min to faint pink colour end-point; and Method 3: 0.4 ml of saturated tetrasodium pyrophosphate solution was added to milk and titrated after 2 min to faint pink colour end-point.

3.5.2.1.2 Formal titration Step 2

This step in formal titration was common to all the three methods and was as follows:

Two millilitres of neutral formalin (one drop of
phenolphthalein indicator was added to 100 ml of 37 to 41 percent (w/v) formaldehyde solution and adjusted to light pink colour by adding 0.1N sodium hydroxide dropwise) was added to the titrated milk in Step 1 and immediately titrated again with 0.1N sodium hydroxide to a faint pink colour end-point. The volume in ml of 0.1 N sodium hydroxide used in Step 2 was recorded as formal titre value of milk.

A blank was prepared by adding 1 ml of rosaniline acetate solution to 10 ml milk to compare the end-point colour during titration in Step 1 and 2. Alternatively a blank can also be prepared by adding 1 ml of fuchsin solution to 10 ml milk. Rosaniline acetate or fuchsin solution was prepared by diluting 1 ml of the stock solution (dissolve 0.12 g of rosaniline acetate or fuchsin in about 50 ml of rectified spirit containing 0.5 ml of glacial acetic acid and making the volume to 100 ml with rectified spirit) to 500 ml with a mixture of rectified spirit and distilled water in equal proportions by volume.

3.5.2.2 Methods of formal titration of casein

Formal titration of casein (obtained from 10 ml of milk in Section 3.3) was done by following the methods described in Section 3.5.2.1 excepting that casein solution was used instead of milk and addition of hydrochloric acid was eliminated in Method 2. Volume in ml of 0.1 N sodium hydroxide required for titration of casein solution in Step 2 was recorded and represented as the formal
titre value of casein.

3.5.2.3 Methods of formal titration of whey

Formal titration of whey (obtained from 10 ml milk in Section 3.3) was done by following the methods described in Section 3.5.2.1 excepting that whey was used instead of milk and addition of hydrochloric acid was eliminated in Method 2. Volume in ml of 0.1 N sodium hydroxide required for titration of whey in Step 2 was recorded and represented as the formal titre value of whey.

3.6 Formal titration of stored samples of milk

Fresh pooled buffalo milk sample was obtained from the same source as described in Section 3.1 and was divided into five lots each of 100 ml. Each lot of milk was taken in separate clean and dry glass bottles of 200 ml capacity and proceeded as described in the following sections. Formal titration of fresh milk at zero period of storage was done as described in Section 3.5.2.1.

3.6.1 Formal titration of milk treated with preservatives and neutralizers.

3.6.1.1 Formal titration of milk stored with preservatives

Formaldehyde (37 to 41 per cent, w/v) and hydrogen peroxide (30 per cent, w/v) were used as preservatives for milk. In one set formaldehyde was added at the rate of 0.4 ml per 100 ml milk and in other set hydrogen peroxide was added at the rate of 0.9 ml per 100 ml milk in separate bottles and securely stoppered and kept at ambient
temperature \((30 \pm 2^\circ C)\) for 24 h.

On completion of the storage period, bottles were inverted 2 to 3 times for proper mixing and 10 ml each of the preserved samples were pipetted in separate porcelain dishes and proceeded for formal titration as described in Section 3.5.2.1. For milk preserved with hydrogen peroxide, 0.5 ml of 0.1N hydrochloric acid was added per 10 ml milk to decolorize the pink tinge developed on addition of 0.4 ml of saturated potassium oxalate and rest of the steps in the procedure were same as in Section 3.5.2.1. For milk preserved with formaldehyde, no such additional step was required.

3.6.1.2 Formal titration of milk treated with neutralizers

Sodium bicarbonate (USP) and sodium hydroxide (GR) were used as neutralizers for stored milk. Two clean and dry 200 ml glass bottles were taken and 100 ml of raw milk was added separately in each bottle and stoppered with rubber stoppers. Bottles were stored at ambient temperature \((30 \pm 2^\circ C)\) for 6 h. On completion of the storage period, 2 ml of 10 per cent \((w/v)\) solutions of sodium bicarbonate and sodium hydroxide were added separately in each bottle so as to give 0.2 per cent \((w/v)\) concentration of the neutralizers. For formal titration, 10 ml of the well mixed sample was taken in a porcelain dish and proceeded as in Section 3.5.2.1 with the following additions:

(i) One ml of 0.1N hydrochloric acid per 10 ml milk to decolourize the pink tinge developed on addition of 0.4 ml
saturated potassium oxalate (Method 2) in milk neutralized with sodium bicarbonate.

(ii) Two ml of 0.1N hydrochloric acid per 10 ml milk to decolourize the pink tinge developed on addition of 1 ml phenolphthalein indicator (Method 1, 2, and 3) in milk neutralized with sodium hydroxide.

(iii) Two ml of 0.1N hydrochloric acid per 10 ml milk to decolourize the pink tinge developed on addition of 0.4 ml of saturated potassium oxalate (Method 2) in milk neutralized with sodium hydroxide, and

(iv) One half ml of 0.1N hydrochloric acid per 10 ml milk to decolourize the pink tinge developed on addition of 0.4 ml of saturated tetrasodium pyrophosphate (Method 3) in milk neutralized with sodium hydroxide.

3.6.2 Correction factors for formal titre values of preserved and neutralized milks

3.6.2.1 Preserved milks

3.6.2.1.1 H₂O₂-preserved milk

To 100 ml of milk sample 0.9 ml of H₂O₂ was added to preserve the sample as recommended by FAO/WHO (1973). Total volume had come to 100.9 ml. To determine the formal titre value of H₂O₂—preserved milk, 10 ml was taken from the total volume of 100.9 ml. This 10 ml represents only 9.9108 ml milk. So the formal titre value obtained from the experiment was of 9.9108 ml milk. To get the correct
formal titre value of 10 ml milk, the value obtained was multiplied with a correction factor of 1.009. The value thus obtained was used to compare with formal titre values of fresh milk.

3.6.2.1.2 HCHO-preserved milk

To 100 ml milk sample, 0.4 ml of formaldehyde (37 to 41 per cent, w/v) solution was added to preserve the sample as recommended by PFA (1990). So for HCHO-preserved milk correction factor used was 1.004.

3.6.2.2 Neutralized milks

3.6.2.2.1 NaHCO₃-neutralized milk

To 98 ml of milk sample, 2 ml of NaHCO₃ solution (10 per cent, w/v) was added to neutralize the sample. Total volume had come to 100 ml. So for NaHCO₃-neutralized milk correction factor used was 1.020.

3.6.2.2.2 NaOH-neutralized milk

To 98 ml of milk sample 2 ml of NaOH solution (10 per cent, w/v) was added to neutralize the sample. So for NaOH-neutralized milk correction factor used was 1.020.

3.7 Determination of Pyne constants

Pyne constants for buffalo milk were calculated according to the method of Pyne (1932) for cow milk described in the following sections.

3.7.1 Pyne constants for determination of total protein in milk
Total protein content of milk is generally expressed on w/w or w/v basis, therefore, values of Pyne constants were calculated for both these expressions and for each of the treatments described in Section 3.5.1. Pyne constants were calculated from formal titre value, specific gravity and total protein content (Kjeldahl method) of the same milk. Formal titre value determination was done according to Methods 1, 2, and 3 described earlier (Section 3.5.2.1).

(i) Pyne constant for calculating total protein on w/w basis of milk

\[
\text{Pyne constant} = \frac{\text{Total protein content by Kjeldahl method (g/100 g milk)}}{\text{Formal titre value (ml of 1N sodium hydroxide per 100 ml milk)}}
\]

(ii) Pyne constant for calculating total protein on w/v basis of milk

\[
\text{Pyne constant} = \frac{\text{Total protein content by Kjeldahl method (g/100 ml milk)}}{\text{Formal titre value (ml of 1N sodium hydroxide per 100 ml milk)}}
\]

Total protein in g/100 ml milk was calculated from the value of total protein in terms of g/100 g milk as in the Kjeldahl method by multiplying with the value of specific gravity of milk.
3.7.2 Pyne constants for determination of casein and whey protein in milk

Casein and whey protein contents of milk are expressed either on w/w or w/v basis, therefore, values of Pyne constants were calculated for both these expressions and for each of the treatments described in Section 3.5.1 from the formal titre values of milk and its fractions.

3.7.2.1 Pyne constants for casein and whey protein from formal titre value of milk

Pyne constant was calculated from the formal titre value, specific gravity, casein and whey protein contents (Kjeldahl method) of the same milk. Formal titre value determination was done according to Methods 1, 2, and 3 described earlier (Section 3.5.2.1).

(i) Pyne constant for calculating the casein content on w/w basis of milk

\[
\text{Pyne constant} = \frac{\text{Casein content by Kjeldahl method (g/100 g milk)}}{\text{Formal titre value (ml of 1N sodium hydroxide per 100 ml milk)}}
\]

(ii) Pyne constant for calculating the casein content on w/v basis of milk

\[
\text{Pyne constant} = \frac{\text{Casein content by Kjeldahl method (g/100 ml milk)}}{\text{Formal titre value (ml of 1N sodium hydroxide per 100 ml milk)}}
\]
(iii) Pyne constant for calculating the whey protein content on w/w basis of milk

Pyne constant = \[ \frac{\text{Whey protein content by Kjeldahl method (g/100 g milk)}}{\text{Formal titre value (ml of IN sodium hydroxide per 100 ml milk)}} \]

(iv) Pyne constant for calculating the whey protein content on w/v basis of milk

Pyne constant = \[ \frac{\text{Whey protein content by Kjeldahl method (g/100 ml milk)}}{\text{Formal titre value (ml of IN sodium hydroxide per 100 ml milk)}} \]

Casein in g/100 ml milk was calculated from the value of casein in g/100 g milk in the Kjeldahl method multiplied by the specific gravity of milk. Similarly whey protein in g/100 ml was calculated from the value of whey protein in g/100 g milk in the Kjeldahl method multiplied by the specific gravity of milk.

3.7.2.2 Pyne constants for casein and whey protein from their formal titre values

Formal titre value determination was done according to methods 1, 2, and 3 described earlier (Section 3.5.2.2 and 3.5.2.3). Pyne constants were calculated from formal titre values of casein and whey, specific gravity of milk, casein and whey protein contents (Kjeldahl method) of milk.
(i) Pyne constant for calculating casein content on w/w basis of milk

\[
\text{Pyne constant} = \frac{\text{Casein content by Kjeldahl method (g/100 g milk)}}{\text{Formal titre value (ml of 1N sodium hydroxide required for casein from 100 ml milk)}}
\]

(ii) Pyne constant for calculating casein content on w/v basis of milk

\[
\text{Pyne constant} = \frac{\text{Casein content by Kjeldahl method (g/100 ml milk)}}{\text{Formal titre value (ml of 1N sodium hydroxide required for casein from 100 ml milk)}}
\]

(iii) Pyne constant for calculating whey protein content on w/w basis of milk

\[
\text{Pyne constant} = \frac{\text{Whey protein content by Kjeldahl method (g/100 g milk)}}{\text{Formal titre value (ml of 1N sodium hydroxide required for whey from 100 ml milk)}}
\]

(iv) Pyne constant for calculating whey protein on w/v basis of milk

\[
\text{Pyne constant} = \frac{\text{Whey protein content by Kjeldahl method (g/100 ml milk)}}{\text{Formal titre value (ml of 1N sodium hydroxide required for whey from 100 ml milk)}}
\]

Casein and whey protein contents in g/100 g milk were converted into g/100 ml milk by multiplication with specific gravity value of milk.
3.8 Statistical analysis of data

Randomized block design, descriptive statistics, hypothesis test for means and factorial randomized block design have been used for statistical analysis of the data. (Steel and Torrie, 1980).
RESULTS
AND
DISCUSSION
CHAPTER 4

RESULTS AND DISCUSSION

This chapter deals with the comparative assessment of Pyne constants for buffalo milk for the estimation of total protein, casein and whey protein by three different methods of formal titration. For this experiment, 15 replications for each method were performed on unpreserved pooled fresh buffalo milk and 5 replications for each method to assess the effect of two preservatives, namely, formaldehyde and hydrogen peroxide and two neutralizers, namely sodium hydroxide and sodium bicarbonate added to sour milk on the Pyne constants and protein content of milk. The gross chemical composition of buffalo milk was also determined so as to define precisely the quality of milk for which these Pyne constants (conversion factors) are applicable. Relative amounts of some important milk constituents likely to influence the formal titre and Pyne constant were determined to elucidate the difference, if any, in the values of Pyne constant for buffalo milk and those reported in the literature for cow milk. Results of these findings were statistically analysed and have been discussed in the light of available information in literature.

4.1 Gross chemical composition of raw pooled buffalo milk

Status of chemical constituents of raw pooled buffalo milk is well known to vary widely in milk from
various species. Breed, stage of lactation and individuality of the animal also contribute appreciably to these variations. However, samples from pooled milk of a species have reasonably consistent levels. In the present study pooled buffalo milk samples were, therefore, used from the same source and 15 replications were performed to obtain representative mean values of chemical constituents. Table 4.1 shows the mean values of total solids, solids-not-fat, total protein, ash, acidity (as per cent lactic acid), pH and specific gravity of buffalo milk. Levels of these milk constituents are similar to those reported for buffalo by several workers (Dastur, 1956; Ghosh and Anantakrishnan, 1963, 1964 and 1965). As expected, however, the levels of total solids, solids-not-fat, total protein and fat were strikingly higher than those for cow milk in the literature (Schneider et al. 1948, and Ghosh and Anantakrishnan, 1964). Thus a systematic and thorough study of milk composition was essential for understanding the effects of milk constituents on the value of Pyne constant for buffalo milk. Pyne (1932) has reported two different conversion factors (Pyne constants) for human and bovine milks for the estimation of protein content by formal titration because of the higher content of whey protein than casein in the former and vice versa.

There is, however, no report in the literature on the effect of composition of buffalo milk on Pyne constant value. For calculating protein content of
Table 4.1: Gross chemical composition of raw pooled buffalo milk

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Constituent</th>
<th>Mean value(^*) + SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total solids (g/100g milk)</td>
<td>17.83 ± 0.2845</td>
</tr>
<tr>
<td>2.</td>
<td>Solids-not-fat (g/100g milk)</td>
<td>9.07 ± 0.2330</td>
</tr>
<tr>
<td>3.</td>
<td>Total protein (g/100g milk)</td>
<td>3.75 ± 0.0768</td>
</tr>
<tr>
<td>4.</td>
<td>Fat (g/100g milk)</td>
<td>8.76 ± 0.3602</td>
</tr>
<tr>
<td>5.</td>
<td>Ash (g/100g milk)</td>
<td>0.70 ± 0.0077</td>
</tr>
<tr>
<td>6.</td>
<td>Acidity (% lactic acid)</td>
<td>0.131 ± 0.0012</td>
</tr>
<tr>
<td>7.</td>
<td>pH</td>
<td>6.629 ± 0.0136</td>
</tr>
<tr>
<td>8.</td>
<td>Specific gravity at 28.9°C</td>
<td>1.026 ± 0.0004</td>
</tr>
</tbody>
</table>

\(^*\) Mean of 15 replications
buffalo milk by formal titration method Singhal and Raj (1989) have, however, used a Pyne constant of 1.75. Walstra and Jenness (1984) reported that titratable protons released by the action of formaldehyde amount to about 0.57 mol. kg of crude protein (often quoted as the reciprocal called "formal factor", that is, 1.75 kg of protein per mole of alkali consumed). Correction to a true protein basis by accounting for 5% nonprotein N makes the value 0.60 mol. H^+ per kg of protein. This is exactly the mean lysine content of the proteins, weighted by their concentrations.

4.2 Level and proportion of some important milk constituents affecting formal titration value

Formal titration method is widely used for the determination of the concentration of amino acids and proteins in pure solvents. However, estimations in a complex system such as milk is tedious because of the opacity of milk due to the colloidal system and the presence of several buffering substances such as carbon dioxide, proteins, phosphates, citrates and lactates which interfere in judgment of correct end point. Physico-chemical properties of caseins are reported to be influenced by their content of phosphorus (Manson, 1973). The disturbing effects of both colloidal and soluble phosphorus were eliminated by addition of potassium oxalate to cow milk during formal titration (Pyne, 1932). Addition of potassium oxalate to milk shifts the ionic equilibria between the micelle external environment and internal one, until a new equilibrium is reached. During the
first titration with alkali, the carboxyl groups of the amino acid residues present (both C-terminal and side chain) on the polypeptide chains and of the free amino acids, bind sodium and release colloidal phosphate and citrate (Woifschoon and Vargas, 1978). For a comparative study of formal titration of buffalo milk for estimation of protein content several compositional factors that distinguish buffalo milk from cow milk, therefore, must be considered for the determination of Pyne constant. These factors include the level and proportion of protein fractions and salt balance in milk. Some important constituents of buffalo milk were investigated and their mean values of 15 replications are compiled in Table 4.2. From the data on buffalo milk it is clear that levels of casein, whey protein, total protein, calcium and phosphorus are higher than those reported for cow milk. Higher levels of protein, calcium and phosphorus in buffalo milk than in cow milk is responsible for the increased opacity of the former (Anantakrishnan et al., 1943; Schneider et al., 1948; Davies and White, 1960; Sabarwal and Ganguli, 1968; Ganguly and Menon, 1971 and Sindhu and Roy, 1973 and 1976). However, the percentage of casein and whey protein in total protein of buffalo milk is nearly the same as reported in the literature for cow milk (Ganguli, 1973 and 1974). This shows that the value of Pyne constant for total protein estimation is unlikely to be affected to great extent as these two species have similar proportions of casein and whey proteins in total protein. The
Table 4.2: Level and proportion of some important milk constituents affecting formal titration value

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Constituent</th>
<th>Level* (g per 100 g milk)</th>
<th>Relative constituents</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Casein</td>
<td>3.00 ± 0.0637</td>
<td>Casein/Total protein(%)</td>
<td>79.98</td>
</tr>
<tr>
<td>2.</td>
<td>Whey protein</td>
<td>0.71 ± 0.0188</td>
<td>Whey protein/Total protein(%)</td>
<td>19.04</td>
</tr>
<tr>
<td>3.</td>
<td>Total protein</td>
<td>3.75 ± 0.0763</td>
<td>Whey protein/Casein</td>
<td>0.2381</td>
</tr>
<tr>
<td>4.</td>
<td>Calcium</td>
<td>173.53 ± 1.1104</td>
<td>Casein/Fat</td>
<td>0.3421</td>
</tr>
<tr>
<td>5.</td>
<td>Phosphorus</td>
<td>127.83 ± 5.5795</td>
<td>Calcium/Phosphorus</td>
<td>1.3574</td>
</tr>
</tbody>
</table>

* Mean ± SD of 15 replications
calcium/phosphorus ratio was found to be 1.3574 in buffalo milk which is higher than that reported for cow milk (1.1367 to 1.3299). Higher proportion of calcium in buffalo milk than cow milk may cause higher precipitation of calcium phosphate, faster fading of pink colour end point as a result of continuous precipitation of calcium phosphate and ratio of calcium to phosphorus may also be altered differently on addition of potassium oxalate. The mean value of casein/fat ratio in buffalo milk was 0.34 which is lower than the value required in milk for good cheese making (Scott, 1981; Price and Germain, 1931; Kosikowski, 1970 and McDowall, 1936). Casein/fat ratio is generally adjusted by first estimating the casein content by formal titration and multiplying by a factor of 1.38 [SP.IS(Part XI)-1981]. Use of this conversion factor has not been specified for milk of a particular species. More reliable estimate of casein in buffalo milk can be made if the conversion factor for casein of this species is used. Caseins in the milks of cow and buffalo differs widely in their chemical compositions, size of micelles and rennet coagulation properties (Shama Sastry and Dastur, 1947; Prodanski and Petrov, 1962; Puri and Prakash, 1962; Dilanyan and Agababyan, 1962 and 1963, and Sabarwal and Ganguli, 1968).

4.3 Titratable acidity and formal titration value of buffalo milk

Titratable acidities and formal titre values of buffalo milk were assessed by three methods (T1, T2 and T3
and their mean values are presented in Table 4.3. It is observed that titratable acidity (first titration value) of milk was reduced when Ca ion chelators were added to bind calcium ion with a view to improve the judgment of end point with phenolphthalein. Effect of potassium oxalate was so great that milk appears pink immediately after addition of potassium oxalate. In order to match the pink shade of test sample with the blank, titration of milk was performed after addition of 0.5 ml N/10 hydrochloric acid. Formal titre values were strikingly similar in T₁ and T₃ methods but higher in T₂ method for the same concentration of total protein determined by Kjeldahl method. Use of potassium oxalate in T₂ method enhanced the formal titre value possibly due to the exposure of titratable groups of micellar casein on removal of colloidal calcium phosphate. According to Ling (1963) oxalate reacts with colloidal calcium to form alkaline phosphate which interferes with subsequent milk titration. However, Wolfschoon and Vargas (1978) suggest that the amount of calcium removed is not enough to destabilize the macromolecule, but is quite enough to expose new titratable groups.

4.4 Titratable acidity and formal titration value of casein isolated from milk

Casein was isolated from fresh pooled milk by acidification with acetic acid and sodium acetate and removal of whey by centrifugation. The sedimented casein was
Table 4.3: Mean values of titratable acidity and formal titre of milk by three methods and corresponding total protein content of the milk

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Treatment</th>
<th>Titratable acidity* (ml of 1N NaOH/100 ml milk)</th>
<th>Formal titre* (ml of 1N NaOH/100 ml milk)</th>
<th>Total protein* (g/100 ml milk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>1.5033 ± 0.0129</td>
<td>2.1267 ± 0.0186</td>
<td>3.85 ± 0.0785</td>
</tr>
<tr>
<td>2</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.0000 ± 0.0000</td>
<td>2.2767 ± 0.0220</td>
<td>3.85 ± 0.0785</td>
</tr>
<tr>
<td>3</td>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.2050 ± 0.0140</td>
<td>2.1300 ± 0.0185</td>
<td>3.85 ± 0.0785</td>
</tr>
</tbody>
</table>

* Mean ± SD of 15 replications

T<sub>1</sub> No Ca ion chelator
T<sub>2</sub> Potassium oxalate as Ca ion chelator
T<sub>3</sub> Tetrasodium pyrophosphate as Ca ion chelator
dispersed in distilled water and proceeded for titration as described in Section 3.5.2.2. Mean values of titratable acidity and formal titre of casein determined by three methods (T₁, T₂ and T₃) are presented in Table 4.4. The values of titratable acidities of casein in the presence of Ca ion chelators were lower than those without any Ca ion chelator. However, formal titre values were similar in T₁ and T₃ and were lower than the values in T₂ for the same concentration of casein determined by Kjeldahl method. From these results it appears that even after leaching of colloidal calcium phosphate from casein micelle and removal of most of soluble titratable substances the effect of potassium oxalate on formal titration is not abolished and a higher formal titre value was obtained. On the other hand removal of calcium by tetrasodium pyrophosphate in T₂ had a negligible increase in formal titre value compared to T₁ where no Ca ion chelator was used. This indicates mere removal of calcium does not affect formal titration and that potassium oxalate increases the formal titre value by exposing more titrable groups in the casein. Wolfschoon and Vargas (1978) have also made similar suggestion on the action of potassium oxalate on formal titration of milk.

4.5 Titratable acidity and formal titration value of whey from milk

Whey prepared from milk by acidification and centrifugation as described in Section 3.5.2.3 was analysed for titratable acidity and formal titre value by three
Table 4.4: Mean values of titratable acidity and formal titre of casein by three methods and corresponding casein content of milk

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Treatment</th>
<th>Titratable acidity*</th>
<th>Formal titre*</th>
<th>Casein* by Kjeldahl method (g/100 ml milk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T1</td>
<td>3.6683 ± 0.0148</td>
<td>1.6200 ± 0.0154</td>
<td>3.07 ± 0.0657</td>
</tr>
<tr>
<td>2</td>
<td>T2</td>
<td>3.1717 ± 0.0248</td>
<td>1.7833 ± 0.0254</td>
<td>3.07 ± 0.0657</td>
</tr>
<tr>
<td>3</td>
<td>T3</td>
<td>3.4663 ± 0.0265</td>
<td>1.6267 ± 0.0181</td>
<td>3.07 ± 0.0657</td>
</tr>
</tbody>
</table>

* Mean ± SD of 15 replications

T1 No Ca ion chelator
T2 Potassium oxalate as Ca ion chelator
T3 Tetrasodium pyrophosphate as Ca ion chelator
methods (T₁, T₂ and T₃) and mean values of 15 replications are presented in Table 4.5. It can be seen from data that the titratable acidities of whey were in the decreasing order of the methods T₂<T₃<T₁. This order of acidities clearly reflects that potassium oxalate depresses titratable acidity of whey greater than tetrasodium pyrophosphate and both these Ca ion chelators suppresses the titratable acidity of untreated whey in a similar way as in whole milk. However, formal titre values of whey were strikingly similar in all the methods for the same whey. Whey protein content was 0.74 per cent (w/v) in all the cases as determined by Kjeldahl method. On comparing the effects of Ca ion chelators on total protein, casein and whey protein in formal titration, it is evident that Ca ion chelators do not expose titratable groups in whey protein unlike the casein since colloidal calcium phosphate and other metal ions are not involved in the quartenary structure of whey proteins. Moreover, lower opacity of whey due to lower protein content may also eliminate the errors in the judgment of pink colour end point in formal titration.

4.6 Comparative assessment of Pyne constants by three methods of formal titration

Randomized block design of statistical analysis was followed for assessment of Pyne constants by three different methods of formal titration.
Table 4.5: Mean values of titratable acidity and formal titre of whey by three methods and corresponding whey protein content of milk

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Treatment</th>
<th>Titratable acidity* (ml of 1N NaOH for Whey/100 ml milk)</th>
<th>Formal titre* (ml of 1N NaOH for Whey/100 ml milk)</th>
<th>Whey protein* by Kjeldahl method (g/100 ml milk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T₁</td>
<td>13.23 ± 0.0455</td>
<td>0.5006 ± 0.0388</td>
<td>0.73 ± 0.0195</td>
</tr>
<tr>
<td>2</td>
<td>T₂</td>
<td>10.58 ± 0.0530</td>
<td>0.5010 ± 0.0417</td>
<td>0.73 ± 0.0195</td>
</tr>
<tr>
<td>3</td>
<td>T₃</td>
<td>11.38 ± 0.1001</td>
<td>0.5006 ± 0.0437</td>
<td>0.73 ± 0.0195</td>
</tr>
</tbody>
</table>

* Mean ± SD of 15 replications

T₁ No Ca ion chelator
T₂ Potassium oxalate as Ca ion chelator
T₃ Tetrasodium pyrophosphate as Ca ion chelator
4.6.1 Pyne constants for total protein, casein and whey protein from the formal titration of milk alone

4.6.1.1 Pyne constants for total protein content of milk

Fifteen replications were done for the evaluation of Pyne constants by three methods (T₁, T₂ and T₃). The range of variations and mean values of Pyne constants calculated from formal titre values and total protein content determined by Kjeldahl method are shown in Table 4.6.1.1. The conversion factors (Pyne constants) were calculated for expressing total protein content of milk on both weight/weight basis and weight/volume basis so as to avoid any confusion in their usage for obtaining protein content in per cent. The mean values of Pyne constants were 1.7620, 1.6482 and 1.7592 for w/w basis and 1.8078, 1.6918 and 1.8051 for w/v basis, respectively in T₁, T₂ and T₃ methods. In either case, the Pyne constant values were in the increasing order of the methods T₁ > T₃ > T₂. This shows that the Pyne constant value is higher in the absence of Ca ion chelator than in its presence and that the type of chelator also influenced its value. However, statistical analysis showed nonsignificant difference in T₁ (no Ca ion chelator) and T₃ (tetrasodium pyrophosphate as Ca ion chelator) in their values of Pyne constant but T₂ (potassium oxalate as Ca ion chelator) showed significant (P<0.05) differences when compared to T₁ treatment. T₂ and T₃ also differed significantly. An increase in formal titre value
Table 4.6.11: Comparative assessment of Pyne constants for determination of total protein content in milk by three methods of formal titration

<table>
<thead>
<tr>
<th>No.</th>
<th>Expression in milk</th>
<th>Mean value of Pyne constant in formal titration method</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>SEM</th>
<th>CD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Weight/</td>
<td></td>
<td>1.7620</td>
<td>1.6486</td>
<td>1.7592</td>
<td>0.0023</td>
<td>0.0066</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>Weight basis</td>
<td>(1.7266-1.7864)</td>
<td>(1.6189-1.7854)</td>
<td>(1.7266-1.7864)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Height/</td>
<td></td>
<td>1.8879</td>
<td>1.6918</td>
<td>1.8851</td>
<td>0.0023</td>
<td>0.0066</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Volume basis</td>
<td>(1.7719-1.8327)</td>
<td>(1.6618-1.7497)</td>
<td>(1.7719-1.8327)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Figures in parentheses are ranges of Pyne constant values
* Mean value of 5 replications
* Nonsignificant difference between T1 and T3
* Significant difference between T1 and T2, and T2 and T3
lowered the Pyne constant value, probably due to exposure of titratable groups in casein. Pyne (1932) also observed higher formal titre values when potassium oxalate was used but reason for this effect was not given by him. Pearson (1971) also reported higher conversion factor when oxalate was omitted confirming the occurrence of higher formal titre value when oxalate is used. Pyne (1932), however, reported that the use of potassium oxalate eliminates the disturbing effects of both colloidal and soluble phosphates in formal titration. On the other hand, Jenness and Patton (1969) reported that potassium oxalate caused an increase in pH due to release of phosphate ion which combined with hydrogen ion. Wolfschoon and Vargas (1978) have proposed the idea that potassium oxalate in milk shifts the ionic equilibria between the micelle external environment and the internal one, until a new equilibrium is reached. Probably, the amount of calcium removed is not enough to destabilize the macromolecule, but is quite enough to expose new titratable groups. A scheme of reactions involved in oxalate method is given in Fig.4.1.

The Pyne constant values standardized by T2 method for determination of total protein on w/w and w/v basis of buffalo milk were found to be higher than the values of bovine milk (1.70, w/w ; 1.74, w/v) reported by Pyne (1932). This indicates that Pyne constants are species specific and are controlled by the genetic apparatus of mammary gland through the synthesis of their own proteins and
A. Reactions during first titration

1. \[
\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_3 + \overset{\circ}{\text{C}}\text{-CH}_2\text{-CH}_2 \xrightarrow{\text{Oxalate}} \overset{\circ}{\text{C}}\text{-CH}_2\text{-CH}_2\text{NH}_3 + \text{CH}_2\text{CH}_2\text{NH}_3
\]

2. \[
\text{Ca}_5(\text{PO}_4)_6 \xrightarrow{} 3\text{Ca}_3(\text{PO}_4)_2
\]

3. \[
\text{Ca}_3(\text{PO}_4)_2 + 3\text{COOK} \xrightarrow{} 3(\text{COO})_2\text{Ca} + 2K_3\text{PO}_4 \quad \text{Alkaline}
\]

4. \[
\text{K}_3\text{PO}_4 + 3\text{HCl} \xrightarrow{} \text{H}_2\text{PO}_4 + \text{HPO}_4 + 3\text{KCl}
\]

5. \[
2\text{H}_2\text{PO}_4 + 3\text{Ca}^{++} \xrightarrow{\text{PH 7,6}} \text{Ca}_3(\text{PO}_4)_2 + 4\text{H}^+
\]

\[
4\text{H}^+ + 4\text{OH}^- \xrightarrow{\text{NaOH}} 4\text{H}_2\text{O}
\]

B. Reactions during second titration

1. \[
\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_3 + \overset{\circ}{\text{C}}\text{-CH}_2\text{-CH}_2 \xrightarrow{+\text{HCHO}} \overset{\circ}{\text{C}}\text{-CH}_2\text{-CH}_2\text{NH}_3 + \text{HCHO}
\]

2. \[
\overset{\circ}{\text{C}}\text{-CH}_2\text{-CH}_2\text{NH}_3 + \text{HCHO} \xrightarrow{} (\text{CH}_2)\text{NH}_2\text{CH}_2\text{OH} + \overset{\circ}{\text{C}}\text{-CH}_2\text{CH}_2
\]

3. \[
\overset{\circ}{\text{C}}\text{-CH}_2\text{CH}_2\text{NH}_3 \xrightarrow{\text{NaOH}} \overset{\circ}{\text{C}}\text{-CH}_2\text{CH}_2\text{NH}_3 + \text{H}_2\text{O}
\]

Fig. 4.1: Reaction mechanisms in oxalate method of formal titration of milk
transporting them into milk along with other milk constituents.

4.6.1.2 Pyne constants for casein content of milk

Estimation of casein by formal titration of milk is a favoured method for adjusting casein/fat ratio of milk in cheese industry, because it is simple and quick method of estimating protein. However, the Pyne constant value for estimation of casein in Indian buffalo milk is not available in the literature. In the present investigation 15 replications for the estimation of casein in milk by formal titration was done and the results are presented in Table 4.6.1.2. As is the customary the casein content is calculated from the formal titration of milk rather than isolated casein, therefore, for buffalo milk also same procedure was adopted in this study. The formal titration of milk was, however, done by three different methods (T₁, T₂ and T₃) but the casein content of the milk was first determined by standard reference method, that is, Kjeldahl method by precipitation of casein from milk as described in Section 3.4.9.2. The Pyne constants were then calculated for each method by dividing the formal titre values (ml of 1N sodium hydroxide per 100 ml milk) obtained separately in each method and the Pyne constants thus calculated were used for expressing the content of casein in milk on w/w basis. For expressing the casein content on w/v basis the casein content in Kjeldahl method was multiplied by specific gravity of milk and then proceeded for calculation of Pyne constants. The
Table 4.6.1.2: Comparative assessment of Pyne constants for determination of casein content in milk by three methods of formal titration of milk

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>System of expression</th>
<th>Mean value of Pyne constant in formal titration method</th>
<th>SEM</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Weight/Height</td>
<td>1.4093</td>
<td>1.3163</td>
<td>1.4878</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td>Weight basis</td>
<td>(1.3874-1.4249)</td>
<td>(1.3887-1.3299)</td>
<td>(1.3874-1.4249)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Weight/Volume</td>
<td>1.4468</td>
<td>1.3507</td>
<td>1.4437</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td>Volume basis</td>
<td>(1.4255-1.4640)</td>
<td>(1.3345-1.3664)</td>
<td>(1.4235-1.4619)</td>
<td></td>
</tr>
</tbody>
</table>

† Mean value of 5 replications

‡ Figures in parentheses are ranges of Pyne constant values

§§ Nonsignificant difference between T1 and T3

§§§ Significant difference between T1 and T2 and T2 and T3
Pyne constants for casein calculated for both the systems of expression in milk were in the increasing order of the method $T_1 > T_3 > T_2$. Statistical analysis, however, showed that differences in the Pyne constant values were significant ($P < 0.05$) in $T_1$ and $T_2$, and $T_2$ and $T_3$, but there was no significant difference in $T_1$ and $T_3$ methods.

This shows that Pyne constant value was unaffected whether tetrasodium pyrophosphate was used as Ca ion chelator or no Ca ion chelator was used, but the value of Pyne constant in $T_2$ is significantly decreased to 1.3163 (w/w basis) and this value is higher than the value of Pyne constant (1.28) reported for bovine milk (Pyne, 1932). BIS [SP. IS. (Part XI)-1981] has reported a higher value of the Pyne constant (1.38) than found in the present investigation using the same method (oxalate method) of formal titration. In the BIS method it has not been clarified whether the Pyne constant value of 1.38 is applicable to cow milk or buffalo milk or mixed milk. Since in the present investigation the Pyne constant value of 1.3183 has been determined for buffalo milk by following the same procedure as outlined for bovine milk by Pyne (1932), it appears that the differences may be real due to the differences in the casein makeup or it may be due to the interference by higher level of calcium and phosphorus in buffalo milk. On comparing the three methods it is surprising that only $T_2$ method in which potassium oxalate had been used as Ca ion chelator that the Pyne constant value was drastically lowered. This is due to the
increase in the formal titre value by virtue of its action to expose more titratable groups from the casein micelle by chelation of colloidal calcium. The interference by oxalate in titration of milk has been reported by several workers (Ling, 1963; Jenness and Patton, 1969 and Wolfschoon and Vargas, 1977).

El-Sokkary and Hassan (1953) reported a conversion factor of 0.2166 for casein nitrogen by formal titration of buffalo milk without the use of Ca ion chelators. This factor when multiplied by 6.38 gives a casein conversion factor of 1.3819 which is almost the same as given by BIS [SP.IS. (Part XI)-1981] but is slightly lower than the casein conversion factor (1.4093) in T1 treatment in the present investigation. From these reports in the literature it appears that more extensive work is needed not only for the procedure to be adopted but also to determine the variations due to the breeds of animals, stage of lactation, seasonal variation and regional variations.

4.8.1.3 Pyne constants for whey protein content of milk

Pyne constants for whey protein determination were calculated from the formal titre value of milk rather than whey using three methods. For this the whey protein content was first determined by separating whey from milk and then proceeding for its determination by Kjeldahl method as described in Section 3.4.9.3. Pyne constants determined by dividing the whey protein content, as
determined by Kjeldahl method, by formal titre values of milk found in each method. The range of variation in Pyne constants and their mean values of 15 replications determined by three methods are presented in Table 4.6.1.3. The mean value for Pyne constants were 0.3348, 0.3130 and 0.3343 on w/w basis of milk and 0.3436, 0.3209 and 0.3430 on w/v basis of milk, respectively in T1, T2 and T3 methods. The increasing order of Pyne constant values in either case was in the order of T1 > T2 > T3 methods. Statistical analysis of the Pyne constants obtained by three methods showed significant difference (P<0.05) between T1 and T2, and T3 methods. However, differences in Pyne constant values between T1 and T3 were nonsignificant. These differences clearly reflects the behaviour of formal titre values of milk obtained by titration of milk by three methods as described in Section 4.3. It appears from Table 4.5 that formal titre value of whey is not influenced by the method of formal titration used. Therefore, the differences that exist in Pyne constants actually reflects interference by added potassium oxalate in milk by virtue of its effect of destabilization of colloidal calcium phosphate in the casein micelles.

No literature value of Pyne constant for whey protein is available either for cow or buffalo milk determined by any of the methods so far.

4.6.2 Pyne constants for casein and whey proteins from the formal titration of milk fractions
Table 4.6.1.3: Comparative assessment of Pyne constants for determination of whey protein content in milk by three methods of formal titration of milk

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Expression in milk</th>
<th>Mean value of Pyne constant in formal titration method</th>
<th>SEM</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T₁</td>
<td>T₂</td>
<td>T₃</td>
<td>SEM</td>
</tr>
<tr>
<td>1.</td>
<td>Weight/Height</td>
<td>0.3348</td>
<td>0.3132</td>
<td>0.3343</td>
<td>0.0003</td>
</tr>
<tr>
<td>(0.3225-0.3509)</td>
<td>(0.3017-0.3275)</td>
<td>(0.3194-0.3509)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Weight/Volume</td>
<td>0.3436</td>
<td>0.3289</td>
<td>0.3430</td>
<td>0.0002</td>
</tr>
<tr>
<td>(0.3321-0.3600)</td>
<td>(0.3100-0.3360)</td>
<td>(0.3282-0.3600)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean value of 5 replications
** Figures in parentheses are ranges of Pyne constant values
*** Nonsignificant difference between T₁ and T₃
   Significant difference between T₁ and T₂, and T₂ and T₃
4.6.2.1 Pyne constants for casein content of milk from formal titration of casein solution

An accurate estimation of casein from the formal titration of milk is complicated when Ca ion chelators are used to improve the judgment of end point. This has been observed that several substances such as colloidal calcium phosphate and soluble calcium phosphate are the prominent interfering substances. In an attempt to eliminate these interfering effects, formal titration was performed on isolated casein obtained from milk. The formal titration of casein solutions was carried out by three methods (T₁, T₂ and T₃) and the casein content in the isolated wet casein was determined by Kjeldahl method. From the formal titre values of casein solution and content of casein in wet casein by Kjeldahl method, the Pyne constants were calculated. The range and mean values of 15 replications of Pyne constants are represented in Table 4.6.2.1 for calculating casein content in milk on w/w and w/v basis. On either basis, the Pyne constant values in the increasing order were for methods T₃ > T₂ > T₁. However the differences between T₁ and T₃ were nonsignificant indicating the value of Pyne constant is unaffected whether tetrasodium pyrophosphate was used as Ca ion chelator or no Ca ion chelator was used in the procedure. Pyne constant in method T₂ differed significantly from those in T₁ and T₃ indicating that effect of potassium oxalate on the formal titre value of casein solutions is possibly due to
Table 4.6.2.1: Comparative assessment of Pyne constants for determination of casein content in milk by three methods of formal titration of casein solution

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>System of expression in milk</th>
<th>Mean value of Pyne constant in formal titration method</th>
<th>SEM</th>
<th>CO</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Weight/ 1.5499</td>
<td>1.7045</td>
<td>1.8424</td>
<td>0.0076</td>
<td>0.0106</td>
</tr>
<tr>
<td></td>
<td>Volume 1.5892</td>
<td>1.7490</td>
<td>1.8905</td>
<td>0.0036</td>
<td>0.0095</td>
</tr>
</tbody>
</table>

Figures in parentheses are ranges of Pyne constant values.

Mean value of 5 replications

Nonsignificant difference between T<sub>1</sub> and T<sub>2</sub>, and T<sub>2</sub> and T<sub>3</sub>

Significant difference between T<sub>1</sub> and T<sub>3</sub>
exposure of charged groups from casein structures. It is well known that upon acidification colloidal calcium phosphate migrate from the casein micelles to the soluble state and possibly almost all is lost in the whey. The precipitated casein may still retain the submicellar structure involving Ca ion bridges but not involving colloidal calcium phosphate and may expose charged groups of $\alpha_{S}$- and $\beta$- caseins on treatment with potassium oxalate and causing an increase in formal titre value and hence lowered Pyne constant.

4.6.2.2 Pyne constants for whey protein content of milk from formal titration of whey

The formal titration of isolated whey fraction of milk was done in order to investigate the accuracy of the method when whey proteins are estimated by direct titration of milk than when formal titration of isolated whey fraction of milk. It was observed that in all the three methods the Pyne constant values were nearly the same, that is, 1.4087, 1.3852 and 1.3852 on w/w basis and 1.4454, 1.4315 and 1.4315 on w/v basis respectively in $T_1$, $T_2$ and $T_3$ treatments. The range of Pyne constants and their mean values are shown in Table 4.6.2.2. From the statistical analysis of Pyne constant values, it was observed that there was nonsignificant effect of Ca ion chelators on the Pyne constant values. This indicates that all the soluble constituents of milk are without any affect on the formal titration of whey proteins and the differences in Pyne constants when calculated from formal titration of milk were
Table 4.6.2.2: Comparative assessment of Pyne constants for determination of whey protein content in milk by three methods of formal titration of whey

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>System of expression</th>
<th>Mean value of Pyne constant in formal titration method</th>
<th>T&lt;sub&gt;1&lt;/sub&gt;</th>
<th>T&lt;sub&gt;2&lt;/sub&gt;</th>
<th>T&lt;sub&gt;3&lt;/sub&gt;</th>
<th>SEM</th>
<th>CD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Weight/Height basis</td>
<td>1.4087&lt;br&gt;(1.3316-1.4738)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.3952</td>
<td>1.3952</td>
<td>0.0050 NS***</td>
<td>1.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4454&lt;br&gt;(1.3682-1.5122)</td>
<td>1.4315</td>
<td>1.4315</td>
<td>0.0051 NS***</td>
<td>1.38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean value of 5 replications
** Figures in parentheses are ranges of Pyne constant values
*** Nonsignificant difference between T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub>
mainly due to exposure of certain titratable groups possibly in the casein submicelles.

4.7 Descriptive analysis of Pyne constants

Coefficient of variations of Pyne constants for total protein, casein and whey protein in each method of formal titration were evaluated to check the precision of each Pyne constant in 15 replications of buffalo milk and have been discussed in the following sections.

4.7.1 Coefficient of variations of Pyne constants determined from formal titre values of milk

Coefficient of variations and mean values of Pyne constants for total protein, casein and whey proteins determined from formal titration of milk by three methods are presented in Table 4.7.1. From the data it is clear that in all the methods the coefficient of variations of Pyne constants for total protein, casein and whey protein were quite lower than 14 per cent. Therefore, the Pyne constants determined have high precision and can be used for accurate determination of each class of protein from the formal titration of milk by any of the three methods.

4.7.2 Coefficient of variations of Pyne constants determined from formal titre values of milk fractions

Coefficient of variations and mean values of Pyne constants for casein and whey proteins determined from formal titration of milk fractions by three methods are
Table 4.7.1: Comparative appraisal of Pyne constants of buffalo milk (Calculated from formal titre values of milk alone) by three methods of formal titration

<table>
<thead>
<tr>
<th>Sr. NO.</th>
<th>Class of milk protein</th>
<th>Mean value* of Pyne constant in formal titration method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T₁</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total protein</td>
</tr>
<tr>
<td>A. Weight/Weight:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Total protein</td>
<td>1.7620(1.0556)</td>
</tr>
<tr>
<td>2</td>
<td>Casein</td>
<td>1.4093(0.8444)</td>
</tr>
<tr>
<td>3</td>
<td>Whey protein</td>
<td>0.3348(2.6583)</td>
</tr>
<tr>
<td>B. Weight/Volume:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Total protein</td>
<td>1.8079(1.0509)</td>
</tr>
<tr>
<td>2</td>
<td>Casein</td>
<td>1.4460(0.8056)</td>
</tr>
<tr>
<td>3</td>
<td>Whey protein</td>
<td>0.3436(2.5902)</td>
</tr>
</tbody>
</table>

* Mean value of 15 replications
** Figures in parentheses are values of CV% of respective treatment
presented in Table 4.7.2. From the data it is clear that in all the methods the coefficient of variations of Pyne constants for casein and whey protein were quite lower than 14 per cent. Therefore, the Pyne constants determined have high precision and can be used for accurate determination of casein and whey protein from the formal titration of milk fractions by any of the three methods.

4.8 Comparative appraisal of formal titration methods with Kjeldahl method

4.8.1 Comparative appraisal of formal titration methods with Kjeldahl method for estimation of total protein, casein and whey proteins determined from formal titre values of milk alone

The reliability of each method of formal titration for its accuracy for protein estimates was determined by comparing with the Kjeldahl method. The mean values of Pyne constants of 15 replications determined for total protein, casein and whey protein from formal titre value of same milk by T₁, T₂ and T₃ methods separately were multiplied by the formal titre value of milk in each replication in each method separately to calculate the contents of total protein, casein and whey proteins in each replication by each method. Mean values and standard error of the estimates of total protein, casein and whey protein in T₁, T₂ and T₃ methods were analysed statistically and presented in Table 4.8.1. The contents of total protein,
Table 4.7.2: Comparative appraisal of Pyne constants for buffalo milk casein and whey protein (calculated from formal titre values of casein solution and whey) determined by three methods of formal titration

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Class of milk protein</th>
<th>A. Weight/weight:</th>
<th>B. Weight/Volume:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean value* of Pyne constant in formal titration method</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>1</td>
<td>Casein</td>
<td>1.8499 (0.8325)</td>
<td>1.7045 (1.4902)</td>
</tr>
<tr>
<td>2</td>
<td>Whey protein</td>
<td>1.4087 (2.7543)</td>
<td>1.3952 (2.9130)</td>
</tr>
<tr>
<td>1</td>
<td>Casein</td>
<td>1.8982 (0.8482)</td>
<td>1.7490 (1.5084)</td>
</tr>
<tr>
<td>2</td>
<td>Whey protein</td>
<td>1.4454 (2.7328)</td>
<td>1.4315 (3.1322)</td>
</tr>
</tbody>
</table>

* Mean value of 15 replications
** Figures in the parentheses are values of CV% of respective treatment
Table 4.8.1: Comparative appraisal of formal titration methods with Kjeldahl method for estimation of total protein, casein and whey proteins determined from formal titre values of milk alone

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Class of milk protein</th>
<th>Mean value* of protein content (%)</th>
<th>Formal titration method</th>
<th>Kjeldahl method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>A. Weight/Weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Total protein</td>
<td>3.7472(0.0103)</td>
<td>3.7447(0.0094)</td>
<td>3.7471(0.0143)</td>
</tr>
<tr>
<td>2.</td>
<td>Casein</td>
<td>2.9971(0.0066)</td>
<td>2.9969(0.0059)</td>
<td>2.9968(0.0057)</td>
</tr>
<tr>
<td>3.</td>
<td>Whey protein</td>
<td>0.7116(0.0046)</td>
<td>0.7125(0.0045)</td>
<td>0.7120(0.0046)</td>
</tr>
<tr>
<td>B. Weight/Volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Total protein</td>
<td>3.8448(0.0105)</td>
<td>3.8517(0.0095)</td>
<td>3.8449(0.0103)</td>
</tr>
<tr>
<td>2.</td>
<td>Casein</td>
<td>3.0752(0.0068)</td>
<td>3.0751(0.0062)</td>
<td>3.0751(0.0059)</td>
</tr>
<tr>
<td>3.</td>
<td>Whey protein</td>
<td>0.7307(0.0048)</td>
<td>0.7306(0.0047)</td>
<td>0.7306(0.0050)</td>
</tr>
</tbody>
</table>

* Mean value of 15 replications

** Figures in parentheses are standard errors of protein values of that treatment when those were compared to protein values of Kjeldahl method
casein and whey protein have been presented on both w/w and w/v basis of milk for comparison with the estimates by Kjeldahl method. Statistical analysis of the estimates by formal titration showed nonsignificant difference in each of the estimates for total protein, casein, whey proteins between formal titration methods and Kjeldahl method. Therefore, any of the method can be used with reasonable accuracy for the estimation of total protein, casein and whey proteins provided that Pyne constant calculated for each of the protein by the particular method adopted is used.

4.8.2 Comparative appraisal of formal titration methods and Kjeldahl method of casein and whey protein estimation in milk fractions

Milk was fractionated by acidification into casein and whey. Casein was dispersed in 20 ml distilled water while whey was used as such for formal titration by three methods (T₁, T₂ and T₃). Formal titre values of casein solutions and whey were separately multiplied by their respective Pyne constants previously standardized (Section 4.6.2.1 and 4.6.2.2) to obtain casein and whey protein contents. Fifteen such replications were analysed statistically and their mean values with standard error are presented in Table 4.8.2. and compared with the estimates of casein and whey proteins by Kjeldahl method. The contents of casein and whey protein have been presented on both w/w and w/v basis of milk. Differences in the estimates of casein and whey protein between formal titration methods and Kjeldahl
Table 4.8.2: Comparative appraisal of formal titration methods with Kjeldahl method for estimation of casein and whey protein from formal titre values of casein solution and whey respectively.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Class of milk protein</th>
<th>Mean value* of protein content (%)</th>
<th>Formal titration method</th>
<th>Kjeldahl method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T₁</td>
<td>T₂</td>
</tr>
<tr>
<td>A.Weight/Weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Casein</td>
<td>2.9968(0.0064)</td>
<td>2.9971(0.0310)</td>
<td>2.9970(0.0076)</td>
</tr>
<tr>
<td>2.</td>
<td>Whey protein</td>
<td>0.7138(0.0052)</td>
<td>0.7136(0.0056)</td>
<td>0.7139(0.0059)</td>
</tr>
<tr>
<td>B.Weight/Volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Casein</td>
<td>3.0751(0.0063)</td>
<td>3.0753(0.0366)</td>
<td>3.0752(0.0077)</td>
</tr>
<tr>
<td>2.</td>
<td>Whey protein</td>
<td>0.7323(0.0053)</td>
<td>0.7325(0.0057)</td>
<td>0.7325(0.0060)</td>
</tr>
</tbody>
</table>

* Mean value of 15 replications
** Figures in parentheses are standard errors of protein values of that treatment when those were compared to protein values of Kjeldahl method.
method were nonsignificant. This indicates that each method of formal titration for estimation of casein or whey protein in milk fractions is as accurate as Kjeldahl method of protein estimation.

On comparing the estimates of casein and whey protein by formal titration methods in Tables 4.8.1 and 4.8.2, it was observed that almost similar values were obtained whether estimated by formal titration of milk alone or its fractions separately. There were small differences in the standard errors of means between the two sets of formal titration methods compared to the estimates by Kjeldahl method.

4.9 Effect of storage of milk in the presence of chemical preservatives on formal titre values and total protein estimates by three methods of formal titration

Formal titration method of total protein estimation is usually performed on fresh and unpreserved milk. In the present investigation, therefore, stored H_{2}O_{2} - preserved and HCHO-preserved milk samples were analysed for assessing total protein content by three methods of formal titration. Formal titre values and total protein contents of fresh raw buffalo milk were compared with the stored and preserved milk samples by each method as described in the following sections.
4.9.1 Effect of preservatives on formal titre values of stored milk

Each of the H$_2$O$_2$ and HCHO-preserved milk samples which had been stored for 24 h at ambient temperature (30 ± 2 °C) were analysed for formal titre values by three methods and compared with the formal titre values of unpreserved fresh buffalo milk. Five replications of formal titrations by three methods each were done and analysed statistically by Factorial Randomized Block Design (FRBD). Data on formal titre values and statistical analysis were collected and presented in Table 4.9.1. Mean formal titre values of milk for unpreserved (P₀), H$_2$O$_2$— preserved (P₁) and HCHO— preserved (P₂) were 2.2283, 2.2381 and 1.1663 respectively. Lowered formal titre values in P₂ than either P₀ or P₁ is most likely due to the reaction of amino groups of proteins with formaldehyde action as preservative and thus partly enhancing the titration of carboxyl groups in first titration (titratable acidity) and when more of formaldehyde is added to milk for second titration (formal titration) the remaining part of titratable groups are titrated. Effect of formaldehyde addition in enhancing titratable acidity of milk has also been shown by other workers (Venkateswara Rao et al., 1950; Bejambes et al., 1956, and Ruiz and Part, 1957). Statistical analysis showed nonsignificant difference in the formal titre values of P₀ and P₁ but significant difference between P₂ and P₀ or P₁. This indicates that Pyne constants which have been standardized for unpreserved milk can be used only for
Table 4.9.1: Influence of preservatives on formal titre values of milk in three methods of formal titration

<table>
<thead>
<tr>
<th>A. Effect of Preservative</th>
<th>Mean formal titre value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No preservative (P₀)</td>
<td>2.2283</td>
</tr>
<tr>
<td>Hydrogen peroxide (P₁)</td>
<td>2.2381</td>
</tr>
<tr>
<td>Formaldehyde (P₂)</td>
<td>1.1683**</td>
</tr>
<tr>
<td>SEM</td>
<td>0.0036</td>
</tr>
<tr>
<td>CD</td>
<td>0.0105</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Effect of method</th>
<th>Mean formal titre value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No calcium ion chelator (T₁)</td>
<td>1.8463</td>
</tr>
<tr>
<td>Potassium oxalate (T₂)</td>
<td>1.9434**</td>
</tr>
<tr>
<td>Tetrasodium pyrophosphate (T₃)</td>
<td>1.8447</td>
</tr>
<tr>
<td>SEM</td>
<td>0.0036</td>
</tr>
<tr>
<td>CD</td>
<td>0.0105</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Interaction of preservative and method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preservative</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>P₀</td>
</tr>
<tr>
<td>P₁</td>
</tr>
<tr>
<td>P₂</td>
</tr>
<tr>
<td>SEM</td>
</tr>
<tr>
<td>CD</td>
</tr>
<tr>
<td>CV%</td>
</tr>
</tbody>
</table>

* Mean value of 5 replications
** Significant difference
H₂O₂—preserved milk but not for HCHO-preserved milk for calculating total protein content.

The study of the effects of method of titration showed mean formal titre values of milk as 1.8383, 1.9350 and 1.8383 respectively for T₁, T₂ and T₃ methods. Statistical analysis of data showed nonsignificant difference between T₁ and T₃ but significant difference between T₂ and T₁ or T₃ methods.

Interaction of preservative and method of formal titration was significant. This indicates that trend of treatments was same only in P₀ and P₁ samples but not in P₂. In P₀ and P₁ milks there is significant difference between T₂ and T₁ or T₃ methods and nonsignificant difference between T₁ and T₃ methods. But in P₂ milk there is no significant difference between T₁, T₂ and T₃ methods.

4.9.2 Comparative assessment of the recalculated Pyne constants by three methods of total protein determination by formal titration of HCHO-preserved milk (0.4 ml per 100 ml milk)

The results of the formal titration of HCHO-preserved milk (Section 4.9.1) have shown a significant decrease in formal titre values compared to formal titre values of unpreserved milk. Therefore, the Pyne constants calculated for unpreserved milk cannot be used for determination of total protein determination of HCHO-preserved milk. Hence, for calculating the total protein
content in HCHO-preserved milk the values of Pyne constants in each of the three methods were recalculated by dividing the protein content as in unpreserved milk by the formal titre values of HCHO-preserved milk. A comparative assessment of these recalculated Pyne constants have been presented in Table 4.9.2. From the data it is clear that quite high values of recalculated Pyne constants were obtained compared to the Pyne constant values calculated for unpreserved milk by each of the methods of formal titration. However, there were nonsignificant differences in recalculated Pyne constant values between three methods of formal titration.

4.9.3 Descriptive analysis of the recalculated Pyne constants for HCHO-preserved milk

Descriptive analysis of recalculated Pyne constants for total protein determination in HCHO-preserved milk was given in Table 4.9.3. From the values of coefficient of variation of the recalculated Pyne constants it is clear that the value in each of the method is quite lower than 14 per cent. Therefore, the recalculated Pyne constants for total protein determination in HCHO-preserved milk can be used for the precise determinations.

4.9.4 Comparative assessment of formal titration methods of total protein determination using recalculated Pyne constant for HCHO-preserved milk and unmodified Pyne constant for unpreserved milk

The reliability of each method of formal titration of HCHO-preserved milk for its accuracy for protein
Table 4.9.2: Comparative assessment of the recalculated Pyne constants by three methods of total protein determination by formal titration of HCHO-preserved milk (0.4 ml per 100 ml milk)

<table>
<thead>
<tr>
<th>Sr. System of No. expression</th>
<th>Mean value of recalculated Pyne constant in formal titration method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_1$</td>
</tr>
<tr>
<td>1. Weight/Weight basis</td>
<td>3.3018</td>
</tr>
<tr>
<td></td>
<td>(3.2175-3.4283)***</td>
</tr>
<tr>
<td>2. Weight/Volume basis</td>
<td>3.3877</td>
</tr>
<tr>
<td></td>
<td>(3.3012-3.5175)***</td>
</tr>
</tbody>
</table>

$\$ Mean value of 5 replications
$\$\$ Figures in parentheses are ranges of Pyne constants
$\$\$\$ Non-significant difference between $T_1$, $T_2$ and $T_3$
Table 4.9.3: Descriptive analysis of recalculated Pyne constants for total protein determination in HCHO-preserved milk

<table>
<thead>
<tr>
<th>Sr. System of expression</th>
<th>Mean value* of recalculated Pyne constant in formal titration method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T₁</td>
</tr>
<tr>
<td>1. Weight/Weight basis</td>
<td>3.3018 (2.7187)</td>
</tr>
<tr>
<td>2. Weight/Volume basis</td>
<td>3.3877 (2.7187)</td>
</tr>
</tbody>
</table>

* Mean value of 5 replications
** Figures in parentheses are the values of coefficient of variation of the recalculated Pyne constants
estimation was compared with the same method of formal titration of unpreserved milk. For this, the values of total protein content of HCHO-preserved milk (obtained with recalculated Pyne constants) were compared with the values of total protein content of unpreserved milk (obtained with unmodified Pyne constants) in each method of formal titration in Table 4.9.4. Statistical analysis by paired t-test showed that there is no significant difference between the protein values of HCHO-preserved milk and unpreserved milk in each method of formal titration. Therefore, any of the method can be used with reasonable accuracy for the estimation of total protein in HCHO-preserved milk.

4.10 Effect of neutralizers on formal titre values and total protein estimates by three methods of formal titration

Neutralization of high acid milk by sodium bicarbonate or sodium hydroxide is sometimes practised in dairy industry. In the present investigation, therefore, NaHCO₃-neutralized and NaOH-neutralized milk samples were analysed for assessing total protein content by three methods of formal titration. Formal titre values and total protein contents of fresh raw buffalo milk were compared with stored and neutralized milk samples by each method as described in the following sections.

4.10.1 Effect of neutralizers on formal titre values of stored milk

Each of the NaHCO₃- and NaOH - neutralized
Table 4.9.4: Comparative assessment of formal titration methods of total protein determination using recalculated Pyne constant for HCHO-preserved milk and un-modified Pyne constant for unpreserved milk

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>System of expression in milk</th>
<th>Method</th>
<th>Mean value* of total protein from formal titration (0.4 ml per 100 ml milk)</th>
<th>HCHO-preserved milk</th>
<th>Unpreserved milk (Fresh milk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. Weight/Weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>T₁</td>
<td>3.8453 (0.0458)**</td>
<td>3.8412</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>T₂</td>
<td>3.8479 (0.0611)</td>
<td>3.8417</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>T₃</td>
<td>3.8343 (0.0717)</td>
<td>3.8351</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. Weight/Volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>T₁</td>
<td>3.9454 (0.0470)</td>
<td>3.9412</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>T₂</td>
<td>3.9461 (0.0627)</td>
<td>3.9419</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>T₃</td>
<td>3.9342 (0.0736)</td>
<td>3.9351</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean value of 5 replications
** Figures in parentheses are standard errors of protein values of HCHO-preserved milk when these were compared to protein values of unpreserved milk
milk samples were analysed for formal titre values by three methods and compared with formal titre values of unpreserved fresh buffalo milk. Five replications of formal titration by three methods each were done and analysed statistically by FRBD. Data on formal titre values and statistical analysis were collated and presented in Table 4.10.1. Mean formal titre values of milk for unneutralized (N0), NaHCO3 neutralized (N1) and NaOH - neutralized (N2) were 2.2283, 2.0484 and 2.1403 respectively. Lowered values of formal titre in N1 and N2 than N0 are evidently due to lesser amount of hydrogen ions released from amino groups or available carboxyl groups or serine phosphate groups during second titration (formal titration) of milk. Mulay and Ladkani (1973) also observed lowered formal titre values in both cow and buffalo milks neutralized by sodium bicarbonate. Lowered formal titre value in neutralized soured milk than in titrated fresh milk with sodium hydroxide is probably due to migration of colloidal calcium phosphate into soluble state during souring of the former milk and thus exposing the hidden titratable groups of casein submicelles upon disintegration of micellar structure. Loss of micellar structure on removal of colloidal calcium phosphate has been reported by Schmidt and Buchheim (1970). Thus, it is quite possible that those exposed titratable groups are the charged serine phosphate residues of αs- and β-caseins in the submicelles and forms linkages with colloidal calcium phosphate which acts as a cementing agent between two or more
Table 4.10.1: Influence of neutralizers on formal titre values of milk in three methods of formal titration

<table>
<thead>
<tr>
<th>Effect of neutralizer</th>
<th>Formal titre value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No neutralizer ((N_0))</td>
<td>2.2283</td>
</tr>
<tr>
<td>Sodium bicarbonate ((N_1))</td>
<td>2.0484</td>
</tr>
<tr>
<td>Sodium hydroxide ((N_2))</td>
<td>2.1403</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td><strong>0.0057</strong></td>
</tr>
<tr>
<td><strong>CD</strong></td>
<td><strong>0.0166</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effect of method</th>
<th>Formal titre value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No calcium ion chelator ((T_1))</td>
<td>2.0799</td>
</tr>
<tr>
<td>Potassium oxalate ((T_2))</td>
<td>2.2523</td>
</tr>
<tr>
<td>Tetrasodium pyrophosphate ((T_3))</td>
<td>2.0849</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td><strong>0.0057</strong></td>
</tr>
<tr>
<td><strong>CD</strong></td>
<td><strong>0.0166</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Interaction of neutralizer and method</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SEM</strong></td>
<td><strong>0.0099</strong></td>
</tr>
<tr>
<td><strong>CD</strong></td>
<td><strong>NS</strong></td>
</tr>
</tbody>
</table>

**CV% 1.04**

NS: Nonsignificant
submicelles in the formation of micelles (Schmidt, 1980). In soured milk these serine phosphate residues get titrated during the first titration and remain unaffected on addition of formaldehyde, while serine phosphate residues in fresh milk are titrated after the disruption of ionic linkages between charged carboxylic groups of acidic amino acid residues and epsilon amino groups of lysine residues after the reaction of amino groups with formaldehyde; thus lowering the formal titre value in the soured milk. The lowered formal titre value in neutralized milks may also be due to reaction of neutralizer with protein when it is added. The neutralizer (alkali) will react with lysine and forms lysinoalanine (LAL) which was observed by Bohak (1964), Manson and Carolan (1980) and DeKoning and Rooijen (1982). Due to this reaction, availability of lysine during second titration may be lowered and thus lowered formal titre value may be obtained.

The study of the effects of the method of titration showed mean formal titre values of milk as 2.2300, 1.1663 and 2.2381 respectively for $T_1$, $T_2$ and $T_3$ methods. Statistical analysis of data showed nonsignificant difference between $T_1$ and $T_3$ but significant differences between $T_2$ and $T_1$ or $T_3$ methods. Use of potassium oxalate as calcium ion chelator in milk is responsible for lowered formal titre value in $T_2$ than in $T_3$ methods. Shift in the ionic equilibria between the micelle external environment and internal one probably occurs on addition of potassium oxalate (Wolfschoon and Vargas, 1978). It is quite possible that observed
difference in formal titration in \( T_2 \) method is attributed to the neutralization of serine phosphate residues in the first titration rather than in second titration as a result of destabilization of micelle by oxalate.

The interaction of neutralizers and methods of formal titration was found nonsignificant. This indicates that Ca ion chelators are behaving in the same way in fresh milk and neutralized stored milks.

4.10.2 Comparative assessment of the recalculated Pyne constants by three methods of total protein determination of \( \text{NaHCO}_3 \) and \( \text{NaOH} \)-neutralized milks

The results of the formal titration of \( \text{NaHCO}_3 \) and \( \text{NaOH} \)-neutralized milks (Section 4.10.1) have shown a significant decrease in their formal titre values compared to formal titre values of unneutralized milk. Thus, the Pyne constants calculated for unneutralized milk can not be used for accurate determination of total protein determination of \( \text{NaHCO}_3 \)-and \( \text{NaOH} \)-neutralized milks. Hence, for calculating the total protein content in \( \text{NaHCO}_3 \) and \( \text{NaOH} \)-neutralized milks, the values of Pyne constant in each of the three methods were recalculated by dividing the protein content as in unneutralized milk by the formal titre values of \( \text{NaHCO}_3 \) and \( \text{NaOH} \)-neutralized milks have been presented in Tables 4.10.2.1 and 4.10.2.2, respectively. From the data it is clear that higher values of recalculated Pyne constants for the neutralized milks were obtained compared to the values of unmodified Pyne constants for unneutralized milk by
<table>
<thead>
<tr>
<th>Sr. System of expression</th>
<th>Mean value of recalculated Pyne constant in formal titration method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T₁</td>
</tr>
<tr>
<td>1. Weight/Height basis</td>
<td>1.9424 (1.8767-1.9887)</td>
</tr>
<tr>
<td>2. Weight/Volume basis</td>
<td>1.9929 (1.9255-2.0323)</td>
</tr>
</tbody>
</table>

* Mean value of 5 replications
** Figures in parentheses are ranges of Pyne constants
### Significantly different from T₁ and T₃
Table 4.10.2.2: Comparative assessment of the recalculated Pyne constants by three methods of total protein determination by formal titration of NaOH - neutralized milk (0.2 per cent, w/v)

<table>
<thead>
<tr>
<th>Sr. Systea of</th>
<th>No. expression</th>
<th>Mean value of recalculated Pyne constant in formal titration method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T₁</td>
</tr>
<tr>
<td>1. Height/</td>
<td>Weight</td>
<td>1.8466</td>
</tr>
<tr>
<td>(1.8291-1.8885)</td>
<td>(1.7853-1.7116)</td>
<td>(1.7845-1.8775)</td>
</tr>
<tr>
<td>basis</td>
<td>Volume</td>
<td>1.8946</td>
</tr>
<tr>
<td>(1.8767-1.9294)</td>
<td>(1.7497-1.7561)</td>
<td>(1.8314-1.9264)</td>
</tr>
</tbody>
</table>

* Mean value of 5 replications
** Figures in parentheses are ranges of Pyne constants
### Significantly different from T₁ and T₃
each method of formal titration. Statistical analysis of data showed nonsignificant difference between recalculated Pyne constant values in $T_1$ and $T_3$ methods, whereas significant difference in $T_2$ and $T_1$ or $T_3$ methods. This shows that for calculating the total protein of neutralized milk by $T_2$ method a different recalculated Pyne constant is required while in $T_1$ and $T_3$ methods same recalculated Pyne constant can be used. The values of recalculated Pyne constants, however, also differed in NaHCO$_3$- and NaOH-neutralized milks.

4.10.3 Descriptive analyses of the recalculated Pyne constants for NaHCO$_3$- and NaOH-neutralized milks

Descriptive analyses of recalculated Pyne constants for total protein determinations in NaHCO$_3$- and NaOH-neutralized milks are presented in Tables 4.10.3.1 and 4.10.3.2, respectively. The values of coefficient of variation of recalculated Pyne constants in each method are quite lower than 14 per cent indicating that recalculated Pyne constants can be used for precise determination of total protein content in NaHCO$_3$- and NaOH-neutralized milks.

4.10.4 Comparative assessment of formal titration methods of total protein determination using recalculated Pyne constants for NaHCO$_3$- and NaOH-neutralized milks and unmodified Pyne constants for unneutralized milk

Accuracy of each of the formal titration methods for total protein determination in NaHCO$_3$- and NaOH-
Table 4.10.3.1: Descriptive analysis of recalculated Pyne constants for total protein determination in NaHCO₃ - neutralized milk

<table>
<thead>
<tr>
<th>Sr.</th>
<th>System of expression</th>
<th>Mean value * of recalculated Pyne constant in formal titration method</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>in milk</td>
<td>T₁</td>
</tr>
<tr>
<td>1.</td>
<td>Weight/Weight basis</td>
<td>1.9424(2.2549)</td>
</tr>
<tr>
<td>2.</td>
<td>Weight/Volume basis</td>
<td>1.9929(2.2580)</td>
</tr>
</tbody>
</table>

* Mean value of 5 replications
** Figures in parentheses are the values of coefficient of variation of the recalculated Pyne constants
Table 4.10.3.2: Descriptive analysis of recalculated Pyne constants for total protein determination in NaOH - neutralized milk

<table>
<thead>
<tr>
<th>Sr. System of expression¹</th>
<th>Mean value* of recalculated Pyne constant in formal titration method</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. in milk</td>
<td>T₁</td>
</tr>
<tr>
<td>1. Weight/Weight basis</td>
<td>1.8466(1.2401)</td>
</tr>
<tr>
<td>2. Weight/Volume basis</td>
<td>1.8946(1.2404)</td>
</tr>
</tbody>
</table>

* Mean value of 5 replications
** Figures in parentheses are the values of coefficient of variation of the recalculated Pyne constants
neutralized milks was compared with the unneutralized milk using recalculated and unmodified Pyne constants, respectively. The values of total protein content determined by formal titration of NaHCO$_3$- and NaOH - neutralized milks (obtained with recalculated Pyne constants) and those of unneutralized milk (obtained with unmodified Pyne constants) are presented in Tables 4.10.4.1 and 4.10.4.2, respectively. Statistical analysis showed no significant difference between protein values of NaHCO$_3$- neutralized milk and unneutralized milk (Table 4.10.4.1) and also between the protein values of NaOH— neutralized milk and unneutralized milk (Table 4.10.4.2), in each method of formal titration. Thus, any one of the formal titration methods gives reasonable accuracy of total protein content in NaHCO$_3$- and NaOH- neutralized milks as in unneutralized milk using the appropriate Pyne constant.
Table 4.10.4.1: Comparative assessment of formal titration methods of total protein determination using recalculated Pyne constant for NaHCO₃ - neutralized milk and unmodified Pyne constant for unneutralized milk

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Method of expression</th>
<th>Mean value* of total protein from formal titration</th>
<th>NaHCO₃- neutralized milk</th>
<th>Unneutralized milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in milk</td>
<td></td>
<td>(0.2%, w/v)</td>
<td>(Fresh milk)</td>
</tr>
<tr>
<td><strong>A. Weight/Weight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>T₁</td>
<td>3.8436 (0.0395)**</td>
<td>3.8412</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>T₂</td>
<td>3.8414 (0.0145)</td>
<td>3.8417</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>T₃</td>
<td>3.8280 (0.0361)</td>
<td>3.8351</td>
<td></td>
</tr>
<tr>
<td><strong>B. Weight/Volume</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>T₁</td>
<td>3.9435 (0.0405)</td>
<td>3.9412</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>T₂</td>
<td>3.9416 (0.0149)</td>
<td>3.9419</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>T₃</td>
<td>3.8278 (0.0370)</td>
<td>3.8351</td>
<td></td>
</tr>
</tbody>
</table>

* Mean value of 5 replications

** Figures in parentheses are standard errors of protein values of NaHCO₃ - neutralized milk when those were compared to protein values of unneutralized milk
Table 4.10.4.2: Comparative assessment of formal titration methods of total protein determination using recalculated Pyne constant for NaOH-neutralized milk and unmodified Pyne constant for unneutralized milk.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>System of expression in milk</th>
<th>Method</th>
<th>Mean value* of total protein from formal titration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NaOH-neutralized milk (0.2%, w/v)</td>
</tr>
<tr>
<td>A. Weight/Weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>T₁</td>
<td>3.8424 (0.0211)**</td>
<td>3.8412</td>
</tr>
<tr>
<td>2.</td>
<td>T₂</td>
<td>3.8419 (0.0240)</td>
<td>3.8417</td>
</tr>
<tr>
<td>3.</td>
<td>T₃</td>
<td>3.8258 (0.0309)</td>
<td>3.8351</td>
</tr>
<tr>
<td>B. Weight/Volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>T₁</td>
<td>3.9419 (0.0215)</td>
<td>3.9412</td>
</tr>
<tr>
<td>2.</td>
<td>T₂</td>
<td>3.9417 (0.0240)</td>
<td>3.9419</td>
</tr>
<tr>
<td>3.</td>
<td>T₃</td>
<td>3.9277 (0.0317)</td>
<td>3.8351</td>
</tr>
</tbody>
</table>

* Mean value of 5 replications
** Figures in parentheses are standard errors of protein values of NaOH-neutralized milk when those were compared to protein values of unneutralized milk.
SUMMARY AND CONCLUSION
CHAPTER 5

SUMMARY AND CONCLUSIONS

Fifteen replications of pooled raw buffalo milk were done for the determination of Pyne constants for total protein, casein and whey protein by 3 methods of formal titration of milk. Formal titration of casein solution and whey of buffalo milk were also done to find out the Pyne constants for casein and whey protein, respectively. Five replications of buffalo milk containing, hydrogen peroxide, formaldehyde, sodium bicarbonate and sodium hydroxide were done separately by three methods of formal titration of milk to investigate the effects of additives, if any, on formal titration of milk and total protein content calculated by multiplying with the standardized Pyne constants for buffalo milk. Chemical analyses of buffalo milk for which Pyne constant determinations have been carried out were also investigated and studied for their influence on formal titration and Pyne constants. Statistical analyses of data were done by randomized block design, factorial randomized block design, hypothesis test for means and descriptive statistics.

Mean values of 15 replications of chemical analysis of buffalo milk showed 17.83, 9.07, 8.76, 3.75, 3.00, 0.71, and 0.7 per cent (w/w), respectively.
of total solids, solids-not-fat, fat, total protein, casein, whey protein and ash. Mean values (mg/100g) of calcium and phosphorus content of milk were 173.53 and 127.83, respectively. Mean values of 1.026, 0.131 and 6.629 respectively were obtained for specific gravity, titratable acidity (per cent lactic acid) and pH of milk.

The formal titration of milk and its fractions was carried out by three methods varying in treatments to milk, namely, (i) no addition of Ca ion chelator (T₁), (ii) addition of 0.4 ml saturated potassium oxalate (T₂), and (iii) addition of 0.4 ml saturated tetrasodium pyrophosphate. Formal titre values of milk, casein solution and whey were compared by three methods and with the total protein, casein and whey protein contents of milk using Kjeldahl method as the standard method. Results of 15 replications showed that mean formal titre values were strikingly similar in T₁ and T₃ methods but higher in T₂ method for the same concentration of total protein determined by Kjeldahl method. Similar pattern of formal titre values were observed with casein solution by three methods. However, formal titre values of whey were strikingly similar in all the methods for the same whey.

Pyne constants were calculated by dividing the protein contents as in the Kjeldahl method by the
respective formal titre values of milk, casein solution and whey in each of the methods. It was found that there was significant difference between the Pyne constants of T₂ and T₁ or T₃ methods, while nonsignificant difference between the Pyne constant of T₁ and T₃ methods when formal titre values of milk were used to calculate the Pyne constants for total protein, casein and whey proteins. For expressing the total protein content of buffalo fresh milk on w/w basis, the Pyne constants for T₁, T₂ and T₃ methods were found to be 1.7620, 1.6488 and 1.7572, respectively, whereas for expressing protein content of milk on w/v basis, the Pyne constants were 1.8079, 1.6918 and 1.8051, respectively. For expressing the casein content of milk on w/w basis, the Pyne constants for T₁, T₂ and T₃ methods were: 1.4093, 1.3183 and 1.4070, whereas, for expressing the casein content of milk on w/v basis, the Pyne constants for T₁, T₂ and T₃ were: 1.4460, 1.3507 and 1.4437, respectively. For expressing the whey protein content of milk on w/w basis the Pyne constants for T₁, T₂ and T₃ methods were: 0.3348, 0.3130 and 0.3343, whereas, for expressing the whey protein content on w/v basis, the Pyne constants were: 0.3436, 0.3209 and 0.3430, respectively.

It was also found that there was significant difference between the Pyne constants for casein in T₂ and T₁ or T₃ methods, while nonsignificant difference
between \( T_1 \) and \( T_3 \) methods using casein solutions for the determination of formal titre values. For expressing the casein content of milk on w/w basis, the Pyne constants for \( T_1 \), \( T_2 \) and \( T_3 \) methods were found to be 1.8499, 1.7045 and 1.8424, whereas, for expressing the casein content on w/v basis, the Pyne constants were 1.8982, 1.7490 and 1.8905, respectively.

There was nonsignificant difference between Pyne constants for whey protein of \( T_1 \), \( T_2 \) and \( T_3 \) methods when formal titre values of whey were used to calculate Pyne constants for whey protein. For expressing the whey protein content of milk on w/w basis, the Pyne constants were 1.4087, 1.3952 and 1.3952, respectively; whereas, for expressing the whey protein content of milk on w/v basis, the Pyne constants were: 1.4454, 1.4315 and 1.4315, respectively for \( T_1 \), \( T_2 \) and \( T_3 \) methods.

The mean values of total protein content of milk determined by three methods of formal titration were compared with the mean values of protein content by Kjeldahl method. From this comparison, no significant difference was observed between three methods of formal titration and Kjeldahl method for determination of total protein, casein and whey protein contents of milk.

It was also found that there was no significant differences in the values of casein and
whey protein contents when these were calculated from formal titration of milk or its fractions using respective Pyne constants.

By comparing the formal titre values of fresh milk with formal titre values of milks containing additives, it was found that there was no significant differences between formal titre values of fresh milk and $H_2O_2$- preserved milk in each method of formal titration. But there were significant differences between formal titre values of fresh milk, HCHO-preserved, $NaHCO_3$- and NaOH- neutralized milks. So recalculated Pyne constants were determined for total protein determination in HCHO-preserved, $NaHCO_3$ and NaOH-neutralized milks.

The recalculated Pyne constants for HCHO-preserved milk, for expressing total protein content of milk on w/w basis were: 3.3018, 3.3040 and 3.2782, whereas, for expressing total protein content of milk on w/v basis were: 3.3877, 3.3901 and 3.3637, respectively in $T_1$, $T_2$ and $T_3$ methods.

The recalculated Pyne constants for $NaHCO_3$-neutralized milk, for expressing the total protein content of milk on w/w basis were 1.9424, 1.7840 and 1.9246, respectively; whereas for expressing total protein content of milk on w/v basis were 1.9929, 1.8100 and 1.9748, respectively in $T_1$, $T_2$ and $T_3$ methods.
The recalculated Pyne constants for NaOH-neutralized milk, for expressing the total protein content of milk on w/w basis were 1.8486, 1.7082 and 1.8307, whereas, for expressing the total protein content of milk on w/v basis were 1.8946, 1.7526 and 1.8784, respectively in T₁, T₂ and T₃ methods.

Comparison of the total protein values of HCHO-preserved, NaHCO₃-neutralized and NaOH-neutralized milks (obtained with recalculated Pyne constants) with the values of fresh milk (obtained with unmodified Pyne constants) showed that there was no significant difference between protein values of fresh milk and other milks.

Following conclusions can be drawn from the findings of present study:

1. Formal titration of milk by method 1 (T₁) can be used with reasonable accuracy for determination of total protein, casein and whey protein contents of milk as it is easy, economical and rapid method out of the three methods tried in the present investigation. The formal titre value of buffalo milk obtained should be multiplied by Pyne constants of values 1.7820, 1.4093 and 0.3348, respectively to obtain total protein, casein and whey protein contents on w/w basis of milk, and 1.8079, 1.4460 and 0.3436, respectively to obtain the same on w/v basis of milk.
2. Formal titration of milk by method 2 ($T_2$) and method 3 ($T_3$) can also be used with reasonable accuracy for determination of total protein, casein and whey protein contents of milk. But addition of 0.4 ml saturated potassium oxalate solution and 0.5 ml hydrochloric acid (0.1N) in method 2, and 0.4 ml saturated tetrasodium pyrophosphate solution in $T_3$ method will increase the cost and time requirement, thus making these methods relatively laborious than method 1.

3. Formal titration of milk fractions by three methods can also be used with reasonable accuracy for determination of casein and whey protein contents of milk. However, fractionation procedure is costly, time consuming and can not be performed by an unskilled operator. Obviously these methods are unsuitable as rapid methods but quite useful for understanding mechanism of formal titration of casein and whey protein when separately titrated by three methods.

4. Hydrogen peroxide (0.9 ml per 100 ml milk) was most suitable for preservation of buffalo milk as it did not affect total protein determination by formal titration methods.

5. When formaldehyde (0.4 ml per 100 ml milk) was used for preservation of buffalo milk, recalculated Pyne constants should be used for determination of total protein content of milk by formal titration.
methods.

6. When soured and neutralized milks have to be analysed for determination of total protein content by formal titration, recalculated Pyne constants of NaHCO₃— and NaOH—neutralized milks should be used, respectively.

7. Formal titre values and Pyne constants for whey protein were nonsignificantly affected by the method of titration for whey but this was not the case with casein solutions.

8. Formal titre values and Pyne constants for total protein, casein and whey protein determined from formal titre value of milk were affected by the method of titration.

9. Removal of colloidal calcium phosphate from casein micelle structure by way of precipitation with potassium oxalate increased the formal titre values of milk and casein solution. Thus exposing the titratable serine phosphate and other negatively charged groups of casein submicelles during the first titration of milk or casein solution and getting protonated on addition of formaldehyde in second titration. On the other hand partial migration of colloidal calcium phosphate from casein micelle structure during souring of milk and subsequent addition of neutralizers lowered the formal titre values probably due to attachment of sodium ions to serine phosphate residues and consequently reducing
protonation of acidic residues on addition formaldehyde. The lowering of formal titre value in the presence of neutralizers may be due to reaction of neutralizers with lysine, which is major constituent responsible for second titration. Due to reaction of neutralizers with lysine, lysinoalanine (LAL) might have been formed and which ultimately reduced the availability of lysine in second titration and thus lowered the formal titre value.

10. The Pyne constant values determined by oxalate method (T₂) for the determination of total protein on w/w and w/v basis of buffalo milk were higher than the values of bovine milk (1.70, w/w; 1.74, w/v) reported by Pyne (1932).

11. The Pyne constant value determined by oxalate method (T₂) for the determination of casein on w/w basis of buffalo milk was also higher than the value of Pyne constant for casein (1.28) reported for bovine milk by Pyne (1932) but was lower than the value (1.38) recommended by BIS [SP.IS (Part XI)-1981].

12. Fuchsin dye can be used instead of rosaniline acetate for the preparation of blank.
REFERENCES
REFERENCES


