LABORATORY MANUAL

on

Milk carbohydrates, minerals and water-soluble vitamins

Rajan Sharma
Bimlesh Mann
Darshan Lal
Y. S. Rajput
Kiran Lata

Dairy Chemistry Division
NATIONAL DAIRY RESEARCH INSTITUTE (ICAR)
KARNAL-132001 (HARYANA), INDIA, 2012
Laboratory Manual

On

Milk Carbohydrates, Minerals and Water-Soluble Vitamins

Dr. Rajan Sharma
Senior Scientist, Dairy Chemistry Division

Dr. (Mrs.) Bimlesh Mann
Principal Scientist, Dairy Chemistry Division

Dr. Darshan Lal
Head, Dairy Chemistry Division

Dr. Y. S. Rajput
Head, Animal Biochemistry Division

Ms. Kiran Lata
Research Scholar, Dairy Chemistry Division

NATIONAL DAIRY RESEARCH INSTITUTE
(Deemed University) (ICAR)
KARNAL-132 001 (HARYANA) INDIA
Preface

The practical manual “Milk carbohydrates, minerals and water soluble vitamins” is designed to fulfill the requirement of undergraduate, postgraduate students and PhD students from Dairy Chemistry and other allied disciplines. The course content and syllabus covers practicals identified in Course DC-612 which is being offered by all Dairy Science Colleges in the Country. The basic purpose of the DC-612 practical course is to make students aware of various techniques in wet chemistry and to prepare the students to take up challenges during their research work. It covers various methods for estimation of lactose in milk, estimation of minerals and ascorbic acid in milk. Further, estimation of brown coloring matter and burnt particles/scorched particles in milk powder has also been included. Images of various results obtained in experiments are also included for the help of students. The basic principle, theory, chemical reaction mathematical calculations have been incorporated in each protocol. The information given in the manual would help students in understanding the concept of preparation of various reagents, stoichiometric calculation, preparation of standard curve etc. would be useful later during their research work.

Support and encouragement of Director, National Dairy Research Institute, Karnal served a key role in this manual and authors are highly thankful. The authors are also thankful to Joint Director (A) and Academic Coordinator, Dairy Science Collage, NDRI for providing financial support for the publication of this manual. Support and encouragement from Head, Dairy Chemistry Division for this venture is duly acknowledged.

Authors
## Contents

<table>
<thead>
<tr>
<th>Chapter no.</th>
<th>Topic</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Carbohydrates in milk</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Determination of lactose content in milk by Lane-Eynon method (volumetric method)</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Estimation of lactose in milk by Polarimetric method</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Estimation of lactose in milk by Colorimetric method</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>Lactose estimation in milk by Enzymatic method</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>Lactose estimation in milk by HPLC method</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>Minerals and salts in milk</td>
<td>33</td>
</tr>
<tr>
<td>8</td>
<td>Determination of total ash content in milk</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>Determination of chloride content in milk</td>
<td>42</td>
</tr>
<tr>
<td>10</td>
<td>Estimation of calcium and magnesium in milk by EDTA method</td>
<td>44</td>
</tr>
<tr>
<td>11</td>
<td>Determination of calcium content in milk by titrimetric method (IDF method)</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>Estimation of total phosphorus content in milk by Fiske &amp; Subbarow method</td>
<td>54</td>
</tr>
<tr>
<td>13</td>
<td>Estimation of total phosphorus in milk by BIS method</td>
<td>58</td>
</tr>
<tr>
<td>14</td>
<td>Estimation of iron content in milk by 1, 10 orthophenanthroline method</td>
<td>61</td>
</tr>
<tr>
<td>15</td>
<td>Determination of the iron content in milk</td>
<td>65</td>
</tr>
<tr>
<td>No.</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>-----</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>16</td>
<td>Estimation of copper in milk by sodium diethyl-dithio-carbamate method</td>
<td>73</td>
</tr>
<tr>
<td>17</td>
<td>Estimation of citric acid content in milk by Furth and Hermann method</td>
<td>76</td>
</tr>
<tr>
<td>18</td>
<td>Estimation of citric acid content in milk by gravimetric method</td>
<td>79</td>
</tr>
<tr>
<td>19</td>
<td>Determination of sodium and potassium in milk by flame photometric method</td>
<td>82</td>
</tr>
<tr>
<td>20</td>
<td>Vitamins in Milk</td>
<td>86</td>
</tr>
<tr>
<td>21</td>
<td>Estimation of ascorbic acid (vitamin C) in milk by dye reduction method</td>
<td>93</td>
</tr>
<tr>
<td>22</td>
<td>Determination of 5-hydroxymethylfurfural (HMF) in milk powder</td>
<td>96</td>
</tr>
<tr>
<td>23</td>
<td>Estimation of brown coloring matter in milk powder</td>
<td>99</td>
</tr>
<tr>
<td>24</td>
<td>Determination of scorched particles content in dried milk</td>
<td>101</td>
</tr>
</tbody>
</table>
1. Carbohydrates in milk

Milk contains various carbohydrates, among them lactose is principal carbohydrate in the milk. Lactose concentration in milk varies from species to species (Table 1). Other sugars are also present in lower concentration like glucose, fructose, galactosamine, neuraminic acid, glucosamine in milk. Lactose is only one fifth as sweet as sucrose.

Lactose occurs in milk (true solution) either in free form or in form of lactose containing oligosaccharides. Lactose serves two important functions in milk: it is a ready source of energy for the neonate (it provides 30% of the caloric value of bovine milk) and is responsible for about 50% of the osmotic pressure of milk, which is isotonic with blood and hence is essentially constant.

Table 1. Concentration (%) of lactose in milk of selected species

<table>
<thead>
<tr>
<th>Species</th>
<th>Lactose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water buffalo</td>
<td>4.8</td>
</tr>
<tr>
<td>Cow</td>
<td>4.9</td>
</tr>
<tr>
<td>Goat</td>
<td>4.2</td>
</tr>
<tr>
<td>Camel</td>
<td>3.3</td>
</tr>
<tr>
<td>Mare</td>
<td>6.1</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>7.0</td>
</tr>
<tr>
<td>Human</td>
<td>6.9</td>
</tr>
<tr>
<td>Baboon</td>
<td>7.3</td>
</tr>
<tr>
<td>Elephant</td>
<td>4.7</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2.1</td>
</tr>
</tbody>
</table>

(Source: Fox P.F and McSweeney, P.L.H, Dairy Chemistry and Biochemistry, 1998, pp. 21)

Physico-chemical properties

Lactose is a disaccharide. It consists of D-glucose and D-galactose, linked by β-1, 4 glycosidic linkages. Both moieties occur predominately in the pyranose form. Thus the compound is systematically called 4-O-β-D galactopyranosyl-D glucopyranose.
Mutarotation

Lactose is a disaccharide and exhibits mutarotation. It occurs in two crystalline forms α-hydrate and β-anhydrous form. It also exists in an amorphous or glassy state. The most common form is the α-hydrate (with one H₂O molecule). When a supersaturated solution of lactose is allowed to crystallize at temperature less than 93.5°C, the α-hydrate crystallises out.
Crystallization of a super saturated solution of lactose (above 93.5°C) result in the formation of crystals of β-anhydrous type. When either of the two form are dissolved in water and the solution is allowed to equilibrate at 20°C the specific rotation \([\alpha]^{20}_D\) is 55.40. The α and β anomers of lactose have different properties as mentioned in Table 2.

**Crystallization**

Crystallization of lactose is of great practical importance. It is one of the most important stage in the manufacture of lactose from whey. However, when crystallization of lactose occurs in some milk products particularly sweetened condensed milk and ice cream, it affects the body and texture of these products. The nucleation for the crystallization is very slow process, which leads to the formation of crystals, and these crystals become large with the passage of time. If crystals more than 10 µm in size are present, they can be felt in mouth and the product is said to be sandy. This can be prevented by the addition of numerous tiny seed crysrals before crystallization to prevent the development of sandiness.

Amorphous lactose is formed when a solution is dried rapidly, as in a spray dryer or during freezing. If the water content of the amorphous lactose is low, say 3%, crystallization may be postponed almost indefinitely. Nucleation rate is negligible because of the extremely high viscosity of the solution. The product is, however, very hygroscopic, and when moisture content rise to almost 8%, α-lactose hydrate starts crystallizes out. This is a way to make very small crystals. However, when crystallization of lactose is caused by moisture uptake by milk and whey powder, the result is caking. Powder particles are connected together by crystalline lactose, forming large and strong lumps.

**Fermentation**

Lactose is metabolized by various microorganisms to compounds of lower molecular weight. Lactic acid bacteria are used as starter in the manufacture of butter, yoghurt, dahi, cheese and other cultured product. Homofreumentative bacteria are those which produce only lactic acid while
heterofermentative bacteria are those which produce acetic acid, alcohol, and CO₂ as well as lactic acid. Besides the end products of the fermentation some intermediates are also formed in the milk. Homofermentative bacteria of the so called N streptococci have phosphotransferase system in the cell membrane that phosphorylates lactose at C-6 of galactose moiety as it enters the cell. Heterofermentative organisms, including some member of genera lactobacillus and leuconostoc, lack an aldolase like the one that cleaves six carbon to two three carbon unit in the embden Meyerhof and entner-doudoroff pathway. They use a pathway known as hexose monophosphate shunt in which 6-phospho gluconic acid is formed from glucose and then is decarboxylated, and resulting 5-carbon unit, ribulose 5-phosphate is split to acetyl phosphate and glyceraldehyde 3-phosphate. The former yields acetic acid and/or ethanol and the latter yields pyruvic acid and then lactic acid.

**Thermal reaction of lactose**

- Reducing carbohydrate undergoes a fast anomerization during heating in solid state which is a primary thermal transformation proceeds with no competing reactions. Different form of α-lactose (stable and unstable anhydrous α-lactose) mutarotate to β-lactose in presence of water, but in anhydrous conditions mutarotation can be achieved only at near melting point temperature.
- At intermediate temperature, between anomerization and melting, different isomerization products such as lactulose (4-O-β-D-galactopyranosyl-D-fructose) and epilactose (4-O-β-D-galactopyranosyl-D-mannopyranose) form in small amount. Near melting point lactosan is also formed.
- The presence of organic or inorganic additives even at trace levels, strongly catalyse the thermal degradation of carbohydrates. The main volatile components formed during heating are acetic acid, furfural, methyl furfural, levulinic acid, hydroxyl methyl furfural, CO₂, CO.
- During heat treatment of lactose at ultra-high temperature (UHT), lactulose (disaccharide of D-galactose and D-fructose) is formed. Galactooligosaccharides (GOS) are produced from lactose by glycosyl
transfer of one or more d-galactosyl units onto the d-galactose moiety of lactose, catalyzed by β-galactosidase.

Maillard reaction

Heat treatment of milk and milk products primarily involves interaction between lactose and proteins, resulting in maillard browning, loss of available lysine and nutritive value and off flavour development. Classically the maillard reaction occurs between an aldo or a keto sugar and the amino group of an amino compound. The overall reaction has been divided into 3 stages:

a. The early reaction involves the formation of amodari compound, 1-amino-1-deoxy-2-ketose, which is uncoloured but biologically unavailable and loss of nutritive value occurs without any observable colour or flavour changes.

b. The second stage involves dehydration of the Amodari compound by the loss of water molecules to form furfurals and reductones; fission mainly by dealdolization and strecker degradation, i.e., the interaction of amino acids and dicarbonyls.

c. The final stage consists of the conversion of furfurals, fission products, reductones and strecker aldehydes into melanoidins pigments with further involvement of amines.

Lactose derivatives

Lactitol - a synthetic sugar alcohol produced on reduction of lactose, usually using Raney nickel. It can be crystallized as a mono- or di-hydrate. Lactitol is not metabolized by higher animals; it is relatively sweet and hence has potential as a non-nutritive sweetener.

Figure 4. Structure of Lactitol
Figure 2. Sugar-amine condensation to form N-substituted glycosylamine

Figure 3. Amadori rearrangement
Lactulose - is sweeter than lactose and 48-62% as sweet as sucrose. It is not metabolized by oral bacteria and hence is not cariogenic. Lactulose has attracted considerable attention as a means of modifying the intestinal microflora, reducing intestinal pH and preventing the growth of undesirable putrefactive bacteria. Lactulose is usually used as a 50% syrup but a crystalline trihydrate, which has very low hygroscopicity, is now available.

Lactulose contents increases with the intensity of heating and can be used as the index of heat treatment for the dairy products. Its levels in In-container sterilized milk, indirect UHT, direct UHT was repoted as 744, 341 and 165 mg/l respectively and UHT milk produced by mild technologies such as milk treated by infusion, high-temperature pasteurized milk, and low temperature pasteurized milk (107, 58, 3.5 mg/l and unheated milk have practically nil. (Marconi, 2004)

Lactobionic acid - The free aldehyde group of lactose can be oxidized to a carboxylic acid or lactobionic acid. This important oxidation reaction occurs when reducing power of lactose is measured using Fehling reagent.

Figure 5. Structure of Lactulose

Figure 6. Conversion of Lactose to Lactobionic acid
Various food applications of lactose

Humanized baby foods, Instantizing/free-flowing agent in foods, Confectionery products, Determinalized whey powder or lactose, Anticaking agent at high relative humidity, Maillard browning, if desired, Flavour enhancement etc.

Some cheeses are almost lactose-free, because during the manufacturing process, most of the lactose is removed with the whey, and the remaining lactose in the cheese is gradually consumed in the fermentation process. However, in fresh cheeses and those made by coagulating fresh milk or cream with rennet or an acid, lactose remains unchanged, and therefore, these products may contain appreciable amounts of lactose.

Lactose determination

Lactose may be determined by various methods based on different principles:

- **Polarimetric** - Lactose solution when placed in the path of plane polarized light, rotate the plane of polarized light and the extent of change in the plane of the polarized light is measured by polarimeter.
- **Oxidation-reduction titration** - Lactose is capable of reducing the oxidizing agents, i.e. alkaline copper sulphate (CuSO₄, in sodium potassium tartrate; Fehling’s solution) or chloroamine-T, so on this basis lactose can be determined quantitatively
- **Colorimetric** - In strongly acidic solution lactose react with phenol or anthrone at high temperature and give a coloured solution. The concentration of lactose is determined from a standard curve prepared using a range of lactose concentrations
- **Chromatography** - On the basis of change in refractive index lactose can be determined by gas liquid (GLC) and high performance liquid chromatography (HPLC).
- **Enzymatically** - Enzymatic methods are very sensitive but are rather expensive. Lactose is first hydrolysed by β-galactosidase to glucose and galactose which can be further determined by using different enzymes.
e.g. Glucose oxidase, Glucose-6-phosphate dehydrogenase, Galactose dehydrogenase.

- **Picric acid method** – This method is based on the fact that reducing sugar in alkaline solution reduce yellow picric acid to mahogany red picramic acid. Recent modification of the method have been adapted it to photoelectric colorometry.

**References:**


2. Determination of lactose content in milk by Lane-Eynon method (volumetric method)

Lactose is the characteristic carbohydrate in milk (approximately 48-50 g/L in cow milk) of all the mammals. Milk contains only trace amounts of other sugars, including glucose (50 mg/L), fructose, glucosamine, galactosamine etc. Lactose is a disaccharide consists of glucose and galactose moieties linked through \( \beta, 1-4 \) glycosidic bond. The hemiacetal group of the glucose moiety is potentially free \( i.e. \) lactose is a reducing sugar and thus it is capable of reducing appropriate oxidizing agents.

Lane-Eynon method for the estimation of lactose is based on the reducing property of lactose present in milk. Reducing sugars, are able to function as reducing agents because of free aldehyde group present in the molecule. The reducing properties of these sugars are usually observed by their ability to reduce metal ions notably copper, iron or silver in alkaline solution. This very property of sugars has been used in this method using Fehling solution. Fehling solution is a mixture of Fehling A (CuSO\(_4\)) and Fehling solution B (alkaline sodium-potassium tartrate). When CuSO\(_4\) is made alkaline (during mixing of Fehling A and Fehling B), the Cu(OH)\(_2\) gets precipitated but in the presence of sodium-potassium tartrate, it forms a soluble complex with copper compound and prevents the precipitation of Cu(OH)\(_2\). The complex is soluble and behaves/reacts as if it is alkaline Cu(OH)\(_2\) solution. The Fehling solution when heated gives rise to cupric oxide (CuO) which in turn reacts with reducing sugar and gets reduced to cuprous oxide (Cu\(_2\)O), brick-red precipitates, resulting into the oxidation of sugars to corresponding acids.

For lactose estimation by this method, milk sample is treated with acetic acid to precipitate protein and fat. The filtrate so obtained is treated with alkaline copper sulfate solution (Fehling’s reagent) while heating. During the reaction, Cu\(_2\)O precipitates are formed which are brick red in colour. The end point of the titration can be detected using methylene blue as an indicator, which on reduction, changes colour of the solution from blue to
colourless. At this stage complete precipitation of Cu₂O occurs in the form of brick-red precipitation.

**Reaction**

\[
\text{CuSO}_4 + 2\text{NaOH} \rightarrow \text{Cu(OH)}_2 + \text{Na}_2\text{SO}_4
\]

\[
\text{Cu(OH)}_2 + \text{HO-CH-COO} \rightarrow \text{Cu} + \text{O-CH-COO} \rightarrow \text{Cu(OH)}_2 + 2\text{H}_2\text{O}
\]

\[
\text{Heat} \rightarrow \text{CuO} + \text{H}_2\text{O}
\]

**Reagents**

1. **Fehling’s Solution**: It is freshly prepared by mixing equal volumes of the following two solutions, termed as Fehling’s A and Fehling’s B.
   - **Fehling’s A**: Dissolve 34.639 g CuSO₄·5H₂O in sufficient distilled water, add a few drops of 1 N sulphuric acid to prevent formation of cloudy appearance or turbidity (use a minimum quantity of acid). Dilute the solution with distilled water to 500 ml. If necessary, filter through prepared asbestos, on Gooch crucible.
   - **Fehling’s B**: Dissolve 173 g Rochelle salt (sodium potassium tartrate; KNaC₄H₄O₆·4H₂O) in distilled water. Add to it 50 g of sodium hydroxide and dilute the mixture to 500 ml with distilled water. Allow to stand in a rubber or polyethene stoppered container for two days and filter through prepared asbestos.
2. **Acetic acid solution**: 10% (w/v; aqueous) solution.
3. **Methylene blue indicator**: 1% (w/v; aqueous) solution.
4. **Standard lactose solution**: Weigh accurately 5 g of this dried lactose and dissolve in freshly boiled and cooled distilled water. Transfer the solution quantitatively to a 1 L volumetric flask and dilute up to mark with.
distilled water. Mix well and store it in a cool place. One ml of this solution contains 5 mg lactose.

Procedure

A. Preparation of milk filtrate

1. Pipette 10 ml of the prepared sample of milk into a 100 ml volumetric flask.
2. Add 30 to 40 ml distilled water and the content of the flask are warmed to 40 to 45°C, immediately add 1.5 ml acetic acid (10%). Mix well and make the final volume with distilled water up to mark and mix again. Keep the flask undisturbed for 30 min.
3. After 30 min filter the content of the flask through a Whatman filter paper Grade 42. Discard the first few ml of the filtrate and collect the rest in a clean dry Erlenmeyer flask fitted with stopper.

B. Standardization of Fehling's solution

1. Pipette 5 ml of Fehling’s solution A and 5 ml of Fehling’s solution B using two separate pipettes in a 250 ml Erlenmeyer flask. Fill up a burette with the standard lactose solution and connect the burette end with an offset tube to keep the burette tube out of steam.
2. Heat the content of the flask to boiling over burner or heater and maintain moderate boiling for 2 min. To prevent bumping add some inert boiling chips. Add 3 to 4 drops of methylene blue indicator without removing from the flame. Titrate the content of the flask against standard lactose solution from the burette until the blue colour disappears and the bright brick-red colour of precipitated Cu₂O appears (at the end point the Cu₂O suddenly settles down giving a clear supernatant). Note the volume of lactose solution required for the standardization of Fehling’s solution. After this preliminary titration, further titration or titrations should be carried out, adding practically the whole of the standard lactose solution volume (one ml less than required as observed in first titration) required before commencing the heating and continuing the
titration as before. The titration must be completed within 3 min from the commencement of boiling. Let $V_1$ ml be the titer for this experiment.

**Note:**

Carefully note the first disappearance of blue colour. Once missed, it is difficult to ascertain the end point. Maintain the boiling at a uniform rate during the titration.

**C. Determination in milk**

Here also the titration is taken up in two stages. In the first stage, an approximate estimation is made, while in the second stage more accurate result is obtained.

**Stage 1**

1. Pipette 5 ml of Fehling’s solution A and 5 ml of Fehling’s solution B using two separate pipettes in a 250 ml Erlenmeyer flask. Fill up a burette with the prepared milk filtrate and connect the burette end with an offset tube to keep the burette tube out of steam.

2. Add 10 ml of the milk filtrate from the burette into flask containing Fehling’s solution and heat to boiling on wire gauge over burner. Boil for about 15 sec and rapidly add further amount of lactose filtrate until only faint perceptible blue colour remains. Add 3-4 drops of methylene blue indicator and complete the titration to the first disappearance of blue colour by adding lactose filtrate drop-wise.

**Stage 2**

Repeat the experiment of Stage 1, but add almost the entire prepared milk filtrate (one ml less than required as observed in first titration) required to reduce all copper, as determined by Stage 1, and complete the titration as described under standardization of Fehling’s solution. Let $V_2$ ml be the titer for this experiment.

**Calculation**

$$\% \text{ lactose in milk} = 5 \times \frac{V_1}{V_2}$$

Where
\[ V_1 = \text{volume in ml, of standard lactose solution taken to reduce 10 ml of Fehling's solution; and} \]

\[ V_2 = \text{volume in ml, of prepared milk filtrate taken to reduce 10 ml of Fehling's solution.} \]

**Note:**

1 ml of standard lactose solution contains = 0.005 g of lactose

Let \( V_1 \) ml = amount of standard lactose solution taken to reduce 10 ml of Fehling's solution (5 ml each of Fehling's A and B solution)

Let \( V_2 \) ml = amount of milk filtrate solution taken to reduce 10 ml of Fehling's solution (5 ml each of Fehling's 1 and 2 solution)

10 ml of Fehling's solution = \( V_1 \) ml of standard lactose solution

= \( V_2 \) ml of milk filtrate

\( V_2 \) ml of milk filtrate \( \equiv \) \( V_1 \) ml of standard lactose solution

= \( V_1 \times 0.005 \) g of lactose

100 ml of milk filtrate = \( \frac{V_1 \times 0.005}{V_2} \times 100 \)

Since 100 ml of milk filtrate is obtained from 10 ml of milk

Lactose present in 100 ml of milk = \( \frac{V_1 \times 0.005}{V_2} \times 100 \times \frac{100}{10} \)

% lactose in milk = \( 5 \times \frac{V_1}{V_2} \)
3. Estimation of lactose in milk by Polarimetric method

Certain organic substances (notably those containing asymmetric carbon atoms) possess the property of rotating the plane of polarized light. Such substances are called as optically active substances. A compound is considered to be optically active if linearly polarized light is rotated when passing through it. The amount of optical rotation is determined by the molecular structure and concentration of chiral molecules in the substance. Ordinary light propagates itself through spherical waves in all directions in space, but when such light is allowed to pass through specially designed prism, like Nichol's prism, (prepared from particular substance like naturally occurring crystalline calcium fluoride, calcite etc.) the emerging light travels in one plane only. Such light is called "plane polarized light". Solution of substances like lactose, glucose, etc., when placed in the path of plane polarized light; rotate the plane of polarized light and such compounds are known as "optically active". The extent of change (rotation) in the plane of the polarized light depends on factors like the specific nature of the optically active compound, its concentration in the solution and temperature of the solution etc. The quantitative relationship between these variables is given by the following equation:

\[ [\alpha]_{D}^{20^\circ C} = 100 \frac{R}{LC} \]

Where

- \( R \) = the observed angular rotation in degree;
- \( L \) = length of tube in decimeters (1 decimeter = 10 cm);
- \( C \) = concentration of the compound in solution in g/100 ml; and

\[ [\alpha]_{D}^{20^\circ C} \] is the specific rotation at 20°C using D line of sodium (589.3 nm).

Thus using the above equation the value of \( C \), \( \alpha \) or \( R \) can be determined if any two of them are known.

The specific rotation \([\alpha]_{D}^{20^\circ C}\) of lactose in solution at equilibrium is \(+55.4^\circ\) expressed on an anhydrous basis (+ 52.6° on a monohydrate basis). The specific rotation is defined as the optical rotation of a solution containing 1 g/ml in a 1 dm polarimeter tube; it is affected by temperature (20°C is...
usually used; indicated by superscript) and wavelength (usually the sodium D line (589.3 nm) is used; indicated by subscript. In this method, the milk sample is first defatted and deproteinated, usually by treatment with mercuric nitrate \([\text{Hg (NO}_3\text{)}_2]\) or mercuric iodide (Hgl).

**Reagents**

1. Acid mercuric nitrate solution: Dissolve a known weight of metallic mercury (Hg) in twice of its weight of concentrated nitric acid, cool and dilute with 5 volumes of water.
2. Mercuric iodide solution: Dissolve 33.2 g potassium iodide (KI) and 13.5 g mercuric chloride (HgCl₂) in 200 ml glacial acetic acid and add 640 ml distilled water.
3. Phosphotungstic acid solution: 5% solution (w/v, aq.).

**Apparatus**

1. Polarimeter: 100 mm or 200 mm tubes and sodium lamp (D line 589.3 nm).
2. Volumetric flasks: 100 ml capacity.
4. Funnels.
5. Whatman filter paper Grade 42.

**Procedure**

1. Weigh accurately 65.8 g of given sample of milk into 100 ml volumetric flask, add 30 ml of acidic mercuric nitrate solution or 30 ml of mercuric iodide.
2. Make up the volume to 100 ml with phosphotungstic acid, shake the contents and keep for 15 minutes. Filter the solution through a Whatman no 42 filter paper. Adjust the temperature of filtrate to 20°C and pour in a polarimetric tube to take the reading.

**Calculations**

\[
C = \frac{100 \times a}{L \times \left[\alpha\right]^{20^\circ} D} 
\]

\(C\) - Conc. of solute in g/100 ml solution
a - Observed rotation in degrees
L - Length of tube in decimeters
$[\alpha]_D^{20}^\circ$ - Specific rotation at 20°C using D line of sodium (+ 52.6°)

Quantity of milk taken = 65.8 g

Therefore, 65.8 g milk contains = C g/100 ml of lactose

100 g milk contains = (C X 100)/ 65.8 % lactose.

The accuracy of the polarimeter can be checked with sucrose solution. A 10% sucrose solution should give an optical rotation of 13.30° when measured using a 200 mm polarimeter tube at 20 ± 1°C.

Note:
Specifically, for carbohydrates, the configuration at the last (highest numbered) stereo center determines whether it’s D or L form. D means it has the same configuration as (+)-glyceraldehyde; L means it has the same configuration as (−)-glyceraldehyde.

D and L (capital letters) are not at all the same as d and l (lower letters). The capital letters relate the configuration to glyceraldehyde, the lower case letters specify whether the substance is dextrorotatory or levorotatory. For glyceraldehyde, D is d (+) and L is l (−), but this is not always the case for other molecules.
4. Estimation of lactose in milk by Colorimetric method

Solution of lactose and those of other reducing sugars, reduce the yellow picric acid (2-hydroxy-1, 3, 5-trinitrobenzene) in alkaline medium to mahogany red picramic acid (2 amino, 4, 6 dinitrophenol); the reaction can be described by the following equation.

**Reaction**

\[
\text{Picric acid (Yellow colour)} + \text{Aldehyde group of lactose} \rightarrow \text{Picramic acid} + \text{Oxidized group of lactose}
\]

In this method a known quantity of milk is diluted to specific volume with saturated picric acid solution. The well mixed solution is filtered and measured volume of clear filtrate is made alkaline with sodium carbonate in a sugar tube. The tube is then placed in boiling water-bath for development of colour which is measure at 480 nm in spectrophotometer.

The developed colour can be compared to that formed in a solution containing known concentration of lactose (standard lactose solution) using a spectrophotometer, whence concentration of lactose in unknown is calculated (Perry and Doan, 1950).

**Reagents**

1. Picric acid: Saturated solution (aq.).
2. Sodium carbonate (Na$_2$CO$_3$) solution: 25% solution (w/v; aq.), filter if required.
3. Lactose standard solution (0.5%): Weigh accurately 5 g of lactose and dissolve in freshly boiled and cooled distilled water. Transfer the solution
quantitatively to a 1 L volumetric flask and dilute up to mark with distilled water. Mix well and store in a cool place.

**Apparatus**

1. Spectrophotometer: Suitable for reading at wavelength of 480 nm
2. Test tubes: 25 ml graduated
3. Volumetric flasks: 25 ml
4. Pipettes 1 ml and 2 ml
5. Boiling water-bath
6. Funnels
7. Whatman filter paper Grade 40

**Procedure**

**A. Preparation of standard curve for lactose**

1. Take six clean, dry test tubes and pipette accurately through graduated pipette 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of standard lactose solution separately into each test tube. Add saturated picric acid solution to make the volume up to mark 2 ml in all test tubes.
2. Add 1 ml sodium carbonate solution (25%) to each test tube, mix well and stopper loosely the test tubes.
3. Heat the test tubes for 20 min by dipping in boiling water-bath. Cool to room temperature and compensate any volume loss by adding water up to 3 ml mark.
4. Dilute the content of each tube to 25 ml with distilled water.
5. Measure absorbance of the brown coloured solution (figure 1) in a spectrophotometer at 480 nm setting the zero of the instrument using the blank.
6. Draw a graph by plotting concentrations of lactose on abscissa versus absorbance of the solution on ordinate, which should be a straight line passing through the origin.
B. **Estimation of lactose in milk**

1. Pipette accurately 1 ml of prepared milk sample separately into two 25 ml volumetric flasks (analysis in duplicate). Make the volume up to mark with saturated picric acid solution.

2. Mix well, allow to stand for 5 to 10 min and filter through Whatman filter paper 40. Collect the filtrate after discarding first few drops.

3. Transfer 2 ml filtrate to each of two graduated test tubes, and add 1 ml of sodium carbonate solution (25%). Mix well and stopper loosely the test tubes.

4. Heat the test tubes for 20 min by dipping in boiling water-bath. Cool to room temperature. Dilute the content of each tube to 25 ml with distilled water.

5. Measure the absorbance after adjusting zero against a blank prepared (figure 1) by taking 1 ml of distilled water instead of milk sample.

   Read from the standard curve the concentration of lactose in milk filtrate corresponding to its absorbance. Take the average of duplicate.

**Calculation**

\[
\text{Lactose} \% = 1.25 \times W
\]

Where \( W \) = concentration of lactose corresponding to the absorbance obtained from the standard curve.

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Milk Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose solution (ml) 5 mg/ml - lactose conc.</td>
<td>0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
<td>2 ml milk filtrate</td>
</tr>
<tr>
<td>Lactose in mg/tube</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Picric acid soln (ml)</td>
<td>2.0</td>
<td>1.8</td>
<td>1.6</td>
<td>1.4</td>
<td>1.2</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Sodium carbonate son. (25%) (ml)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Heat the test tubes for 20 min by dipping in boiling water-bath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cool to room temperature &amp; compensate for any water loss by adding DW up to 3 ml mark</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Calculations

From the standard curve:

Let the concentration of lactose corresponding its OD is x mg

Then 2 ml of milk filtrate contains x mg lactose

Therefore, 25 ml of milk filtrate contains \((x/2)\times25\) mg lactose

As 25 ml of milk filtrate was obtained from 1 ml milk, therefore 1 ml of milk contains \((x/2)\times25\) mg lactose or \([((x/2)\times25]/1000\) g lactose

Therefore, 100 ml milk contains \([[(x/2)\times25]/1000]\) g lactose or \(1.25 \times x\) g lactose

Precautions

1. Allow the tubes to cool to room temperature. Do not cool the tube in chilled water as sometimes crystals are formed.

2. Use Saturated Picric acid only. Diluted picric acid is not able to precipitate the milk and thus clear filtrate is not formed.

3. As the browning red colour formed is very intense, final dilution of the content is required. Dilution may be done to final volume to 25 ml. It is convenient to do the experiment in 25 ml graduated stoppered tubes.

Reference

Figure 1: The colour development in blank, standard curve and sample during lactose estimation in milk by picric acid method
5. Lactose estimation in milk by Enzymatic method

In this method, lactose is enzymatically hydrolysed to its components, monosaccharides (D-glucose and D-galactose) at pH 6.6 in the presence of the enzyme β-galactosidase and water.

\[
\text{β-galactosidase} \quad \text{Lactose} + H_2O \rightarrow \text{D-glucose} + \text{D-galactose}
\]

D-galactose is oxidised at pH 8.6 by nicotinamide-adenine dinucleotide (NAD) to D-galactonic acid in the presence of the enzyme β-galactose dehydrogenase (Gal-DH).

\[
\text{D-galactose} + \text{NAD}^+ \rightarrow \text{D-galactonic acid} + \text{NADH} + H^+
\]

The amount of NADH formed in the above reaction is stoichiometric to the amount of lactose, and D-galactose, respectively. The increase in NADH is measured by means of its light absorbance at 334, 340 or 365 nm.

An enzymatic kit for the determination of lactose/D-galactose in food stuff and other materials for approximate 30 determinations (per one kit) is available with Roche-Biopharm GmbH Boehringer Mannheim, Dolivostraße 10, 64293 Darmstadt, Germany.

**Reagents**

1. Potassium hexacyanoferrate (II) solution (Carrez I solution): Dissolve 3.60 g of potassium hexacyanoferrate (II) trihydrate \([K_4Fe(\text{CN})_6.3\text{H}_2\text{O}]\) in water and dilute to 100 ml with water and mix.
2. Zinc sulfate solution (Carrez II solution): Dissolve 7.20 g of zinc sulfate heptahydrate \((\text{ZnSO}_4.7\text{H}_2\text{O})\) in water and dilute to 100 ml with water and mix.
3. Sodium hydroxide solution: 0.1 N.
4. Assay reagents:

Solution I: 610 mg lyophilizate consisting of citrate buffer (pH 6.6); NAD (35 mg); MgSO₄ and stabilizer. Dissolve lyophilizate in 7.0 ml redistilled water before use.
Solution 2: About 1.7 ml enzyme suspension of β-galactosidase (approximately 100 Units).
Solution 3: 34 ml solution consisting of potassium diphosphate buffer (0.51 mol/L, pH 8.6) and stabilizer.
Solution 4: About 1.7 ml enzyme suspension of galactose dehydrogenase (approximately 35 units).

Apparatus

1. Spectrophotometer: Suitable for reading at wavelength of 480 nm provided with cuvettes (1.0 cm light path).
2. Pipettes: capillary pipettes for transfer of volumes less than one ml.
3. Stirrer: Plastic or glass rod, 1 mm diameter.
4. Volumetric flask: 100 ml capacity.
5. Whatman filter paper: Grade 42.
6. Funnels.

Procedure

A. Sample preparation

1. Accurately weigh about 2 g of prepared sample of milk into 100 ml volumetric flask, add about 60 ml distilled water, and mix.
2. Add 5 ml Carrez I solution and mix. Add 5 ml Carrez II solution and mix. Add 10 ml of 0.1 N sodium hydroxide solution and mix the content vigorously.
3. Dilute the content of the flask up to the mark with distilled water, mix, and filter through Whatman filter paper Grade 42. Use clear filtrate for assay. This procedure breaks emulsions, absorbs some colours, and precipitate proteins.

B. Determination

1. Label one cuvette as “blank” and one cuvette as “sample”.


2. Pipette 0.2 ml Solution I into each cuvette. Pipette 0.05 ml Solution 2 into each cuvette. Pipette 0.10 ml sample solution (filtrate) into sample cuvette.

3. Mix the content of both cuvettes with stirrer, and hold for 10 min at 20 to 25°C.

4. Pipette 1.0 ml of Solution 3 into each cuvette. Pipette 2.0 ml of distilled water into blank cuvette and 1.9 ml distilled water into sample cuvette.

5. Mix the content of both cuvettes with stirrer, and hold for 2 min at 20 to 25°C.

6. Determine absorbance \( (A_1) \) of each solution at 340 nm.

7. Add 0.05 ml of Solution 4 to each cuvette. Mix and hold at 20 to 25°C until reaction has stopped (about 10 to 15 min). Determine absorbance \( (A_2) \) of both solutions. If reaction has not stopped after 15 min, continue to read at 2 min intervals until absorbance remains constant for 2 min.

**Calculation**

Convert absorbance readings to anhydrous lactose, \( C \), by the following equation:

\[
C \text{ (g/L sample solution)} = \frac{(V \times MW \times \Delta A)}{(\epsilon \times d \times v \times 1000)}
\]

Where

- \( V \) = final volume (ml);
- \( v \) = sample volume (ml);
- \( MW \) = molecular weight of anhydrous lactose (342.3);
- \( d \) = light path (cm);
- \( \epsilon \) = absorption coefficient of NADH at 340 nm = 6.3 \( (1 \times \text{mmol} \cdot 1 \times \text{cm}^{-1}) \); and
- \( \Delta A \) = \( (A_{S2} - A_{S1}) - (A_{B2} - A_{B1}) \), i.e., absorption differences for blank and samples.

Thus, for anhydrous lactose, equation becomes:

\[
C \text{ (g/L)} = \frac{(3.30 \times 342.3 \times \Delta A)}{(6.3 \times 1 \times 0.1 \times 1000)} = 1.793 \times \Delta A
\]

Since sample was diluted before analysis, multiply result by dilution factor, \( F \), in general case, 2 g milk to 100 ml, \( F = 50 \).
6. Lactose estimation in milk by HPLC method

In this method (ISO 22662: 2007), an internal standard [D (+)-melezitose] is added to a known volume of milk and to lactose standards. A chemical reagent (Biggs-Szijarto solution) is added to precipitate out the fat and the protein component fractions of milk. The sample is filtered twice prior to injection, first through paper filter and then through a 0.45 \( \mu m \) nylon filter. The lactose and the internal standard are separated by a cation exchange column in the lead form and detected by a differential refractometer detector or other suitable detector. As mobile phase, HPLC grade water is used.

Apparatus

1. HPLC ion exchange resin column: Length 300 mm, of internal diameter 7.8 mm, with 8% cross-linked copolymer, based on polystyrene-divinylbenzene cation exchange resin and packed in the lead form.
2. Guard column: In order to prolong ion exchange resin column life, replace the guard column after about 200 injections.
4. Column heater: Capable of maintaining a constant temperature of 85±1°C.
5. HPLC pump: Capable of maintaining a flow rate of between 0 ml/min and 10 ml/min.
6. HPLC auto sampler.
Note: Manual injection can also be used.
Note: Other detectors, e.g. an evaporative light scattering detector, can also be used.
8. Software, capable of: automating injections, performing data acquisition, processing, and managing chromatographic information.
9. Water purification unit, capable of providing water complying with grade 1 requirements of ISO 3696, with a resistivity of between 10 MΩ cm and 18 MΩ cm.
10. Solvent filtration unit, including a vacuum source, with a membrane filter of 0.45 μm pore size and of diameter 47 mm.
11. Analytical balance, capable of weighing to the nearest 1 mg, with a readability of 0.1 mg.
12. Water bath, capable of maintaining a temperature of between 38 - 40°C.
13. Accurate dispenser, accurate automatic pipette, or one-mark pipettes: Class A, of capacity 2 ml.
14. Filter funnel: Diameter 75 mm.
15. Filter paper: Diameter 110 mm, Whatman1) No 1 or equivalent.
16. Nylon syringe filter: Porosity 0.45 μm.
   Note: An in-line filter of the same porosity may also be used.
17. Syringe: With Luer-lock, of capacity 5 ml.
18. HPLC vials, with caps.
19. One-mark volumetric flasks: Capacity 10 ml ± 0.02 ml.
   Note: Flasks with a capacity of more than 10 ml can also be used by taking into account the concentration factor.s

Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.
1. Degassed HPLC grade water: Filter the water, conforming to the requirements of ISO 3696, Grade 1, obtained from the water purification unit using the solvent filtration unit. To improve the pump performance and to obtain a stable baseline, degas the mobile phase daily by selecting one of the available techniques such as sparging with helium, sonication, vacuum or in-line degassing system.
2. D(+)—Melezitose hydrate solution (C_{18}H_{32}O_{16}·H_{2}O) (50 mg/ml): Dissolve an amount of D(+)-melezitose hydrate in water to give a final concentration equivalent to 50 mg/ml of the anhydrous form. The D(+)—melezitose solution can be stored at 4°C for no longer than 1 week.
3. α—Lactose monohydrate (C_{12}H_{22}O_{11}·H_{2}O): Before use, dry the α-lactose monohydrate at 70°C for 4 h. Cool it to room temperature in a desiccator.
   Note: After drying, the lactose remains in the monohydrate form.
4. Biggs-Szijarto solution: Dissolve 25 g of zinc acetate dihydrate, Zn(CH₃COO)₂·2H₂O and 12.5 g of phosphotungstic acid monohydrate (W₁₂O₃₆·H₃PO₄·H₂O) in about 100 ml of HPLC grade water in a 200 ml one-mark volumetric flask. Add 20 ml of glacial acetic acid (CH₃COOH). Dilute to the 200 ml mark with HPLC grade water and mix. After use, the solution may be stored at 4°C for no longer than 1 week.

Procedure

1. Preparation of test sample

   For fluid milk and cream, warm the test sample in the water bath to between 38°C and 40°C. Gently mix the test sample by repeatedly inverting the bottle. Cool the sample quickly to 20 ±1°C while gently mixing the sample immediately prior to weighing the test portion. Prepare milk powder and other samples as the case.

2. Preparation of standard solution

   A. In a 10 ml one-mark volumetric flask, weigh, to the nearest 1 mg, the appropriate amount of α-lactose monohydrate to give the equivalent of a 20 mg/ml anhydrous α-lactose solution.

   B. Dissolve the α-lactose monohydrate in about 5 ml of HPLC grade water. Add 2 ml of D(+) melezitose solution, used as internal standard, to the flask. Make up the volume to the mark with HPLC grade water and mix by inverting the flask. Express the final α-lactose concentration in milligrams of the anhydrous form per millilitre.

   C. Filter the standard solution through a pleated filter paper using a filter funnel. Aspirate the filtrate into a syringe. Screw the nylon syringe filter to the syringe and then transfer each filtrate into an HPLC vial. Inject each standard solution at least twice. The standard solution thus prepared can provide three sets of calibration solutions. Use each set once only to calibrate the HPLC column. Store non-used sets of lactose standard solution at 4°C for no longer than 1 week. Before use, bring all refrigerated standard solutions to approximately 20°C. In order to monitor the calibration, inject the standard solution as unknown sample at the beginning and at the end of the set of the test portions.
3. Preparation of test portion

Depending on the type of sample, treat the sample accordingly as per following

Fluid milk test sample: Weigh, to the nearest 1 mg, about 3 ml of prepared test sample (see Clause 8) into a 10 ml one-mark volumetric flask. Proceed as in Step 4.

Milk powder test sample: Weigh, to the nearest 1 mg, about 0.300 g of test sample into a 10 ml one-mark volumetric flask. Add about 5 ml of HPLC grade water pre-warmed to between 50°C and 60°C. Mix thoroughly until the solution becomes homogenous. Allow the test solution thus obtained to cool to 20 ± 1°C. Proceed as in Step 4.

Cream test sample: Weigh, to the nearest 1 mg, about 1 g of prepared test sample into a 10 ml one-mark volumetric flask. Proceed as in Step 4.

Preparation of filtrate

Add 2 ml of D(+)-melezitose internal standard solution and 1.2 ml of Biggs-Szijarto solution to the content of the flask obtained above (See Step 3), as appropriate. Dilute to the mark with HPLC grade water. Gently mix the contents by inverting the flask five times. Allow to stand at room temperature for 10 min. Repeat the mixing and standing process two more times. Filter the contents of the flask through a pleated filter paper using a filter funnel. Collect the filtrate with a syringe. Screw the nylon syringe filter to the syringe and then transfer the filtrate into a HPLC vial. Inject the test solution at least twice.

Note: The filtration step through the filter paper can be replaced by centrifugation of the test sample.

4. HPLC determination

A. Preliminary preparation of HPLC: In order to get a stable baseline, turn on the detector at least 24 h before starting the analysis. Set the internal temperature at 35°C. Set the HPLC pump to deliver a flow rate of 0.2
ml/min for at least 20 min while the column heater is set to room temperature.

Increase the column heater temperature to 85°C. When that temperature is reached, gradually increase the flow rate from 0.2 ml/min to 0.6 ml/min. Allow the system to equilibrate at a flow rate of 0.6 ml/min and at 85°C for 2 h or until a stable baseline is obtained.

**Note:** Checking and recording the pressure of the system from day to day can help to detect whether abnormal pressure changes occur.

**B. Chromatographic conditions:** The chromatographic conditions are as follows:

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>Degassed HPLC grade water</td>
</tr>
<tr>
<td>Internal detector temperature</td>
<td>35°C</td>
</tr>
<tr>
<td>Guard column temperature</td>
<td>Ambient temperature</td>
</tr>
<tr>
<td>Column temperature</td>
<td>85°C</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.6 ml/min</td>
</tr>
<tr>
<td>Volume to be injected</td>
<td>20 µl</td>
</tr>
<tr>
<td>Run time</td>
<td>15 min</td>
</tr>
<tr>
<td>Retention time of D(+)-melezitose</td>
<td>9 min ± 1 min</td>
</tr>
<tr>
<td>Retention time of lactose</td>
<td>11 min ± 1 min</td>
</tr>
</tbody>
</table>

Carefully choose the acquisition and integration parameters such as sensitivity, scale factor, time constant, peak width and threshold. See Figure 1 for an example of a chromatogram.

Measure the column efficiency, also called theoretical plate count, N, at least once per week. A decrease in N is related to the band spreading of the peak which is often due to a loss in column performance. Calculate N by using the following equation:
\[ N = 5.54 \times \left[ \frac{t_R}{w} \right]^2 \]

Where

\( t_R \) is the retention time, in minutes, of the lactose peak;
\( w \) is the width of the lactose peak, equivalent to time difference in minutes, at 50% of its height.

When the theoretical plate count decreases by more than 25% compared to the original measurement, a replacement of the column is recommended.

**Note:** In most cases, a used column performing with low efficiency can be restored to its original form by back washing with an appropriate regenerating solvent described in the manufacturer's documentation.

**Figure 1** - Example of a chromatogram from a raw milk sample containing the internal standard

**Key**
- M  D (+)-melezitose
- L  α-lactose
- \( t_R \)  retention time
- \( U \)  potential difference
5. Calculation and expression of results

A computer performs the calculations as follows:

First, the software generates a curve by plotting the response ratio of the lactose standard peak area, $A_{rs}$, to that of the internal standard, $A_{is}$, multiplied by the internal standard concentration, $C_{is}$ i.e. $(A_{rs}/A_{is}) \times C_{is}$, against lactose concentration, $C_l$. The curve fit is linear through the origin.

To quantify an unknown test sample, the software divides the concentration derived from the calibration curve by the mass of the test sample to calculate the anhydrous lactose mass fraction expressed as a percentage.

Express the test results to three decimal places.

Reference
7. Minerals and salts in milk

The salts of milk include those constituents except hydrogen ions and hydroxyl ions that are present as ions or in equilibrium with ions. A large part of the salt constituents of milk are mineral materials including metals and inorganic acid radicals. In addition, some organic acid radicals are present. Strictly speaking, the proteins of milk should be included as part of the salt system since they carry positively and negatively charged groups and can form salts with counter-ions; however, they are not normally treated as such.

A distinction is usually made between the major salt constituents of milk and the trace elements. The former category includes potassium, sodium, magnesium, calcium, phosphate, citrate, chloride, sulfate and bicarbonate. The latter group includes all other mineral and salt compounds.

Milk salts are important in three principal areas of dairy chemistry. In the first place, some of the salt constituents, particularly calcium and phosphate, are of tremendous importance in human nutrition. Milk is an excellent nutritional source of these minerals. Secondly, the physical state and physical stability of the milk proteins, particularly the caseinate, is very dependent on the composition of the salt system. Thus, problems arising in heat coagulation of evaporated milk and in rennet coagulation of milk stem largely from variations in salt composition. In the third place, certain metallic elements in milk, particularly copper, iron, catalyse oxidation of milk lipids, which leads to undesirable flavour.

Salts versus ash

The mineral content of foods is usually determined from the ash prepared by heating a sample at 500-600°C in a muffle furnace for about 4 h to oxidize organic matter. Ashing or incineration of milk destroys the organic compounds, leaving the mineral constituents behind. Actually, the ash does not truly represent the salt composition of milk for the following reasons.

- Organic radicals such as citrate are destroyed by the incineration process.
Phosphorus and sulfur of the milk proteins and lipids appear in the ash. The ash is a mixture of carbonates and oxides of the element present in the food and not of the original salts. Carbonates appearing in milk ash arise in part from carbon dioxide produced by decomposition of the organic matter. Oxidation during incineration results in the formation of oxides of the metals.

The temperature usually employed in ashing may vaporize certain volatile elements, e.g. sodium and potassium. Therefore, it is difficult or impossible to relate the ash obtained from a food with its salt system, and low values are obtained for certain mineral elements by analysis of the ash compared to direct analysis of the intact food. The ash content gives a rough idea of the total mineral content and of course, ashing is a useful technique of getting rid of organic matter as a prelude to analysis for many of the mineral elements.

**Composition of milk salts**

The ash content of milk remains relatively constant at 0.7 – 0.8%, but the relative concentrations of various ions can vary considerably. The ash content of human milk is only about 0.2%; the concentration of all principal and several minor ions is higher in bovine than in human milk.

**Changes in milk salt composition during infection of udder**

Milk from cow with mastitic infections contains a low level of total solids, especially lactose, and high levels of sodium and chloride, the concentration of which are directly related. The sodium and chloride ions come from blood to compensate osmotically for the depressed lactose synthesis or vice versa. These are related by the Koestler number:

\[
\text{Koestler Number} = \frac{\%Cl}{\%Lactose} \times 100
\]

Which is normally 1.5 – 3.0 but increases on mastitic infection and has been used as an index of such infections. The pH of milk increases (e.g. 6.8 -
6.9) to approach that of blood (7.4) during mastitic infection due to the influx of constituent from blood.

**Partition of milk salts between colloidal and soluble phase**

Certain of the milk salts (e.g. chloride, and the salts of sodium and potassium) are sufficiently soluble to be present almost entirely in the dissolved phase. The concentration of others, in particular calcium phosphate, is higher than can be maintained in solution at the normal pH of milk. Consequently, these exist partly in soluble form and partly in an insoluble or colloidal form associated with casein. The dividing line between soluble and colloidal is somewhat arbitrary, its exact position depending very much on the method used to achieve separation. However, a fairly sharp separation between the two phases is not difficult since the insoluble salts occur mainly associated with the colloidal phase.

**Methods used to separate the colloidal and soluble phases**

The method used must not cause changes in equilibrium between the two phases. The two most important precautions are to avoid change in pH (lowering the pH dissolves colloidal calcium phosphate) and temperature (reducing the temperature dissolves colloidal calcium phosphate and vice versa). Since milk comes from the cow at about 40°C, working at 20°C and especially at 4°C will cause significant shifts in calcium phosphate equilibrium.

Four principal methods used for this purpose are (i) pressure ultrafiltration, (ii) equilibrium dialysis, (iii) high speed centrifugation, and (iv) rennet coagulation. Pressure ultrafiltration involves forcing some of the dissolved phase of a sample of milk through a fine-pored filter by means of pressure. Cellophane or polysulphone membranes, clay, porcelain, collodion filters etc. are used for this purpose. Equilibrium dialysis involves dialyzing a small amount of water against at least 50 times its volume of milk until equilibrium (20°C for 48 h) is established. This can be conveniently done with cellophane membranes. The material then within the sac at the completion of equilibrium dialysis represents a sample of the dissolved phase of milk. It is
essential in using this technique that the amount of water be kept small in comparison to the amount of milk to avoid any undue dilution. High speed centrifugation can be used to sediment the colloidal particles and leave the dissolved phase as a supernatant. By treating milk with a small amount of rennet (rennet coagulation), which coagulates the colloidal caseinate particles, separation can be obtained. The sera obtained by centrifugation or by rennet coagulation contain the whey proteins or serum proteins of milk. On the other hand, the sera obtained by ultrafiltration or dialysis do not contain these serum proteins. All four methods of partition of the system give reasonably concordant results on the distribution of salts between the two phases.

Table 1: Distribution of salts between the soluble and colloidal phases of milk.

<table>
<thead>
<tr>
<th>Constituent (mg/100 ml)</th>
<th>Total concentration in milk</th>
<th>Dissolved</th>
<th>Colloidal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total calcium</td>
<td>114</td>
<td>38 (34%)</td>
<td>76 (66%)</td>
</tr>
<tr>
<td>Magnesium</td>
<td>11</td>
<td>7.4 (67%)</td>
<td>3.6 (33%)</td>
</tr>
<tr>
<td>Sodium</td>
<td>50</td>
<td>46 (92%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Potassium</td>
<td>148</td>
<td>137 (92%)</td>
<td>11 (8%)</td>
</tr>
<tr>
<td>Total Phosphorus</td>
<td>85</td>
<td>38 (43%)</td>
<td>47 (57%)</td>
</tr>
<tr>
<td>Citrate</td>
<td>166</td>
<td>156 (94%)</td>
<td>10 (6%)</td>
</tr>
<tr>
<td>Chloride</td>
<td>106</td>
<td>106 (100%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

The partition of salts between the soluble and colloidal phases is summarized in above Table 1. In general, most or all of the sodium, potassium, chloride and citrate, one-third of the calcium and two-thirds of the magnesium and about 40% of the inorganic phosphate are in soluble phase.

Changes in milk salt equilibria induced by various conditions

The equilibria between the soluble and colloidal salts of milk are influenced by many factors, the more important of which are discussed below, and which consequently modify the processing properties of milk. Milk serum is supersaturated with calcium phosphate, the excess being
present in the colloidal phase. The balance between the colloidal and soluble phases may be upset by various factors, including changes in temperature, dilution or concentration, addition of acid, alkali or salts as follows:

1. **Addition of acid or alkali:**

   Acidification of milk either directly or indirectly by bacterial action is accompanied by a progressive solublization of colloidal calcium phosphate and other colloidal salts from casein. Solublization is complete below about pH 4.9. Addition of alkali has the opposite effect, and at about pH 11 almost all the soluble calcium phosphate occurs in the colloidal phase.

2. **Addition of various salts:**

   Divalent cations: Addition of calcium to milk causes precipitation of soluble phosphate as colloidal calcium phosphate, an increase in ionized calcium, a decrease in the concentration of soluble phosphate and a decrease in pH.

   Phosphate: Addition of secondary Na or K phosphate (i.e. Na₂HPO₄ or K₂HPO₄) causes the precipitation of colloidal calcium phosphate, with concomitant decreases in the concentration of soluble calcium and calcium ion. Polyphosphates, e.g. Na-hexametaphosphate, chelate Ca²⁺ strongly and dissolve colloidal calcium phosphate.

   Citrate: Addition of citrate reduces the concentration of calcium ions and colloidal calcium phosphate and increases the soluble calcium, soluble phosphate and pH.

3. **Effect of changes in temperature:** The solubility of calcium phosphate is markedly temperature dependent. Unlike most compounds, the solubility of calcium phosphate decreases with increasing temperature; therefore heating causes precipitation of calcium phosphate while cooling increases the concentration of soluble calcium and phosphate at the expense of CCP. At low temperature, shifts in the ionic balance are
readily reversible, but after heating at high temperatures, reversibility becomes more sluggish and incomplete.

4. Changes in pH induced by temperature: The pH of milk is changed following heating due to changes in two salt systems. Fresh milk contains 200 mg of CO$_2$ per litre; about 50% of this is lost on standing, with additional losses on heating. This results in a decrease in titratable acidity and an increase in pH. The formation of colloidal calcium phosphate during heating more than compensates for the loss of CO$_2$. The effect of temperature on pH is shown in below Table.

**Table 2. Effect of temperature on the pH of milk**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>6.64</td>
</tr>
<tr>
<td>30</td>
<td>6.55</td>
</tr>
<tr>
<td>40</td>
<td>6.45</td>
</tr>
<tr>
<td>50</td>
<td>6.34</td>
</tr>
<tr>
<td>60</td>
<td>6.23</td>
</tr>
</tbody>
</table>

The change in pH can be explained as follows

$$3\text{Ca}^{2+} + 2\text{HPO}_4^{2-} \rightarrow \text{Ca}_3(\text{PO}_4)_2 + 2\text{H}^+$$

The reaction is reversible on cooling after heating to moderate temperatures but becomes only partly reversible following more severe heating.

5. Effect of dilution and concentration: Since milk is saturated with respect to calcium and phosphate, dilution of milk with water reduces the concentration of Ca$^{2+}$ and HPO$_4^{2-}$ and causes solublization of some colloidal calcium phosphate, making the milk more alkaline. Concentration of milk causes precipitation of colloidal phosphate and shifts the reaction of milk to the acid side. e.g. concentration by a factor of 2:1 reduces the pH by about 0.3 unit and by about 0.5 unit for 3:1 concentration.
Dilution: \( \text{Ca}_3(\text{PO}_4)_2 \rightarrow 3\text{Ca}^{2+} + 2\text{HPO}_4^{2-} + 2\text{OH}^- \)

Concentration: \( 3\text{Ca}^{2+} + 2\text{HPO}_4^{2-} \rightarrow \text{Ca}_3(\text{PO}_4)_2 + 2\text{H}^+ \)

6. **Effect of freezing:** Freezing milk causes crystallization of pure water and unfrozen liquid becomes more saturated with respect to various salts. Some soluble calcium phosphate precipitates as \( \text{Ca}_3(\text{PO}_4)_2 \), with the release of \( \text{H}^+ \) and a decrease in pH (e.g. to 5.8 at -20°C). The combination of increased concentration of \( \text{Ca}^{2+} \) and reduced pH causes destabilization of the casein micelles.

**Table 3. Minerals of milk of different species**

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Contents in milk (per 100 g fluid milk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cow milk</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>114</td>
</tr>
<tr>
<td>Chloride (mg)</td>
<td>106</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>11</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>85</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>148</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>50</td>
</tr>
<tr>
<td>Citrate (mg)</td>
<td>166</td>
</tr>
<tr>
<td>Sulphur (mg)</td>
<td>30</td>
</tr>
<tr>
<td>Iron (µg)</td>
<td>50</td>
</tr>
<tr>
<td>Copper (µg)</td>
<td>20</td>
</tr>
<tr>
<td>Zinc (µg)</td>
<td>350</td>
</tr>
</tbody>
</table>
8. Determination of total ash content in milk

Ash refers to the inorganic residue remaining after either ignition or complete oxidation of organic matter in any foodstuff. There are three major types of ashing (a) dry ashing which is generally done for majority of food samples (b) wet ashing or oxidation, for food samples with high fat content (c) low temperature plasma dry ashing used for samples when the volatile elemental analysis are carried out. High fat products need to be dried and fat extracted before ashing. For milk, dry ashing is carried out.

Milk contains soluble salts mainly the phosphates, citrates, sulphates, chlorides, carbonates and bicarbonates of calcium, magnesium, potassium, sodium etc. Heating milk at higher temperatures (500 to 600°C) decomposes organic matter and soluble inorganic salts are left behind in the form of ash. The milk ash does not truly represent the salt composition of milk for the following reasons (a) organic radicals such as citrates are destroyed during incineration process (b) phosphorus and sulfur of milk proteins and lipids appear in ash (c) the temperature usually employed in ashing may vaporize certain volatile elements e.g sodium and potassium

**Apparatus**

1. Platinum or silica crucible: Crucibles of about 100 ml capacity with lids.
2. Muffle furnace: Capable of being controlled at 550 ± 20°C.
4. Desiccator: Containing an efficient desiccant.
5. Safety tongs having long arms.

**Procedure**

1. Heat the crucible with its lid alongside in an oven maintained at 100 ± 2°C in order to remove any moisture from it.
2. Place the lid on the crucible, immediately transfer the crucible to the desiccator, allow to cool to room temperature, and weigh the crucible with lid to the nearest 0.1 mg.
3. Pipette into the crucible about 10 ml of the prepared sample of milk and weigh quickly, with the lid on the crucible. Place the uncovered crucible containing milk on a boiling water-bath and evaporate the milk sample to dryness avoiding spurting of the milk sample.

4. Transfer the uncovered crucible containing milk sample to a muffle furnace maintained at a temperature 550 ± 20°C, placing the lid along its side, using safety tongs. Incineration is carried out until grey ash is obtained. It takes about 4 to 6 h to obtain carbon free ash.

5. Remove the covered crucible from the muffle furnace and quickly transfer the crucible to a desiccator and allow the crucible to cool to room temperature. Take the weight of the covered crucible containing ash.

6. Repeat the process of heating in the furnace (for 30 min) followed by cooling and weighing until the difference between two consecutive weighing is less than 1 mg. Record the lowest mass.

**Calculation**

Ash content, % by mass = \[
\frac{M_2 - M_0}{M_1 - M_0} \times 100
\]

Where,

M₀ = mass in g, of empty crucible along with lid
M₁ = mass in g, of the crucible, lid and test portion of liquid milk taken for the test and
M₂ = mass in g, of the crucible, lid and ash
9. Determination of chloride content in milk

The normal range of chloride content in cow’s milk is 80-140 mg/100 ml and in buffalo’s milk is 60-70 mg/100 ml. Determination of chloride content in milk can be used as a means of detecting abnormal milks such as infected udders, colostrum and late lactation milk which are usually high in chloride content. Values of chloride content higher than 0.14% (140 mg/100 ml) in milk indicate the presence of abnormal milk.

Estimation of chloride content in milk is based on argentometric titration (Volhard’s method). In this method a known excess quantity of standard AgNO₃ solution is added to the known volume of milk. The chloride present in milk reacts with silver nitrate and forms white precipitate of insoluble silver chloride. The unused AgNO₃ (which is present in excess) is back titrated against standard potassium thiocyanate (KCNS) in presence of concentrated HNO₃ using ferric alum as indicator. End point of titration is the first appearance of orange-red colour which persists for at least 15 seconds. End point of titration is not clearly visible in sample titration with potassium thiocyanate (KCNS). HNO₃ is added to the system to dissolve the interfering casein and also to make the solution acidic so as to keep the AgCl in the precipitated form.

**Reaction**

\[
\text{Cl}^- + \text{Ag}^+ \rightarrow \text{AgCl (until all Cl}^- \text{ is complexed)}
\]

\[
\text{Ag}^+ + \text{SCN}^- \rightarrow \text{AgSCN (to quantitate silver not complexed with chloride)}
\]

\[
\text{SCN}^- + \text{Fe}^{+3} \rightarrow \text{FeSCN (orange-red when there is any SCN}^- \text{ not complexed to Ag}^+)
\]

**Reagents:**

1. Ferric alum indicator: In 10% nitric acid, prepared by boiling excess of iron alum, cooling and filtering.
2. Potassium thiocyanate: 0.05 N, standardized against standard potassium chloride.
3. Silver nitrate: Approximately 0.05 N.
4. Concentrated nitric acid.

**Apparatus**

Titration flask, burette, pipettes etc.

**Procedure**

1. Take 10 g of the sample, accurately weighed in a 250 ml of Erlenmeyer flask.
2. To this add 10 ml of silver nitrate solution and 10 ml of concentrated nitric acid. Shake the content.
3. Digest the mixture until reddish brown fumes are evolved.
4. Cool the flask and add 1 ml of saturated iron alum solution.
5. Determine the excess of silver nitrate by titrating with the standard potassium thiocyanate until the first appearance of an orange red colour that persists for 10 seconds.
6. In the same manner, determine the volume of the standard thiocyanate solution equivalent to 10 ml of silver nitrate using the same volumes of reagents and water.

**Calculations**

Chloride, % by weight = 0.01773 (B - A)
Chloride as sodium chloride, % by weight = 0.02923 (B - A)

Where,

B = volume in ml of the standard potassium thiocyanate solution required by the blank, and
A = volume in ml of the standard potassium thiocyanate solution required by the sample.
10. Estimation of calcium and magnesium in milk by EDTA method

The average calcium content in cow milk is around 1200 mg/L (Range 1100–1300 mg/L). 67% of the total calcium in milk is in colloidal form and the remaining (33%) is in soluble form. The average magnesium content in cow milk is around 120 mg/L (Range 90 – 140 mg/L). 33% of the total magnesium in milk is in colloidal form and the remaining (67%) is in soluble form.

In this method which is based on Davis and White (1962) procedure, milk proteins are precipitated with TCA and the protein free filtrate is used for the estimation of calcium and magnesium. EDTA is used as titrant for the estimation of calcium and magnesium. Titrations are carried out in such way that other ions do not interfere during titrations.

For the separate estimation of calcium and magnesium, calcium is first precipitated as calcium oxalate with the help of ammonium oxalate at low pH (pH 4.0) and then the precipitate is separated by centrifugation; the supernatant containing magnesium is decanted. The success of the method depends on the strict conditions for precipitation of calcium as given in the following procedure.

Magnesium in the calcium free supernatant is estimated by titrating the supernatant at pH 10.0 against standard EDTA solution and using Erichrome Black T as indicator.

Calcium is estimated in the precipitates obtained above which are dissolved in HCl. The calcium in the dissolved precipitate is not estimated by direct titration with EDTA due to the interference of phosphate. At high pH, phosphates interferes with titration by precipitating Ca++ as Ca$_3$(PO$_4$)$_2$. This is eliminated by adding the excess of EDTA solution to the dissolved calcium oxalate (at low pH) and the reaction is completed, and then pH is raised. The unused EDTA is then back titrated against standard magnesium acetate using Calmagite as indicator.
From the volume of EDTA used in both the titrations, the quantity of calcium and magnesium can be calculated.

1 ml of 0.004 M EDTA = 0.09728 mg of Magnesium
1 ml of 0.05 M EDTA = 2.004 mg of Calcium

In the above method EDTA is used. EDTA (Ethylene diamine tetra-acetic acid) behaves as dibasic acid and it reacts with different types of metal depending upon the pH of the medium i.e. reaction of EDTA with cations is pH dependent. For practical purpose, Na-salt of EDTA is used because this acid is unstable. The formula for EDTA is as follows:

If we abbreviate the formula of EDTA as H₄V or H₂(H₂V) then its Na-salt will have the formula Na₂H₂V. The Na-salt when in solution supplies anions H₂V⁻² which then react with Ca and Mg as follows:

\[ \text{Ca}^{+2} + \text{H}_2\text{V}^{-2} \rightarrow \text{Ca}_2\text{V}^{-2} + 2\text{H}^+ \]

\[ \text{Mg}^{+2} + \text{H}_2\text{V}^{-2} \rightarrow \text{Mg}_2\text{V}^{-2} + 2\text{H}^+ \]

EDTA is a powerful sequestering or chelating agent for divalent and polyvalent cations. The EDTA is often used as titrant for the estimation of metal ions. EDTA usually binds to a metal cation through its two amines and four carboxylates.
Reagents

A. For calcium estimation

1. Trichloroacetic acid (TCA) solution: 12% solution.
2. Ammonium oxalate (NH₄COO)₂ H₂O solution: 4%, Aq. (if required, dissolve by heating).
3. Methyl red indicator solution: 0.02% solution in 95% ethanol.
4. Sodium hydroxide: 20% solution.
5. Hydrochloric acid: 1 N.
6. Ethylenediamine tetracetic acid disodium salt (EDTA) dihydrate, 0.05 M: Dissolve accurately weighed 1.8612 g of disodium ethylenediamine tetracetate dihydrate in water and dilute to 100 ml in a volumetric flask.
7. NH₄Cl-NH₃ buffer (pH 10): Prepare by mixing 142 ml of liquor ammonia (sp. gr. 0.88) and 17.5 g ammonium chloride, and diluting the mixture with water to 250 ml.
8. Magnesium acetate solution (0.015 M): Weigh 1.6086 g (CH₃COO)₂Mg·4H₂O, dissolve in water and dilute to 500 ml in a volumetric flask.
9. Calmagite solution: Dissolve 50 mg calmagite in 10 ml of 2-methoxyethanol, filter if necessary. Store the solution in dark and use within one week. Alternatively the dye can be dissolved in water and stored in dark.

B. For Magnesium estimation

1. EDTA solution (0.004 M): Weigh accurately 0.1489 g EDTA-disodium dihydrate, dissolve in water and dilute to 100 ml in a volumetric flask.
2. Erichrome black T solution: Dissolve 50 mg of the dye in 10 ml of 2-methoxyethanol, filter if necessary and store in dark; use within one week. Alternatively, 50 mg of the dye is dissolved in a mixture of 7.5 ml of triethanolamine and 25 ml absolute ethanol, or even in 10 ml of pure methanol.
Apparatus

1. Graduated centrifuge tubes rounded conical bottom: 15 ml capacity, graduated.
2. Centrifuge to spin at 1400 g.

Procedure

A. Estimation of calcium

1. Take 20 ml milk in a 50 ml volumetric flask. Make up the volume with 12% TCA solution. Shake and keep for 30 min.
2. Filter it through Whatman No. 40/42 filter paper. Take 2.5 ml of filtrate in 15 ml centrifuge tubes.
3. To each tube add 0.5 ml of ammonium oxalate (to precipitate calcium as calcium oxalate) and add 2 drops of methyl red indicator (the colour of solution will be pink). Then add 20% NaOH drop wise till the colour is pale yellow.
4. Add dilute HCl (0.1 N) till the colour changes to pink. Keep the tubes for 4 h at room temperature to complete the precipitation of calcium oxalate.
5. After 4 h dilute the contents of the tube to about 10 ml with distilled water and then centrifuge at 1400 rpm for 10 min. Decant the supernatant liquid into a tube, allowing sufficient time for thorough drainage, and retain it for magnesium estimation. The precipitate is used for calcium estimation.
6. Dissolve the calcium oxalate precipitate in 0.5 ml of HCl and transfer to a beaker. Add 1 ml of 0.05 M EDTA. After 5 min add 1 ml of ammonia buffer and 2-3 drops of calmagite indicator.
7. Immediately titrate (the excess of EDTA) the solution against magnesium acetate (0.015 M) till the colour changes from blue to pinkish red/wine red
8. For a blank determination, repeat the whole procedure by taking distilled water instead of milk. Retain the supernatant liquid for the blank determination for magnesium estimation.
B. **Estimation of Magnesium**

1. To the supernatant obtained in the step A-5 (for blank use supernatant obtained at step A-8), add 0.5 ml of ammonia buffer of pH 10.0 and add 2-3 drops of Erichrome Black T indicator.
2. Titrate the solution against 0.004 M EDTA, till the colour is bluish green (colour change is from red to bluish green). Similarly conduct the blank titration also.

**Calculation**

**For calcium**

Volume of magnesium acetate (0.015 M) for Blank = B ml
Volume of magnesium acetate (0.015 M) for Sample = S ml
(B-S) ml of magnesium acetate (0.015 M) is equivalent to the amount of EDTA which has combined with calcium in the sample.

Now B ml of magnesium acetate = 1 ml of EDTA (used for blank)
Therefore, 1 ml of magnesium acetate = 1/B ml of EDTA
& (B-S) ml of magnesium acetate = (B-S)/B ml of EDTA

\[ = \frac{B-S}{B} \text{ ml of EDTA} \]

we know 1 ml of EDTA (0.05 M) = 2.004 mg of Calcium
Therefore C ml of EDTA (0.05 M) = 2.004 x C mg of Calcium
i.e. 2.5 ml of milk filtrate contains = 2.004 x C mg of calcium

Or 50 ml of filtrate contains = \(2.004 \times C \times \frac{50}{2.5}\) mg of Calcium

Or 20 ml milk contains = \(2.004 \times C \times \frac{50}{2.5}\) mg of Calcium

Therefore 100 ml of milk has = \(2.004 \times C \times \frac{50}{2.5} \times \frac{100}{20}\) mg of Calcium

\[ = 200.4 \times C \text{ mg of calcium per 100 ml of milk} \]
For magnesium

Volume of EDTA (0.004 M) used for blank = B ml
Volume of EDTA (0.004 M) used for sample = S ml
Therefore, volume of EDTA (0.004 M) used = (S - B) ml

= M ml of EDTA (say)

We know 1 ml of EDTA (0.004 M) = 0.09728 mg of Magnesium
therefore, M ml of EDTA (0.004 M) = 0.09728 x M mg of Magnesium
i.e. 2.5 ml of filtrate contains = 0.09728 x M mg of Magnesium

& 50 ml of filtrate contains = \[
\frac{0.09728 \times M \times 50}{2.5}
\] mg of Magnesium

or 20 ml milk has = \[
\frac{0.09728 \times M \times 50}{2.5}
\] mg of Magnesium

100 ml milk has = \[
\frac{0.09728 \times M \times 50 \times 100}{2.5 \times 20}
\] mg of Magnesium.

= 9.728 \times M \text{ mg of magnesium per 100 ml milk}

Reference

11. Determination of calcium content in milk by titrimetric method (IDF method)

In this method (IDF, 2010), milk proteins are precipitated using trichloroacetic acid, followed by filtration. Calcium in the filtrate is precipitated as calcium oxalate, and is separated by centrifugation. The precipitates obtained after centrifugation are washed, dissolved and titrated using standard potassium permanganate solution. The calcium content in the sample is expressed as percentage by mass.

**Apparatus**

1. Volumetric flask: 50 ml capacity.
2. Pipette: 20 ml capacity.
3. Centrifuge, capable of producing a radial acceleration of 1400 g.
4. Centrifuge tubes: 30 ml capacity, cylindrical and round bottomed, graduated at 20 ml.
5. Pipettes: 2 and 5 ml capacities.
6. Suction device with capillary tube.
8. Burette: 10 ml capacity, graduated to 0.02 ml.

**Reagents**

1. Trichloroacetic acid solution I: 200 g/L.
2. Trichloroacetic acid solution II: 120 g/L.
3. Ammonium oxalate: Saturated solution, cold.
4. Methyl red solution: Dissolve 0.05 g of methyl red in 100 ml of ethanol (96%, v/v).
5. Acetic acid solution: 20 % (V/V) solution.
6. Ammonia solution I: Mix equal volumes of 25% (m/m) ammonia solution and distilled water.
7. Ammonia solution II: Dilute 2 ml of 25% (m/m) ammonia solution to 100 ml with distilled water.
8. Sulphuric acid solution: Add 25 ml of concentrated sulphuric acid (98%, m/m) to 80 ml of distilled water.

9. Standard Potassium permanganate solution, (KMnO₄): 0.02 mol/l.

10. Whatman filter paper: Grade 42, ashless.

Procedure

A. Precipitation of protein substances

Weigh by difference, to the nearest 10 mg, approximately 20 g of the prepared test sample of milk in a 50 ml volumetric flask. Gradually add, while shaking, trichloroacetic acid solution I until a volume of 50 ml is obtained. Shake vigorously for a few seconds and allow to stand for 30 min. Filter through Whatman filter paper Grade 42, taking care that the filtrate obtained is clear.

B. Precipitation and separation of calcium as calcium oxalate

1. Transfer 5 ml of the clear filtrate in a 30 ml centrifuge tube and then add 5 ml of the trichloroacetic acid solution II, 2 ml of the ammonium oxalate solution, two drops of the methyl red solution and 2 ml of the acetic acid solution. Mix the contents of the tube by swirling.

2. Gradually add ammonia solution I drop by drop until the colour becomes pale yellow. Then add a few drops of the acetic acid solution until a pink coloration appears. Allow to stand for 4 h at room temperature.

3. Dilute with water to 20 ml and centrifuge it at 1400 g for 10 min. Remove the clear supernatant liquid with the suction device.

4. Taking care not to disturb the deposit of calcium oxalate, rinse the walls of the centrifuge tube with 5 ml of ammonia solution II. Centrifuge at 1400 g for 5 min, and then remove the supernatant liquid with the suction device. Repeat this washing operation twice.

C. Titration

1. Add 2 ml of the sulphuric acid solution and 5 ml of water to the calcium oxalate deposit in the centrifuge tube.
2. Place the tube in the boiling water-bath. When the calcium oxalate deposit is completely dissolved, titrate with the potassium permanganate solution until a pink colour persists. Take care that during the titration the temperature of the solution is above 60°C.

3. Record the volume, in ml, of potassium permanganate solution used, to the nearest 0.01 ml.

D. Blank test

1. Carry out a blank test in parallel with the determination but using 20 ml of water instead of the milk sample.

2. Record the volume, in ml, of potassium permanganate solution used, to the nearest 0.01 ml.

Calculation

The calcium content, expressed in g of calcium per 100 g of milk, is calculated using the formula:

\[
\text{Calcium content} = 0.0004 \times \frac{(V - V_0) \times 1000}{m} \times f
\]

\[
= 0.4 \times \frac{(V - V_0) \times f}{m}
\]

Where

\(V\) = volume, in ml, of potassium permanganate solution used for the titration of milk sample;

\(V_0\) = volume, in ml, of potassium permanganate solution used in the blank test;

\(m\) = mass in g, of the test portion;

\(f\) = a correction factor for the volume of precipitate resulting from the trichloroacetic acid precipitation, as follows:
For milk having a fat content of \([\% \text{ (m/m)}]\)  

<table>
<thead>
<tr>
<th>Fat Content [% (m/m)]</th>
<th>Correction Factor ((f))</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5 to 4.5</td>
<td>0.972</td>
</tr>
<tr>
<td>3</td>
<td>0.976</td>
</tr>
<tr>
<td>2</td>
<td>0.980</td>
</tr>
<tr>
<td>1</td>
<td>0.985</td>
</tr>
<tr>
<td>&lt; 0.1</td>
<td>0.989</td>
</tr>
</tbody>
</table>

Express the result to the nearest 0.001 g.

**Reference**

Milk -- Determination of calcium content -- Titrimetric method (ISO 12081:2010 (IDF 36:2010))
12. Estimation of total phosphorus content in milk by Fiske & Subbarow method

The average phosphorus content in cow milk is around 950 mg/L (Range 900 - 1000 mg/L). 57% of the total phosphorus in milk is in colloidal form and the remaining (43%) is in soluble form.

There is a possibility of losses of phosphorus during the ashing process of milk, so the more satisfactory procedure to estimate phosphorus in milk is to oxidize the milk with oxidizing agent, such as H$_2$O$_2$, HClO$_4$, HNO$_3$ etc. and digest it with H$_2$SO$_4$ which will effect complete conversion of all the phosphorus to H$_3$PO$_4$ (Phosphoric acid). The acidic solution (digested material) is then treated with excess solution of ammonium molybdate which gives yellow coloured precipitate of ammonium phospho-molybdate. These precipitates on addition of reducing reagent (1-ammino 2-napthol 1, 4 sulphonic acid or ANSA) form blue color complex which is unstable and uncertain in composition. The intensity of the blue colour is proportional to the amount of phosphorus present in the sample and can be estimated spectrophotometrically at 600 nm. Amount of phosphorus in the sample can be calculated from the standard curve.

**Reaction**

Phosphorus under acidic conditions reacts with ammonium molybdate forming the ammonium phophomolybdate which further reacts with reducing reagent (ANSA) to form the molbedanum blue complex.

\[
P^+ (\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O} \rightarrow (\text{NH}_4)_3\text{PO}_4 \cdot 12\text{MoO}_3 \\
\text{(Yellow coloured ppt)}
\]

+ Reducing reagent (ANSA) \rightarrow Blue colour complex

(Molybdenum blue)

Probably the oxides of molybdenum
Reagents

1) 5 N H$_2$SO$_4$
2) 60% HClO$_4$ or 30% H$_2$O$_2$
3) 2.5% ammonium molybdate solution: Dissolve 2.5 g of ammonium molybdate in water. Transfer to a 100 ml vol. flask and fill to the mark and mix.
4) Reducing reagent (1-Amino-2-naphthol-4-sulphponic-acid-ANSA): 29.25 g of sodium bisulphite (anhydrous), 0.5 g of ANSA and 1 g of sodium sulphite (anhydrous) is grounded and kept in a cool and dry place. Before using, 2.5 g of reagent is dissolved in 100 ml of distilled water.
5) Standard Phosphorus Solution: Dissolve exactly 2.192 g of pure KH$_2$PO$_4$ (Potassium dihydrogen phosphate) in 250 ml distilled water which gives a concentration of phosphorus as 2 mg/ml.

Procedure

A. Preparation of samples for standard curve

Take 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 ml of standard phosphorus solution in different volumetric flasks of 50 ml capacity. Dilute the contents in each flask to 50 ml mark with distilled water. Take 2 ml of each of these diluted standard solutions of phosphorus in different Kjeldahl flasks of 30 ml capacity. (2 ml of these solutions will have 0, 40, 80, 120, 160, 200, 240 and 280 µg/2 ml respectively). Put one piece of glass bead in each Kjeldahl flask.

B. Preparation of milk samples

Take 2 ml of milk and dilute to 50 ml with distilled water and take 2 ml of this diluted milk sample also in a separate kjeldahl flask and put one glass bead in this also.

C. Digestion and Estimation

To each Kjeldahl flask containing standard solution of phosphorus and diluted milk sample, add 2.5 ml of 5 N H$_2$SO$_4$. Heat it on the open flame
in a Kjeldahl room till the colour of the solution is dark brown or black. Remove the flasks and cool slightly and then add few ml of perchloric acid dropwise. Again heat till the solution is colourless and no fumes of perchloric acid come out. Add 2-3 ml of distilled water and again heat for 5 min. so that no perchloric acid is remaining.

Now cool the flasks and transfer the contents (washings with distilled Water) to 50 ml vol. flasks. Adjust the volume to about 25 ml. Now add 2.5 ml of Ammonium molybdate solution to each volumetric flask and add 1 ml of reducing reagent (ANSA) in each flask. Make the volume upto the mark (50 ml) with dist. water and mix. Intensity of the colour (figure 1) is measured at 600 nm within 10 minutes. Alternatively, samples may be kept at boiling water bath for about 7 minutes to get the maximum colour development and then cooled and measured colorimetrically. Here colour will also be stable.

Calculations

Let OD for the sample be = X
Corresponding concentration from graph = Y µg
2 ml of diluted sample contains = Y µg of P
1 ml of diluted sample contains = Y/2 µg of P

50 ml diluted sample or 2 ml milk has \( \frac{Y}{2} \times 50 \) µg of P.

Therefore, 1 ml milk has \( \frac{Y}{2} \times 50 \times \frac{1}{2} \)

and 100 ml milk \( \frac{Y}{2} \times 50 \times \frac{1}{2} \times 100 \) µg of P

\[
\text{mg} \% \ P = \frac{Y}{2} \times 50 \times \frac{1}{2} \times 100 \times \frac{1}{1000} \\
= Y \times \frac{5}{4} \text{ mg} \ %
\]

References

I  J. Biol. Chem. 66, 375 (1925)
II Hawk’s Physiological Chemistry by Oser, B.L.
Figure 1: The colour development in blank, standard curve and sample during phosphorus estimation in milk by Fiske & Subbarow method
13. Estimation of total phosphorus in milk by BIS method

The average phosphorus content in cow milk is about 95 mg/100 ml and buffalo’s milk is about 125 mg/100 ml. About one third of the total phosphorus is present in soluble form and rest is in colloidal form. The total phosphorus in milk is distributed in five fractions, as follows:

1. Inorganic salt in solution = 33%
2. Organic ester in solution = 7%
3. Lipid phosphorous = 1.5%
4. Protein phosphorous (Bound with Casein) = 20%
5. Inorganic colloidal phosphate = 38.5%

In this method of estimation, the phosphorous must be present as ortho phosphate (PO$_4^{3-}$). Upon treatment with excess of ammonium molybdate solution at 20-45°C, in the presence of nitric acid, phosphorous present in the ash solution gives a precipitate of ammonium phospho-molybdate with the composition (NH$_4$)$_3$ [PO$_4$.12 MoO$_3$]. The precipitates obtained are suitably washed with dilute NaNO$_3$ or KNO$_3$ solution. The washed precipitates thus obtained are dissolved in known quantity of standard alkali and the excess alkali which remains unreacted is titrated back against standard acid.

The following reaction is generally assumed to take place with the precipitates formed at 20-45°C.

\[
(PO_4) + (NH_4)_6Mo_7O_{24}.4H_2O \rightarrow (NH_4)_3[PO_4.12 MoO_3] \\
(\text{Ammonium molybdate}) \quad \text{(Ammonium phospho-molybdate, Yellow Coloured ppt)}
\]

\[
(NH_4)_3[PO_4.12 MoO_3] + 23 NaOH \rightarrow 11 Na_2MoO_4 + (NH_4)_2MoO_4 + Na(NH_4)HPO_4 + 11 H_2O
\]

**Reagents**

1. Concentrated Nitric Acid - sp. gr 1.42
2. Dilute Nitric Acid (1: 3 by volume)
3. Ammonium Hydroxide - sp. gr 0.88
4. Molybdate Solution - Dissolve 150 g ammonium molybdate in 271 ml of
distilled water. Cool, and pour the solution slowly and with constant stirring into a cool mixture of 489 ml of conc. nitric acid and 1148 ml of distilled water. Keep the final mixture in a warm place for several days or until a portion heated to 40°C deposits no yellow precipitate of ammonium phospho-molybdate. Decant the solution from any sediment and preserve in glass-stoppered vessels. To 100 ml of the molybdate solution, add 5 ml of conc. nitric acid. Filter this solution immediately before using.

5. Standard alkali (Sodium or Potassium Hydroxide) Solution - Dilute 324·03 ml of 1N strength alkali, free from carbonates, to one litre.

6. Standard acid solution (HCl or HNO₃) - Prepare a solution of hydrochloric acid or of nitric acid corresponding to the strength of alkali (0.32403 N) and standardize by titration against that solution using phenolphthalein indicator.

7. Phenolphthalein indicator solution - one percent in rectified spirit.

8. Dilute hydrochloric acid (1:4 by Volume).

**Procedure**

Exactly weigh 10 g of milk in silica or nickel crucible dish, keep it for evaporation to dryness on a burner. Then transfer to muffle furnace and allow for ashing at not more than 550°C. Cool the content in a desiccator and dissolve the ash contents in dilute HCl (1:4).

Use 20 ml of the solution, prepared as above. Add 10 ml of concentrated nitric acid (sp gr 1.42). Add conc. ammonium hydroxide (sp gr 0.88) drop wise until the precipitate that forms dissolves but slowly on stirring vigorously. Dilute to 75 to 100 ml with distilled water. and adjust to a temperature of 25 to 30°C. If the sample does not give a precipitate with ammonium hydroxide as test of neutralization, make the solution slightly alkaline to litmus paper with ammonium hydroxide and then slightly acidic with nitric acid (l: 3). Add 20 to 25 ml of freshly filtered molybdate solution. Place the solution in a shaking or stirring apparatus and shake or stir for 30 minutes at room temperature. Decant at once through a filter
paper (Whatman No. 42 or its equivalent) and wash the precipitate twice by
decantation with 25 to 30 ml portions of water containing 2 percent of
sodium nitrate, agitating thoroughly and allowing to settle. Transfer the
precipitate to the filter and wash with cold water containing 2 percent of
sodium nitrate until the filtrate from the fillings of the filter yields a pink
colour upon the addition of phenolphthalein indicator and one drop of
standard alkali. Transfer the precipitate and the filter paper to a beaker.
Dissolve the precipitate in a small excess of the standard alkali, add a few
drops of phenolphthalein indicator and titrate with standard acid solution.
One ml of the standard sodium or potassium hydroxide solution = 0.4364
mg of phosphorus.

**Calculation**

Phosphorous, % by weight = \[
\frac{0.4364 \times (V_1 - V_2)}{2W_1}
\]

Where

\( V_1 \) = volume in ml of standard alkali used to dissolve the ppt.

\( V_2 \) = volume in ml of standard acid solution required for neutralization of
excess alkali, and

\( W_1 \) = weight in g of the prepared sample taken for the determination of ash.

**Reference**

14. Estimation of iron content in milk by 1, 10 orthophenanthroline method

Iron content in normal cow and buffalo milks is about 50 and 85 µg/100 ml, respectively. Its estimation is based upon the spectrophotometric procedure and the principal involved is as follows:

Iron in ferrous form reacts with orthophenanthroline to form an orange red complex. The colour intensity of the complex formed is independent of pH range 2-9 and is stable for long periods (Below pH 2.0 the colour does not develop/ develops slowly or does not develop fully. Above pH 9.0 the colour may not develop, and also interference and difficulty due to precipitation of metal hydroxides may be observed). To eliminate the interference of copper the pH must be controlled between 2.5 - 4.0. The method is so standardized that the final pH of the solutions and standards will be between 3.4-3.8. Acetate buffer is used to maintain the pH. Hydroxylamine hydrochloride is used as reducing agent to convert all ferric iron to ferrous form. The orange red colour of the ferrous phenanthroline complex is directly proportional to the concentration of iron in the sample and may be measured in a colorimeter at 515 nm against a blank. A standard curve is drawn using ferrous ammonium sulfate.

\[ \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} \text{ (By reduction)} \]

1, 10 orthophenanthroline
Reagents

1. 10% hydroxylamine Hydrochloride (HONH₂HCl): 10 g of hydroxylamine hydrochloride + 90 ml of distilled water. This solution is stable for one week.

2. Acetate Buffer solution (pH 4.6): 16.6 g of sodium acetate + 24 ml of glacial acetic acid diluted to 200 ml with distilled water.

3. 1, 10-orthophenanthroline solution: Dissolve 1 g of this reagent in 300 ml of distilled water, by stirring and heating to 80°C. Discard the solutions, if it darkens. Heating is not necessary, if two drops of conc. HCl are added to distilled water. This solution is stable for at least one week.


5. HCl (1:1).


A. Stock Iron solution (conc.1 mg/ml): Fe (NH₄)₂(SO₄)₂.6H₂O = Mol.Wt 392

56 g iron is present in 392 g of the above salt
1 g = 392/56 =7.0

7.0 g of salt dissolved in 1000 ml water give 1 g of Iron/1000ml or 1mg/ml

Dissolve 07g of ferrous ammonium sulphate in 1000 ml volumetric flask and add 1 drop of conc. HCl and make up the volume to 1000 ml.

B. Working solution (Conc.10 µg/ml): Take 10ml of stock solution and dilute to 1000 ml.

Glassware


2. Silica crucible

3. Pipettes: 1 ml, 2 ml, 5 ml, 10 ml.


5. Muffle furnace.

Note: Wash all glassware with concentrated HCl and rinse with distilled water before use to remove deposit of iron oxide.
Procedure

1. **Preparation of standard curve**

   Take aliquots of 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ml of the standard iron working solution accurately measured in Pyrex test tubes and dilute to 10 ml with distilled water in each test tube. Add the following reagents in the below mentioned sequence and shake well after each additions.
   
   - 2 ml of hydroxylamine hydrochloride solution.
   - 5 ml of acetate buffer solution.
   - 2 ml of 1, 10-orthophenanthroline solution.

   For blank, take 10 ml of Distilled Water and add the above reagents in the same order.

2. **Preparation of sample and estimation of iron**

   Take 25 ml of milk; evaporate to dryness on direct flame and ash in muffle furnace (500-550°C) until carbon free. Remove the dish from the muffle furnace and allow to cool. Place dish on a clay triangle and add 5 ml of HCL (1:1). Cover the sample with watch glass and boil gently for 5 min. (Boiling for 5 min with HCl is done to eliminate the interferences due to pyrophosphates). Cool the dish and watch glass. Remove the watch glass, if any water droplets have condensed and sticking to watch glass, should be washed into the dish with distilled water. Transfer the solution into 50 ml volumetric flask and dilute almost up to the neck with distilled water. Add 1 ml of NH₄OH and make up the volume to 50 ml with distilled water and mix the contents thoroughly. Take 10 ml of the prepared ash solution into the Pyrex test tube and add three above reagents in the same order (2 ml of hydroxylamine hydrochloride solution; 5 ml of acetate buffer solution; 2 ml of 1, 10-orthophenanthroline solution) and shake after each addition (as done in case of the standard curve. A permanent orange red colour develops (figure 1) which is measured at 515 nm in a spectrophotometer and the concentration of iron is calculated from the standard curve.

**Calculations**

Let the reading of the sample be = X and corresponding conc. = Y µg
Since 10 ml ash solution has \( Y \) µg of iron

Therefore, 1 ml will have = \( \frac{Y}{10} \) µg of iron

Therefore, 50 ml will have = \( \frac{Y}{10 \times 50} \) µg of iron

Now 50 ml ash solution = 25 ml milk has \( \frac{Y}{10 \times 50} \)

1 ml milk has = \( \frac{Y}{10 \times 50} / 25 \)

100 ml milk has = \( \left( \frac{Y}{10 \times 50} / 25 \right) \) 100 µg of iron

**Figure 1:** The colour development in blank, standard curve and sample during iron estimation in milk by 1, 10 orthophenanthroline method.
15. Determination of the iron content in milk and milk products photometric method (IDF method)

In this procedure organic material is digested with a mixture of nitric acid and sulphuric acid preceded in the case of cream, and butterfat by removal of the fat. In the case of the butter the serum is separated and digested. This is followed by complexing of the iron (II), obtained by reduction of the iron (III) ions, by means of bathophenanthroline. Iron (II) compound is then extracted with isoamyl alcohol followed by photometric measurement of the absorbance of the red solution.

Reagents

1. Ethanol, about 96% (v/v) (Distill, if necessary, in an iron free distillation unit)
2. Diethyl ether (Distill, if necessary, in an iron free distillation unit).
3. Light petroleum (petroleum ether), boiling range 40 to 60°C (Distill, if necessary, in an iron free distillation unit).
5. Sulphuric acid: concentrated, (ρ₂₀ 1.84 g/ml).
6. Potassium sulfate solution in sulphuric acid: Dissolve 25 g of dry potassium sulfate (K₂SO₄) in sulphuric acid and make up to 100 ml with this acid. Filter the solution without suction through an al glass, iron-free filter crucible, porosity grade P100 (pore diameter 40 to 100 μm).
7. Hydrogen peroxide solution (ρ₂₀ 1099 to 1.103 g/ml). Store in a refrigerator.
8. Sodium acetate solution (Saturated): Dissolve 232.5 g of anhydrous sodium acetate (CH₃COONa) in 500 ml of water. (if the sodium acetate solution available is not iron-free, purify as follows: Dissolve 232.5 g of sodium acetate in 500 ml of water. Filter through a filter paper. Add 3 ml
of the hydroxyl ammonium chloride solution (reagent 9). Extract the solution with 10 ml of the bathophenanthroline solution. Repeat the extraction until the upper layer remains colourless).

9. Hydroxyl ammonium chloride solution: Dissolve, 20 g of hydroxyl ammonium chloride (HO NH₃Cl) in water and make up to 100 ml. Filter through a filter paper. Extract the solution with 5 ml of the bathophenanthroline solution reagent 11). Repeat the extraction until the upper layer remains colourless.

10. Generally five extractions are sufficient. If the has not been prepared on the day of use, extraction with bathophenanthroline solution is advisable. Instead of hydroxyl ammonium chloride solution a freshly prepared solution of ascorbic acid can be used as a reducing agent. This ascorbic acid solution can be made by dissolving 10 g of ascorbic acid in 100 ml water. The solution should be extracted with bathophenanthroline solution in exactly the same way as described for the hydroxyl ammonium chloride solution. It should be stored in a refrigerator. Instead of 3 ml of the hydroxyl ammonium chloride solution, 3 ml of this ascorbic acid solution can be used.

11. Isoamyl alcohol: Distill, if necessary, in an iron-free distillation unit.

12. Bathophenanthroline solution: Dissolve 83.1 mg of bathophenanthroline (4, 7 diphenyl-1, 10-phenanthroline, C₂₄H₁₆N₂) in 100 ml of isoamyl alcohol.

13. Potassium permanganate solution: Dissolve 100 mg of potassium permanganate (KMnO₄) in 50 ml water.

14. Standard iron solution

Stock solution (containing 1.00 g Fe/L)

Dissolve 7.022 g of ammonium iron (II) sulfate hexahydrate [(NH₄)₂Fe(SO₄)2.6H₂O)] in 250 ml of water. Add 8 ml of sulphuric acid and cool to room temperature. Dilute to 1000 ml with water.
Working solution (containing 1 μg Fe/ml)
Prepare this solution on the day of use. Pipette 1 ml of the stock solution into 250 ml of water. Add 1 ml of sulphuric acid and dilute to 1000 ml with water.

Apparatus
Keep the clean glassware, including the glass beads, in 10% (w/w) nitric acid. Rinse before use three times with distilled water and then three times with double distilled water. Dry, if necessary, by successively rinsing with ethanol and diethyl ether.

1. Centrifuge: Capable of producing a radial acceleration of 2500 g, with tubes of at least 150 ml capacity.
2. Glass beads.
4. Graduated pipette: Capacity of 1, 2 and 5 ml with 0.1 ml graduation.
5. One mark pipettes: Capable of delivering 1, 2, 3, 4, 5, 10 and 25 ml.
6. Spectrophotometer: Operating at 533 nm, equipped with cells of 10 mm optimal path length.

Procedure
A. Preparation of the sample: As appropriate.

1. Milk, Skimmed milk, whey, buttermilk and yoghurt: Weigh into a digestion flask 10 g of test sample to the nearest 10 mg. Add 8 ml of the nitric acid and 1.8 ml of the potassium sulfate solution. Continue as Step B.

2. Cream: Weigh into a digestion flask 10 g of the test sample, to the nearest 10 mg. Add ml of the nitric acid. Heat the flask in water bath at 80 to 90°C for 1 h. Shake vigorously every 3 min in order to wash the fat with the nitric acid. Cool to 40°C and remove as much as possible of the fat layer by means of a pipette. Add 15 ml light petroleum ether, swirl carefully, and remove the solvent by mans of a pipette. Repeat twice with fresh 15 ml portions of light petroleum. Remove residual light petroleum
by warming in a water bath at 65°C. Cool to room temperature. Add 1.8 ml of the potassium sulfate solution. Continue as Step B.

3. Evaporated milk and sweetened condensed milk: Weigh into a digestion flask 2.5 g of the test sample to the nearest 1 mg. Add 4 ml of water, 3 ml of the nitric acid and 1.8 ml of the potassium sulfate solution. Continue as Step B.

4. Whole and skimmed milk powder: Weigh into a digestion flask 1 g of the test sample to the nearest 1 mg. Add 4 ml of water and mix well. Then add 3 ml of the nitric acid and 1.8 ml of the potassium sulfate solution. Continue as Step B.

5. Butter: Weigh into a digestion flask 2 g of the butter serum, to the nearest 1 mg. Add 3 ml of the nitric acid and 1.8 ml of the potassium sulfate solution. Continue as Step B.

6. Milk fat (ghee): weigh into a digestion flask 20 g of the liquid test sample to the nearest 10 mg. Add 4 ml of water and 8 ml of nitric acid. Heat the flask in water bath at 80-90°C for 1 h. Shake thoroughly every 3 min in order to wash the fat with nitric acid. Cool to 40°C and remove the fat layer by means of a pipette. Add 15 ml light petroleum ether, swirl carefully, and remove the solvent by means of a pipette. Repeat twice with fresh 15 ml portions of light petroleum. Remove residual light petroleum by warming in a water bath at 65°C. Cool to room temperature. Add 1.8 ml of the potassium sulfate solution. Continue as Step B.

7. Ice cream: Weigh into a digestion flask 2.5 g of the test sample to the nearest 1 mg. Add 4 ml of water, 3 ml of the nitric acid and 1.8 ml of the potassium sulfate solution. Add 15 ml light petroleum ether, swirl carefully, and remove the solvent by means of a pipette. Repeat twice with fresh 15 ml portions of light petroleum. Remove residual light petroleum by warming in a water bath at 65°C. Cool to room temperature. Add 1.8 ml of the potassium sulfate solution. Continue as Step B.
8. Cheese and processed cheese: Weigh into a digestion flask 1 g of the test sample to the nearest 1 mg. Add 4 ml of water, 3 ml of the nitric acid and 1.8 ml of the potassium sulfate solution. Add 15 ml light petroleum ether, swirl carefully, and remove the solvent by means of a pipette. Repeat twice with fresh 15 ml portions of light petroleum. Remove residual light petroleum by warming in a water bath at 65°C. Cool to room temperature. Add 1.8 ml of the potassium sulfate solution. Continue as Step B.

9. Casein, caseinates, co-precipitates: Weigh into a digestion flask, in the case of casein and caseinates 0.75 g and in the case of co-precipitates 0.35 g of the test sample to the nearest 0.1 mg. Add 4 ml of water, 3 ml of the nitric acid and 1.8 ml of the potassium sulfate solution. Add 15 ml light petroleum ether, swirl carefully, and remove the solvent by means of a pipette. Repeat twice with fresh 15 ml portions of light petroleum. Remove residual light petroleum by warming in a water bath at 65°C. Cool to room temperature. Add 1.8 ml of the potassium sulfate solution. Continue as Step B.

B. Digestion

1. Add three glass beads. Operating under a well-ventilated fume hood, place the flask in an inclined position and heat with a micro burner. The height of foam shall be controlled so as to limit the production of foam in the flask. Foaming into the neck of the flask is allowed but the foam shall not be allowed to escape. Keep the mixture gently boiling and avoid local overheating. (Carry out a blank test simultaneously).

2. When the solution turns brown, carefully add 3 to 5 drops of nitric acid. Heat vigorously as soon as possible. Continue heating and adding of the nitric acid, 5 ml to 20 drops at a time, swirling the flask occasionally to remove any material adhering to the wall until the mixture remains colourless. Cool to room temperature.

3. Carefully add 2 ml of water and 1 ml of the hydrogen peroxide solution. Swirl and heat again until white fumes are emitted. Prevent evaporation
losses by allowing the sulphuric acid fumes to reflux I the neck of the flask. If the solution becomes yellow, cool to room temperature. Add a further 0.5 ml of the hydrogen peroxide solution. Continue heating for 45 min after the beginning of the emission of white fumes. Cool to room temperature and add water to give a total volume of approximately 20 ml.

4. Add one or two drops of the potassium permanganate solution, until the digest becomes faintly purple. Then add 3 ml of the hydroxyl ammonium chloride solution and mix well. Add 20 ml of the sodium acetate solution. Make up to the 50 ml mark. Mix well and allow to cool to room temperature.

C. Colour development:

1. Pipette 4 ml of the bathophenanthroline solution to the contents of the digestion flask and close the flask with a stopper.
2. Shake the flask vigorously for 3 min, ensuring that the stopper remains in position. Cool in running tap water for at least 10 min and carefully tilt the flask several times after cooling. Keep the flask in a water bath at 25 ± 1°C for 1 h.

D. Blank test

Simultaneously with the analysis of the test portion, carry out a reagent blank test using all reagent and 10 ml water instead of the test portion. During the digestion period, use the same amount of nitric acid and the hydrogen peroxide solution as for the digestion of the test portion.

E. Photometric measurement

Transfer the isoamyl alcohol (upper) layer by means of a pipette into a 10 mm cell. Measure the absorbance of the isoamyl alcohol layer of the test solution and the reagent blank solution against that of water at a wavelength of 533 nm. Subtract the value for the reagents blank solution from that of the test solution.
F. Calibration Curve

1. Pipette 0 (blank), 1, 2, 3, 5 and 10 ml of the standard iron working solution into a series of six digestion flask. Dilute with water to about 10 ml. Add to each flask 3 ml of the nitric acid and 1.8 ml of the potassium sulfate solution.

2. Carry out the steps as mentioned in Step B, C, D and E.

3. Transfer each isoamyl alcohol (upper) layer by means of a pipette into a 10 mm cell. Measure the absorbance of each isoamyl alcohol layer against that of water at a wavelength of 533 nm. Subtract the blank values from the values obtained for the other solutions.

4. Plot these absorbances against the amounts of iron added.

5. Check the calibration curve weekly.

Calculations

Except in the case of butter, calculate the iron content of the sample, in mg/kg, from the formula:

\[ \frac{M_0}{M_1} \]

Where

- \( M_0 \) is the mass, in g of the test portion
- \( M_1 \) is the mass, in microgram, of iron read from the calibration curve

In the case of butter, calculate the iron content of the butter serum as described above.

For the calculation of iron content of butter, in mg/kg from the formula:

\[ \frac{M_3}{M_2} \]

Where

- \( M_3 \) is the mass, in g of the butter serum
- \( M_2 \) is the mass, in microgram, of iron read from the calibration curve
M$_2$ is the mass, in g of the butter brought into the centrifuge tube.
M$_3$ is the mass, in g of the butter serum,
C is the iron content, in milligram per kg, of the butter serum.

Reference

Milk and milk products -- Determination of iron content -- Spectrometric method
(Reference method) ISO 6732:2010 (IDF 103:2010)
16. Estimation of copper in milk by sodium diethyl-dithio-carbamate method

Copper content in cow as well as buffalo milk is about 22 µg/100 ml. Estimation of copper is based upon the colorimetric procedure. The principle involved is given as follows:

Sodium-diethyl-dithio-carbamate reacts with a slightly alkaline solution of copper (II) in low concentration to produce a yellow or brown colloidal suspension of the cupric-diethyl-dithio-carbamate. The yellow or brown coloured copper carbamate complex can be extracted with an organic solvent, such as, carbon tetrachloride, chloroform or n-butyl acetate. The intensity of the coloured extract is measured colorimetrically at 435 nm (if CCl₄ or CHCl₃ are used) or at 560nm (if n-butyl acetate is used).

Interference due to Iron and precipitation of phosphate is prevented by the addition of citrate to the aqueous solution. Interference due to other metals present is prevented by the addition of chelating agent like EDTA.

**Reaction**

\[
2(\text{C}_2\text{H}_5)_2\text{N} \cdot \text{Na}^+ + \text{Cu}^{++} \rightarrow (\text{C}_2\text{H}_5)_2\text{N} \cdot \text{Cu} \cdot \text{S} \cdot \text{S}^{-} \cdot (\text{C}_2\text{H}_5)_2\text{N} \cdot \text{C} \cdot \text{S} \cdot \text{S}^{-}
\]

sodium-di-ethyl-dithio-carbamate

Cupric-diethyl-dithio-carbamate (yellow or brown color depending upon concentration of copper)

**Reagents**

1. Sodium diethyl-dithio-carbamate solution 1%.
2. **Citrate-EDTA solution:** Dissolve 20 g dibasic-ammonium citrate and 5.0 g of sodium salt of EDTA in distilled water and dilute to 100 ml.

3. **Standard Copper Solution:**
   A. **Stock Solution:** (0.2 mg/ml): Dissolve 0.393 g of CuSO₄·5H₂O in 500 ml of vol. flask containing water and 2 ml H₂SO₄. Dilute to 500 ml with distilled water.
   B. **Working Solution:** (2 µg/ml): Take 1 ml of stock solution & dilute it to 100 ml.

4. NH₄OH Sol.-5 N
5. CCl₄ - AR quality
6. H₂SO₄ – 2 N Solution
7. Thymol blue – Indicator

**Procedure**

(A) **Preparation of sample:** Take 10 g of milk in a silica dish and evaporate to dryness and convert to ash in a muffle furnace at a temperature not more than 550°C. Dissolve the ash in (1:4) HCl and make up the volume to 100 ml with HCl (1:4).

(B) **Preparation of Standard curve and Determination of Copper:** Transfer 0, 1, 2.5, 5.0, 10.0, 15.0, 20.0 and 25.0 ml of standard copper solution (2 µg/ml) to different separating funnels of 100 or 250 ml capacity and add 2 N H₂SO₄ to make total volume to 25 ml in each of the separator. Take 25 ml of the ash solution (sample) in another separating funnel. Take 25 ml of the ash solution (sample) in another separating funnel.

To each separating funnel, add 10 ml citrate-EDTA-solution. Then add 2 drops of Thymol blue indicator and 6 N-NH₄OH drop wise until solution turns green or bluish green. Cool and add 1 ml of carbamate solution and 15 ml CCl₄. Shake vigorously for 2 minutes (yellow or brown colour in CCl₄ layer will appear only after vigorous shaking).

Allow to stand for some time till the layers separate clearly. Take the Carbon-tetra-chloride layer and measure the colour at 435 nm (using...
blue filter) against the reagent blank. Draw the standard curve and calculate the copper content in the sample using the standard curve.

**Calculations**

Let the optical density (O.D.) for the sample be =X

Corresponding concentration =Y µg

25 ml of ash solution has = Y µg of copper

1ml of ash solution will have = $\frac{Y}{25}$ µg of copper

100 ml of ash solution will have = $\frac{Y}{25} \times 100$ µg of copper

That means, 10 g of milk has = $\frac{Y}{25} \times 100$ µg of copper

Therefore, 100 g of milk has = $\frac{Y}{25} \times \frac{100}{10} \times 10$ µg of copper
17. Estimation of citric acid content in milk by Furth and Hermann method

Citric acid content in cow and buffalo milk is about 170 mg and 245 mg/100 ml, respectively. Citric acid is present in milk to a large extent in the form of polyvalent citrate ions, although a small fraction of the total citric acid in milk is present in the insoluble protein bound phase in milk. However, treatment of milk with deproteinising agent, such as phosphotungstic acid, brings the entire citric acid in aqueous phase. The method of estimation is based upon the colour reaction of Furth and Hermann (1935) in which citric acid heated with a mixture of pyridine and acetic anhydride, develops a permanent yellow, which may be measured photometrically at 420 nm.

Reaction

In this method/procedure, the milk is deproteinised with the help of TCA. Then in the protein free filtrate the citric acid is estimated by utilizing the Furth and Hermann reaction which at the experimental condition is probably a condensation reaction between pyridine and citraconic anhydride (which is produced from citric acid via aconitic acid and citraconic acid). After this condensation a yellow colour is produced which is proportional to the
concentration of citric acid in solution. Intensity of colour is measured and citric acid content is calculated from the standard curve. Acetic anhydride used in the system helps to remove the moisture from the solution because the reaction must be carried out under anhydrous condition.

Reagents

1. TCA 24% solution and 12% soln.
2. Pyridine (AR grade)
3. Acetic anhydride AR grade
4. Standard citric acid solution (conc.1 mg/ml): Dissolve 0.140 g of trisodium citrate in 100 ml of distilled water which gives conc. of 1mg/ml

Apparatus

1. Volumetric flask 50 ml capacity
2. Graduated pipettes of 1, 2 & 10 ml capacities
3. Standard joint glass stoppered test tubes – 50 ml capacity
4. Warm water bath
5. Filter paper –Whatman no 42/40

Procedure:

1. Preparation of sample: Take 5 ml of milk in a 50 ml volumetric flask and make the volume up to about 25 ml with distilled water and then make up to 50 ml with 25 ml of 24% TCA solution. Mix the contents well. Keep it for 30 min. and then filter through Whatman No. 42/40 filter paper and collect the filtrate.

2. Preparation of standard curve and estimation of citric acid: Take 0 ml (blank), 2.5, 5.0, 7.5, 10.0, and 12.5 ml of standard citric acid solution (which contain 1 mg/ml citric acid) in separate 50 ml volumetric flask and add 25 ml of distilled water in each flask. Then finally make up to 50 ml mark with 24% TCA solution. On addition of distilled water and TCA, the concentration of citric acid in different flask will be as 0, 50, 100, 150, 200, and 250 µg /ml, respectively. Take 1 ml of each of these standard solutions and also sample
filtrate (1 ml) in a standard joint glass stoppered test tube. Keep the test tubes in a constant temperature water bath at 32°C. Add 1.3 ml of pyridine in each test tube and shake. Then add 5.7 ml of acetic anhydride in each tube, shake again and after closing with stoppers place the tubes immediately back at 32°C. Wait for 30 min. for maximum colour development. After this, read the intensity of colour in a spectrophotometer at 420 nm within next 30 min. colour development is complete after 30 min. either in water bath at 32°C or in air at a temperature of 22-29°C). Draw a standard curve by plotting conc. Vs OD and from the standard curve, calculate the conc. of citric acid in the given sample of milk.

Precautions

1. Do not inhale and pipette (with mouth) the pyridine and acetic anhydride. Keep the pipettes in respective bottles of pyridine and acetic anhydride and let the reagent rise by itself and then adjust the volume with the help of finger.
2. While adding 1.3 ml pyridine and 5.7 ml acetic anhydride in a standard joint glass tube give half a minute gap after each addition, mix and then close with stoppers. If it is closed immediately after addition, vapour pressure develops due to the acetic anhydride and the released CO₂ may result into an explosion.

Calculations

Let the OD for sample (1ml) be X and the corresponding conc. from the standard cure/graph be Y µg.
1ml sample filtrate contains = Y µg of citric acid
50 ml sample filtrate will contain = 50 Y µg of citric acid
Since 50 ml sample filtrate = 5 ml milk = 50 Y µg of citric acid
Therefore 100 ml milk will have \( \frac{50Y \times 100}{5 \times 100} \) mg /100 ml milk.

References:

18. **Estimation of citric acid content in milk by gravimetric method**

Citric acid content in cow’s milk is about 170 mg/100 ml and in buffalo milk it is about 245 mg/100 ml. There are two methods for the estimation of citric acid content in milk.

Citric acid is present in milk to a large extent in the form of polyvalent citrate ions, although a small fraction of the total citric acid in milk is present in the insoluble, protein bound phase. However, treatment of milk with deproteinizing agent, such as phosphotungstic acid, brings the entire citric acid in aqueous phase. This soluble form of the citric acid is converted through reaction with suitable reagents into solid pentabromacetone, which is dried and weighed. The amount of citric acid is subsequently calculated from that of pentabromacetone (BIS, 1961).

**Reagents**

1. Tartaric acid: AR grade.
2. Sulphuric acid: concentrated.
3. Dilute sulphuric acid: 1 N.
4. Phosphotungstic acid solution: 20% (w/v).
5. Potassium bromide.
7. Potassium permanganate: Dissolve 5 g of potassium permanganate in water and dilute to 100 ml.
8. Ferrous sulfate solution: Dissolve 40 g of ferrous sulfate (FeSO₄.7H₂O) in 100 ml of water containing 1 ml of concentrated sulphuric acid.
10. Diethyl ether.

**Procedure**

1. Weigh accurately about 50 g of the prepared sample in a 150 ml beaker. Add about 100 mg of tartaric acid and 6 ml of dilute sulphuric acid and heat on a steam bath for a 15 min.
2. Immediately add 3 ml of phosphotungstic acid solution, mix well, and return to steam bath for a few min.

3. Transfer quantitatively to a 250 ml graduated flask with ethyl alcohol, cool and make up the volume with ethyl alcohol. Mix well and filter through a folded filter paper.

4. Take 200 ml filtrate thus obtained using 50/100 ml pipette into a large beaker and evaporate to about 20 ml.

5. Transfer quantitatively this material to a previously weighed, glass stoppered Erlenmeyer flask, using water to rinse, and make the weight of the collected solution to about 40 g with water.

6. Add 2 g of potassium bromide and 5 ml of concentrated sulphuric acid. Heat the content to about 50°C and allow to stand for 5 min.

7. Add 20 ml of potassium permanganate solution from a pipette of burette slowly (1 or 2 ml portions) mixing the contents for a few sec after each addition by swirling the flask.

8. Allow to stand for 5 min and then cool to about 15°C.

9. Add ferrous sulfate solution slowly with constant agitation until mixture starts to clear. Shake for 1 min and continue addition of ferrous sulfate until the brown manganese oxide is dissolved. Add a few ml of ferrous solution is excess.

10. Add 20 g anhydrous sodium sulfate and swirl the flask to dissolve it (if sodium sulfate remains substantially undissolved, repeat the experiment). Cool to about 15°C and shake vigorously for about 5 min.

11. Prepare a Gooch crucible with asbestos pad and filter the precipitate formed in the cold mixture. Transfer the precipitate quantitatively to the Gooch by rinsing with the filtrate obtained. Finally, wash the precipitate with about 50 ml cold water and allow the suction to continue for few min. remove the crucible along with the precipitate and place to dry overnight in a desiccator with concentrated sulphuric acid as desiccant. Weigh the crucible with precipitate to a constant weight (say \(W_1\)g).

12. Remove the pentabromacetone precipitate from the crucible 3 times with each solvent. Dry the crucible for 10 min at 98 – 100°C, cool in the
desiccator and, weigh (say \(W_2\) g). The difference between the two weighing gives the weight of pentabromacetone.

**Calculations**

a) Weight of citric acid in aliquot = \(X = 0.424\ (W_1-W_2)\)

b) Anhydrous citric acid (\%, \(w/w\)) in milk = \((12\ X)/W\)

\(W\) = Weight of milk taken for the test.

**Reference**

19. Determination of sodium and potassium in milk by flame photometric method

In this method (BIS, 1989) sample as well as standard solutions are atomized directly into the flame of a flame emission spectrometer followed by the spectrometric measurement of the intensity of the emitted light.

Reagents

All reagents should be of analytical grade. The water used shall be distilled water or water of at least equivalent purity.

1. Hydrochloric acid (about 4 N): Dilute 300 ml of concentrated (37% (m/m) hydrochloric acid to 1000 ml with water and mix.
2. Standard solutions: Store the standard solutions in vessels of hard polyethylene of other material of at least equivalent quality.
   a. Sodium standard solution (0.4 mg/ml): Dissolve 1.0168 g of sodium chloride (NaCl), dried to constant mass at 110 to 120°C, in water, dilute to 1000 ml with distilled water and mix.
   b. Potassium standard solution (1mg/ml): Dissolve1.9068 g of potassium chloride (KCl), dried to constant mass at 110 to 120°C, in water, dilute to 1000 ml with distilled water and mix.
   c. Calcium standard solution (1mg/ml): Dissolve 2.4972 g of calcium carbonate (CaCO₃), dried to constant mass at 110 to 120°C, in 15 ml of the hydrochloric acid, dilute to 1000 ml with distilled water and mix. One ml of this standard solution contains 1 mg of calcium.
   d. Phosphorus standard solution (2.5 mg/ml): Dissolve 10.660 g of diammonium monohydrogen orthophosphate [(NH₄)₂HPO₄] in water, dilute to 1000 ml with distilled water and mix.

Apparatus

1. Glass beaker: 50 ml capacity
2. Volumetric flasks: 100, 500 and 1000 ml capacities
3. Graduated measuring cylinder: 50 ml capacity
4. Pipettes to deliver 10, 15, 20, 25, 30, 40, 45, 50 and 60 ml (burette may also be used)

5. Flame emission spectrometer: With a burner fed with a mixture of either acetylene and air or propane and air, and provided with filters with maximum transmittance at about 589 and 768 nm for sodium and potassium respectively, or fitted with a monochromator

**Procedure**

1. Prepare the sample as appropriate
   
a. For dried milk dissolve about 1.25 g of the test sample in about 20 ml of warm water (40 to 50°C), while stirring with glass rod. Transfer the contents of the glass beaker by rinsing with water quantitatively into a 500 ml volumetric flask. Cool to about 20°C and dilute to the mark with distilled water. Mix the contents of the flask thoroughly.
   
b. For liquid milk and other products, prepare sample so that the final dilute solution contains 10 mg of sodium and 40 mg of potassium per litre.

2. Preparation of sodium and potassium reference solutions
   
Pipette successively into seven 1000 ml volumetric flasks the volumes of the standard solutions (solutions 2-a, 2-b and 2-c) indicated in the table, and dilute to 900 ml with water. Add to each flask 10 ml of the phosphorus standard solution (solution 2-d), dilute to the mark with distilled water and mix.

3. Determination

3.1 Sodium content
   
Atomize the reference solutions (solutions 2), starting with the solution with the lowest sodium content, and the test solution (solution 1), alternatively into the flame of the flame emission spectrometer, following the manufacture’s instructions and using the sodium filter, or the monochromator, adjusted to 589 nm. Note the readings.
3.2 Potassium content

Atomize the reference solutions (solutions 2), starting with the solution with the lowest potassium content, and the test solution (solution 1), alternatively into the flame of the flame emission spectrometer (figure 1), following the manufacturer instructions and using the potassium filter, or the monochromator, adjusted to 768 nm. Note the readings.

3.3 Dilution

If the flame emission spectrometric reading for the test solution (1) exceeds that of the reference solution with the highest concentration, repeat the spectrometric measurement using an appropriate dilution of the test solution (1) and the appropriate reference solutions. Prepare, for this purpose, reference solutions having concentrations of sodium, potassium, calcium and phosphorus which are as close as possible to the expected concentrations in the diluted test solution.

Calculations

The sodium and potassium contents of the sample, expressed as percentage by mass, are given by the formula

\[
\% = \left[ \frac{I_x - I_1}{I_2 - I_1} (C_2 - C_1) + C_1 \right] \frac{f}{m}
\]

Where

- \( I_x \) is the reading of the flame emission spectrometer for the test solution (1)
- \( I_1 \) is the nearest lower reading of the flame emission spectrometer for the reference solution of concentration \( c_1 \)
- \( I_2 \) is the nearest higher reading of the flame emission spectrometer for the reference solution with concentration \( c_2 \)
- \( C_1 \) is the concentration, in mg/L, of the reference solution giving reading \( I_1 \)
- \( C_2 \) is the concentration, in mg/L, of the reference solution giving reading \( I_2 \)
\( f \) is the conversion factor for expressing the results as a percentage by mass  
(for sodium and potassium, \( f = 0.04 \))

\( m \) is the mass, in g, of the test solution.

**Reference:**


**Fig. 1 Schematic diagram of a Flame photometer**
20. Vitamins in Milk

A vitamin may be defined as an organic compound which is required in small amounts for satisfactory growth and maintenance and is not synthesized within the body. Most of the vitamins predominantly acts as coenzyme for other metabolic enzymes. The principal functions of vitamins are to facilitate transfer of energy and to regulate metabolisms in the body. They do not serve as source of energy or as structural units for the body to any significant extent.

Table 1. Levels of vitamins in milk and their recommended dietary allowances (RDA)

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>Levels in milk (per litre)</th>
<th>RDA per day for Indians</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cow milk</td>
<td>Buffalo milk</td>
</tr>
<tr>
<td>Vitamin D (I.U.)</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin K (µg)</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin B₁ (µg)</td>
<td>450</td>
<td>580</td>
</tr>
<tr>
<td>Vitamin B₂ (µg)</td>
<td>1750</td>
<td>1430</td>
</tr>
<tr>
<td>Niacin (µg)</td>
<td>900</td>
<td>1280</td>
</tr>
<tr>
<td>Vitamin B₆ (µg)</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td>Pantothenic acid (µg)</td>
<td>3500</td>
<td>2400</td>
</tr>
<tr>
<td>Biotin (µg)</td>
<td>35</td>
<td>106</td>
</tr>
<tr>
<td>Folic acid (µg)</td>
<td>55</td>
<td>42</td>
</tr>
<tr>
<td>Vitamin B₁₂ (µg)</td>
<td>4.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>20</td>
<td>21</td>
</tr>
</tbody>
</table>

*Not fully established. Given values are considered adequate.

They are generally divided into two major groups: fat soluble and water soluble. Vitamins A, D, E and K are generally found in the lipid fractions of food and are termed as fat soluble vitamins. The water soluble vitamins
include vitamin C and the vitamins of the B complex: thiamin (B1), riboflavin (B2), niacin, pantothenic acid, pyridoxine (B6), folic acid, biotin and vitamin B12. Milk and milk products have played an important role in the recognition and early development of knowledge of the vitamins. By now, it is well established that milk contains all the known vitamins, although some are present in small quantities, but for others, milk is very rich source – indeed one of the best (Table 1).

**Note:**

1. **Conversion IU of vitamin A into various other units**
   
   1 IU of vitamin A = 0.30 μg retinol
   
   = 0.344 μg retinyl acetate
   
   = 0.60 μg of β- carotene

   1 μg RE in milk = 2 μg of β- carotene

   Or 3.3 IU = 1.98 μg of β- carotene = 2 μg of β- carotene = 1 μg RE

   i.e. 1 μg RE = 3.3 IU

2. **Conversion IU of vitamin D into other units**

   1 I.U = 0.025 μg D₃

**Nutritional Importance of Vitamins**

Vitamins play a very important role in the human health and nutrition, and their deficiency in the diet is responsible for numerous diseases like beri-beri, pellagra, pernicious anaemia, scurvy, night blindness, rickets, hemorrhages, etc. Some details of vitamin deficiency diseases are given in Table 2.

**Changes in Vitamins during Processing of Milk**

**Effect of heat treatments**

Water soluble vitamins: Among the various water soluble vitamins, riboflavin, pantothenic acid, biotin and nicotinic acid are relatively stable to heat and there are generally no losses of these vitamins when milk is heated.
### Table 2. Diseases due to vitamin deficiency

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Vitamin</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vitamin A</td>
<td>Night blindness, conjunctivitis of the eye, defects in teeth, disturbance in bone growth.</td>
</tr>
<tr>
<td>2</td>
<td>Vitamin D</td>
<td>Rickets in children and osteomalacia in older people, defective teeth formation leading to dental caries.</td>
</tr>
<tr>
<td>3</td>
<td>Vitamin E</td>
<td>No known deficiency in man. In rats and other animals, deficiency causes sterility in males and miscarriage in females.</td>
</tr>
<tr>
<td>4</td>
<td>Vitamin K</td>
<td>Defective blood clotting due to a fall in the prothrombin content of the blood leading to haemorrhagic tendency.</td>
</tr>
<tr>
<td>5</td>
<td>Thiamin</td>
<td>Beri-beri, cardiovascular disorder, rough tongue, nervous imbalance etc.</td>
</tr>
<tr>
<td>6</td>
<td>Riboflavin</td>
<td>Lesions of the skin and eye, cheilosis condition of the mouth, increased sensitivity to light etc.</td>
</tr>
<tr>
<td>7</td>
<td>Pyridoxine</td>
<td>Skin changes in the areas of nose, eyes and mouth, nervous disorders including peripheral neuritis and epileptiform convulsions especially in growing children.</td>
</tr>
<tr>
<td>8</td>
<td>Folic acid</td>
<td>Poor growth and anaemia.</td>
</tr>
<tr>
<td>9</td>
<td>Niacin</td>
<td>Pellagra (Diarrhoea, Dementia and Dermatitis)</td>
</tr>
<tr>
<td>10</td>
<td>Pantothenic acid</td>
<td>Clear-cut deficiency Symptoms are rarely observed in man.</td>
</tr>
<tr>
<td>11</td>
<td>Biotin</td>
<td>Skin changes, fall in haemoglobin concentration and rise of blood cholesterol.</td>
</tr>
<tr>
<td>12</td>
<td>Vitamin B₁₂</td>
<td>Pernicious anemia and characteristic lesions of the central nervous system.</td>
</tr>
<tr>
<td>13</td>
<td>Vitamin C</td>
<td>Scurvy, bleeding of gums, shrunken teeth, poor wound healing, easy fracture (soft bones etc.)</td>
</tr>
</tbody>
</table>
Only when heating is prolonged or when milk is sterilized, slight reduction in riboflavin occurs. On the other hand, thiamin, pyridoxine, cobalamin, folic acid and ascorbic acid are less stable to heat and increasingly severe heat treatment, therefore, causes increasing losses. The average losses of these vitamins in milk due to different heat processes are indicated below:

<table>
<thead>
<tr>
<th>Processes</th>
<th>Losses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>q</td>
</tr>
<tr>
<td>Pasteurization</td>
<td>&lt;10</td>
</tr>
<tr>
<td>UHT treatment</td>
<td>0-20</td>
</tr>
<tr>
<td>Boiling</td>
<td>10-20</td>
</tr>
<tr>
<td>Sterilization</td>
<td>20-50</td>
</tr>
</tbody>
</table>

From these results, it can be concluded that the vitamin losses in pasteurized milk are so small that there is practically no reduction in the nutritive value of milk. The same applies to UHT milk, where the vitamin losses are of the order of 10 to 20 per cent. The indirect UHT treatment produces slightly greater losses of vitamins than the direct one. The vitamin losses produced by pasteurization and UHT treatment are, on the whole, smaller than those occurring in the preparation of food in the home, such as boiling. The reduction in the vitamin potency by conventional sterilization is more serious. The above results also show that the modern methods of sterilization preserve the thermally unstable vitamins better than the conventional processes used in older sterilizing plants, in which the vitamin B12 and C were largely destroyed and half the vitamin B1. B6 and folic acid were inactivated. Similar losses occur in evaporated milk which is subject to a sterilizing process, but in sweetened condensed milk, which does not need to be sterilized, losses are only 10 to 30 per cent.

Losses of ascorbic acid are primarily due to oxidation and only secondarily due to heating. When milk is pasteurized in the absence of oxygen, there are
hardly any losses of vitamin C. In fact, the higher losses of vitamin C, B₁, B₁₂ and folic acid produced by the indirect method of UHT treatment can be considerably reduced by de-gassing the milk, i.e., reducing the oxygen content.

**Fat Soluble Vitamins**

Fat soluble vitamins A, D, E and K are relatively insensitive to heat and generally there are no losses of these vitamins when milk is heated. However, when milk is heated for longer periods or is sterilized, slight reductions in vitamin A and E occurs and it is thought that these losses are mainly due to the effect of oxygen which causes oxidation. Part of vitamin K is reported to be destroyed during pasteurization and evaporation.

**Effect of Fermentation**

Starter cultures used in the fermentation of milk need water soluble vitamins especially B vitamins for their growth and have the ability to synthesize the B-vitamins in excess during the course of fermentation. Microbial biosynthesis of B-vitamins is a useful method for improving the content of B-vitamins in the dairy products.

Generally, there is a decrease in the B vitamins during the initial stages of fermentation followed by an increase at the subsequent stages. The decrease in B-vitamins during fermentation has been attributed to the utilization of these vitamins by the starter organisms, whereas the increase has been ascribed to biosynthesis. This decrease or increase of water soluble vitamins depends upon the temperature and period of ripening.

**Conclusion**

Milk is a rich source of vitamins as majority of the vitamins appears to have high nutrient density (the ratio between nutrient content per joule and recommended intake per joule), which exceeds 100%. Therefore, milk has the potential to compensate the low concentration of vitamins in other foods. Unfortunately, several water-soluble vitamins are sensitive to various processing treatments and environmental factors.
Heating causes a serious threat to the nutritive value of milk in respect of vitamins. Maximum destruction of vitamins occurs during sterilization process. In order to bring down the losses during such severe heat treatments, incorporation of nisin and lowered time-temperature combinations to achieve sterilization have been suggested. Similarly, addition of vitamin C, which acts as an antioxidant, is reported to provide protection of folic acid during processing and storage of milks. HTST pasteurization and UHT sterilization, which involve exposure of, milk constituents to heat for a minimum possible time, causes the least damage to vitamins. The losses caused by these processes could be further reduced by removing oxygen partially or completely either by vacuum treatment or by passing nitrogen in the milk. Similarly, these losses of water-soluble vitamins could be kept at minimum level if the exposure of milk to light is avoided after heat processing. Repeated heat treatments, a situation more prevalent in India, results into cumulative losses of water-soluble vitamins and should therefore, be dispensed with.

References


21. Estimation of ascorbic acid (vitamin C) in milk by dye reduction method

Estimation of vitamin C content in milk is based on the reduction of the dye (2, 6-dichloro-phenol-indophenol) by an acidic solution of ascorbic acid. In this method, the milk is de-proteinized with meta-phosphoric acid and the protein free filtrate is used for vitamin C estimation. First, the dye solution is standardized against the standard ascorbic acid solution, the concentration of which is known. Then the protein free filtrate of unknown sample is titrated against the standardized solution of the dye. Ascorbic acid present in the sample reacts with the dye and reduce the colour of the dye from blue to colourless. End point of titration is detected by the disappearance of pink colour.

Reaction

![Reaction Diagram]

L-Ascorbic acid (Reduced form) + 2, 6 dichlorophenol indophenol (oxidized form of dye) → Dehydro ascorbic acid (oxidized form) + 2, 6 dichlorophenol indophenol (Reduced form of dye)

The titration is conducted in presence of m-phosphoric acid (HPO₃) which serves to precipitate proteins, to inactivate enzymes, to lower pH and to prevent aerobic air oxidation by metallic ions like copper, iron etc. In the absence of interfering substances, the capacity of the sample to reduce a standard solution of dye is directly proportional to the ascorbic acid content. If we know the amount of standard ascorbic acid required to reduce a definite volume of dye (say 1 ml) then we can calculate the concentration of ascorbic acid in the unknown sample.
The average ascorbic acid content in cow milk is about 2 mg/100 ml. Buffalo milk contains slightly higher amount of ascorbic acid.

Reagents

1. 2,6 dichlorophenol indophenol dye solution (aq) – 0.02%, w/v. 100 mg of 2,6 dichlorophenol indophenol sodium salt (dihydrate) is dissolved in distilled water and final volume is made to 500 ml
2. \( \text{\textit{m}} \)-phosphoric acid solution – 4% (w/v).
3. Ascorbic acid solution (1 mg/ml) – Dissolve 100 mg ascorbic acid in 4% meta-phosphoric acid solution and make the volume to 100 ml.

Procedure

A. Standardization of the dye solution against standard solution of ascorbic acid

1. Take 2 ml of standard ascorbic acid solution in a 250 ml conical flask. Add 5 ml of 4% \( \text{\textit{m}} \)-phosphoric acid solution and mix the contents.
2. Titrate against dye solution till the appearance of pink colour. Note down the volume of dye used. Calculate the amount of ascorbic acid required to reduce the definite volume (say 1 ml) of the dye.

B. Estimation of ascorbic acid in the sample

1. Take 50 ml milk in a 100 ml volumetric flask and make up the volume with 4% \( \text{\textit{m}} \)-phosphoric acid solution. Keep it undisturbed for 10 to 15 min. Then filter it through Whatman No. 4 filter paper.
2. Take 10 ml of this filtrate in 250 ml conical flask. Titrate it against the dye in a similar manner as in the case of standard ascorbic acid.

Calculation

Let the volume of dye used for 2 ml of standard ascorbic acid be = \( X \) ml dye

Therefore, volume of dye used for 1 ml of standard ascorbic acid = or \( X/2 \) ml of dye = 1 ml of standard ascorbic acid = 1 mg of standard ascorbic acid
Therefore 1 ml of dye = $\frac{1x2}{X}$ mg of standard ascorbic acid

Now say the volume of dye used for 10 ml of sample filtrate = $Y$ ml dye

Therefore $Y$ ml of dye = $\frac{Yx2}{X}$ mg of standard ascorbic acid

Let $\frac{Yx2}{X}$ mg of ascorbic acid be = $Z$ mg of ascorbic acid

Now 10 ml sample filtrate contains = $Z$ mg of ascorbic acid

Therefore 100 ml sample filtrate contains = $\frac{Zx100}{10}$

i.e. 50 ml milk contains = $\frac{Zx100}{10}$

100 ml milk contains = $\frac{Zx100}{10} \times \frac{100}{50}$ mg of ascorbic acid per 100 ml milk.
22. Determination of 5-hydroxymethylfurfural (HMF) in milk powder

Determination of HMF as employed in the method of Keeney and Bassette (1959) could be classified as an indirect method for the determination of Amadori adducts plus intermediates of the 1, 2-enolization (designated as 1,2,-E pathway), including preformed HMF. The following method is a cheap and relatively simple method for maintaining maillard reactions. The presence of HMF (figure 1) and its precursors in food is an indication of the heat exposure of the food product and is suitable for estimating the flavour stability of whole milk powder. This method measures not only actual HMF in a sample (free HMF) but also the browning intermediates (potential HMF) which are mainly enols and Schiff bases. Application of heat during digestion of milk with oxalic acid converts these browning intermediates into free HMF (O’Brien, 1997).

In the following method (Kenney and Bassette, 1959), HMF reacts with 2-thiobarbituric acid under acidic conditions to produce chromophores with absorption maxima at 443 nm. The method was suggested to be a sensitive detector of real symptoms of browning reaction. The authors had clearly stated that the HMF value determination would help only to detect significant initiation of the Maillard reaction, rather than the quantitative measurement of the extent or intensity of browning reaction.

Figure 1: Formation of HMF
**Apparatus**

1. Test tubes: 50 ml capacity.
2. Funnels.
3. Water-bath: Maintained at 40°C.
5. Volumetric flasks: 100 ml capacities.
6. Whatman filter paper: Grade 42.
7. Spectrophotometer: Suitable for reading at wavelength of 443 nm.

**Reagents**

1. Oxalic acid (0.3 N): Take 18.9 g of oxalic acid (H$_2$C$_2$O. 2H$_2$O) and dilute to 1 L with distilled water.
2. Thiobarbituric acid (0.05 M): Take 0.72 g of 2-thiobarbituric acid (TBA) in distilled water, and dilute to 100 ml. Dissolve TBA by warming slightly and cooling to 25°C before use.
3. Trichloroacetic acid (40%, w/v): Take 40 g trichloroacetic acid (TCA) and make up the volume to 100 ml with distilled water.

**Procedure**

1. Take 10 g skim milk powder in 250 ml beaker, and reconstitute it in 100 ml distilled water using laboratory blender.
2. Pipette 10 ml of above reconstituted milk in a 50 ml test tube, and add 5 ml of 0.3 N oxalic acid. Mix the contents.
3. Cover the tube with inverted beaker (20 ml) and place it in a boiling water-bath for 1 h; after which it is removed and cooled with cold water to room temperature.

   Note: This heating step is omitted if one wishes to estimate the free HMF in the sample.
4. Add 5 ml of 40% TCA, mix, and filter through Whatman filter paper Grade 42.
5. Take 4 ml of the above filtrate into a test tube, and 1 ml of 0.05 M TBA and mix the content.
6. Incubate the tube in a water-bath at 40°C for 35 min.
7. Remove the tube from the water-bath and cool to room temperature.
8. Measure absorbance of the solution at 443 nm in a spectrophotometer against a blank prepared same as sample, substituting water for milk.

**Calculation**

Calculate HMF from absorbance as follows:

1. Sample acidified, but not heated which measure free HMF in test sample:
   \[ \text{HMF} = (\text{absorbance} - 0.015) \times 81 = \mu\text{M HMF/L of milk} \]

2. Sample acidified and digested for 1 h in boiling water-bath which measure free and potential HMF from browning intermediates:
   \[ \text{HMF} = (\text{absorbance} - 0.055) \times 87.5 = \mu\text{M HMF/L of milk} \]

Note: Here, results are expressed in terms of fluid milk equivalent i.e. of reconstituted milk.

The original method of Keeney and Bassette has been modified and improved by replacing oxalic acid by acetic acid in the sample pre-treatment step and replacing the colorimetric reaction by HPLC separation and detection, exploiting the strong absorption of HMF at 280 nm (van Boekel and Rehamn, 1987; Morales *et al.*, 1992).
23. Estimation of brown coloring matter in milk powder

The colour of dried milk is one of the important characteristics by which the consumer judges its acceptance. Dried milks are susceptible to quality losses due to chemical instability which depends on composition and storage conditions. Factors such as extensive exposure to heat during processing, high moisture content and prolonged storage period at high temperature are generally known to promote browning also known as Maillard reaction which may lead to undesirable deterioration due to the formation of chemically stable and nutritionally unavailable derivatives known as Melanoidins. Dried milks are most sensitive to non-enzymatic browning as they contain relatively high concentration of lactose and proteins with high lysine level. In addition, high temperature and water content during processing and prolonged storage are the major factors involved in the high susceptibility of dehydrated dairy products as these are favourable conditions for Maillard reaction.

In the following method, browning in dried milk is expressed as browning index which is defined as the optical density difference measured at 420 nm and 550 nm. The method involves liberation of brown pigments from the protein molecules by means of a proteolytic enzyme pronase (Palombo et al., 1984). For complete proteolysis, the pronase mixture (2.53 mg enzyme/0.1g dry powder) is incubated at 45°C for 2 h. After clarification, the browning index is determined spectrophotometrically at 420 and 550 nm. The procedure is easy to employ, suitable for routine laboratory analysis and shows high accuracy and reproducibility.

Apparatus

1. Water-bath: Maintained at 45°C
2. Test tubes: 10 ml capacity.
3. Centrifuge tubes: 2.5 ml capacity.
5. Whatman filter paper: Grade 1.
6. Funnels, Micro-syringe, Cuvette 1 ml capacity with 1 cm path length.
7. Spectrophotometer: Suitable for reading at wavelengths 420 and 550 nm.
8. pH meter.

Reagents

1. Pronase solution (Calbiochem-Bhring P-53702; 45,000 P.U.K. /mg): 10 mg enzyme/ml buffer Tris, pH 7.00, with 50 mM CaCl₂, yielding ultimately 2.53 mg enzyme/0.1 g dry powder.
Note: Pronase is also available from Sigma Chemical Co., St Louis, Mo, USA.
2. Trichloroacetic acid (TCA).

Procedure

1. Weigh 1 g of test portion of dried milk in a test tube and disperse it in 5 ml water (45°C). The mixture is mixed thoroughly and 1.5 ml of this mixture is transferred to a 2.5 ml test tube containing 0.4 ml pronase solution.
2. Incubate the test tube in a water-bath maintained at 45°C for 2 h. Cool the test tube in ice water and add 150 µl trichloroacetic acid.
3. Centrifuge the tube at 7000 rpm for 20 min. Filter the contents of the tube using Whatman filter paper Grade 1.
4. Measure the optical density (OD) of the clear filtrate obtained in a 1 ml Cuvette with 1 cm path length using a spectrophotometer at two wavelengths i.e. 420 and 550 nm. Use water as a blank for taking readings.

Calculation

Browning index expressed as OD/g dry solids = A₄₂₀ nm - A₅₅₀ nm
Where
A₄₂₀ = OD of the filtrate taken at 420 nm;
A₅₅₀ = OD at the filtrate taken at 550 nm

References:

24. Determination of scorched particles content in dried milk

Scorched particles in dried milk are a physical property which is a product defect. Scorched particles consist principally of burnt powder deposit within the drying chamber, particularly around the atomizer and air dispenser. They can also result from gel deposits within the evaporator calandria which occur as a result of insufficient milk flow or protein destabilization, e.g. high acidity resulting into casein precipitation. The amount of scorched particles in spray dried powder is determined (IS 13500: 1992) by comparison with the ADPI chart “Scorched Particle Standards for Dry Milk”.

Apparatus

1. Photocopies of ADPI:”Scorched Particle Standards for Dry Milks”. This chart can either be procured from American Dairy Products Institute, 130 North Franklin Street, Chicago, Illinois 60606, USA or from Bureau of Indian Standards, Manak Bhavan, New Delhi, India.
2. Mixer: Waring blender or similar type. Waring blender with worn or bent shaft should be replaced immediately since metal particles may be produced, giving false scorched particles reading.
3. Scorched particles filtering discs: Cotton discs 3.8 cm (1.25 inch) diameter or cotton pads mounted on test cards use with the aspirator type tester.
4. Scorched particle tester: Aspirator or pressure type, 3.2 cm (1.125 inch) filtering diameter.

![Figure 1. Scorched particles standards for dry milk](image)

A–7.5 mg  B–15 mg  C–22.5 mg  D–32.5 mg
Reagents

1. Defoaming agent: Diglycol laurate S or octylalcohol or antifoam B emulsion, A 5757 Sigma (Sigma Chemical Co., St Louis, Mo, USA).

2. Water: Sediment free, distilled, temperature between 32.5 and 41°C.

Procedure

1. Measure 250 ml sediment free water in a waring blender jar, start the mixer and add 25 g of non-fat dry milk or dry butter milk or 32.5 g of dry whole milk.

2. Add approximately 0.5 ml of defoaming agent and mix for 60 sec in the blender. Filter the entire solution through a standard cotton disc, using an aspirator or pressure type tester. Rinse the mixing container in tester with approximately 50 ml of sediment free water, also passing this through the cotton disc.

3. If reliquefied sample is allowed to stand before filtering, stir vigorously just before pouring it into the tester. Do not allow samples to stand uncovered.

4. Remove the filter disc, place it in a scorched particle disc test card and dry at 30 to 40°C in a dust free atmosphere.

5. Compare the dry disc, placed on a table and viewed from directly above with the scorched particle standard photo prints under uniform, indirect light.

6. Any test falling between two standard discs should be assigned the higher discs letter.

Example: A disc showing more scorched particles than the standard disc A but less than B should be assigned B, and similarly for the other discs.

References: