Detection of Adulterants in Milk

A Laboratory Manual

Revised Edition

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(मान्य विश्वविद्यालय)

करनाल-132001 भारत

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE

(Deemed University)

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Dr. R.R.B. Singh
Acting Director

FOREWORD

Milk is one of the essential food commodities and plays a crucial role in the diet of human nutrition. The quality of milk has been challenge in this country due to high ambient temperature and also due to man-made menace of adulteration as well as presence of contaminants.

In the recent times, media has highlighted many instances of adulteration of milk and milk products with various kinds of adulterants. In general, every milk industry in India is facing problem of adulterated milk at reception dock. Therefore, milk collection centers needs simple tests for detection of adulteration. NDRI is working proactively for developing various analytical techniques and simpler methodology for the detection of adulteration in milk and milk products.

This manual provides the complete overview of analytical methods covering all the aspects of adulterants being added to milk. The methods have been explained with the help of photographs to avoid any ambiguity in the interpretation of results. Further, most of the methods explained in the manual, have been evaluated at authors' laboratory, thus ensuring their working as well as validation.

I warmly congratulate the authors of the manual titled *"Detection of Adulterants in Milk – A Laboratory Manual (Revised Edition)"* for designing and bringing this manual at right time. I am hopeful that the information compiled here will greatly benefit the requirement of milk industry and legal agencies. It is as an effort to improve the milk quality by providing precise and user friendly methods developed to carryout milk testing at all stages of milk handling till it reaches consumer.

(R.R.B. Singh)
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Head, Dairy Chemistry Division

PREFACE

The practice of adulteration of milk is one of the important problems that stands against the progress of dairying in India and may have detrimental effect on our export of dairy products. The image of milk has been considerably deteriorated due to its adulteration with harmful chemicals. With the analytical methods developed for most of the adulterants, unscrupulous traders are finding more innovative ways to adulterate the milk with cheaper ingredients. In the recent past, the menace of adulteration has taken serious proportion as highlighted by many media reports as well as by the report of FSSAI. Over the years, efforts have been made to develop simpler methods to detect adulteration of milk and milk products.

The Division of Dairy Chemistry has made sincere efforts to develop simple methods for the ascertaining the quality of milk and in this direction many new methods have been developed for detection of adulteration in milk. Many of these methods have been adopted by FSSAI and BIS and dairy industry in general is benefitting from these rapid analytical tests.

In this manual, authors who made diligent effort to explain the milk adulteration tests in a lucid manner. Each rapid test has been explained in details along with the photograph of positive and control milk sample. The manual has been divided into different parts, each covering different methods to detect various adulterants encountered. I am sure this manual will make the task of quality control personnel working in dairy laboratories much more easier. The manual can also be used for the teaching of undergraduate and post-graduate students.

I congratulate the authors for this endeavour.

17/3/17

(Bimlesh Mann)
HEAD

ACKNOWLEDGEMENT

The authors are highly thankful to the Director, National Dairy Research Institute, Karnal for guidance and according the necessary permission. The authors are also thankful to Academic Coordinator, National Dairy Research Institute, for providing financial support for the publication of this manual. Financial support received from NAIP, for developing test method to detect the cheese whey adulteration in milk is also acknowledged. Our acknowledgements are due to Head, Dairy Chemistry and Scientists of Dairy Chemistry Division for their support. Technical supports rendered by students are also appreciated here.

Date: March 17, 2017

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Y.S. Rajput
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Table of Contents

1. Composition of Milk and Chemical Quality Evaluation:

1.1. Introduction:

Milk is a complex, homogeneous fluid containing various components in several state of dispersion. It is a unique product secreted by the mammary glands of mammals under complex hormonal control and is intended for nutrition of infants. The composition of milk determines its nutritive quality, its value as raw material for making food products. Milk is defined as lacteal secretion practically free from colostrum obtained by the complete milking of the healthy animals. It is a white opaque liquid having a typical clean smell and sweetish-salty taste. Immediately after parturition, the udder secrets a fluid known as colostrum which differs considerably in composition from the later secretion. Colostoral milk has a salty taste, viscous, has a yellowish colour, acidic pH (around 6.0) and is subject to flocculation during boiling (due to high content of immunoglobulins which are around 7%). Milk from the middle period of the lactation in its natural composition and characteristics is the most suitable raw material for the manufacture of high quality dairy products. Milk differs widely in composition, the greatest difference being among milk of different species of mammals. The major constituents of milk are water, fat, proteins, lactose and minerals (ash). Water is the medium in which all the other components of milk (total solids) are dissolved or suspended. Milk lipids exists in the form of spherical droplets with average diameter 2 – 20 μm, consisting of a fat core enclosed by a membrane known as milk fat globule membrane. Milk fat is present as oil-in-water type emulsion. Lactose is the predominant carbohydrate in milk. The principal protein of milk i.e. casein makes up 80% of total

protein while α-lactalbumin and β-lactoglobulin also known as whey proteins make up the remaining 20%. The ash content is an analytical value indicating the amount of non-combustible matter in milk. In normal milk this value remains rather constant at about 0.7%. A value much higher than this is indicative of abnormal conditions in the secretary glands. The average gross composition of milk of different species has been presented in **Table 1.1.**

Table 1.1 Average composition of milk from different species*

Component (per 100 ml)	Species				
	Cow	**Human**	**Buffalo**	**Goat**	**Sheep**
Water, g	87.99	87.50	83.39	87.03	80.70
Food energy, kcal	61	70	97	69	108
KJ	257	291	404	288	451
Protein (Nx6.38), g	3.29	1.03	3.75	3.56	5.98
Fat, g	3.34	4.38	6.89	4.14	7.00
Carbohydrate, total, g	4.66	6.89	5.18	4.45	5.36
Ash, g	0.72	0.20	0.79	0.82	0.96
Minerals					
Calcium, mg	119	32	169	134	193
Iron, mg	0.05	0.03	0.12	0.05	0.10
Magnesium, mg	13	3	31	14	18
Phosphorus, mg	93	14	117	111	158
Potassium, mg	152	51	178	204	136
Sodium, mg	49	17	52	50	44
Zinc, mg	0.38	0.17	0.22	0.30	-
Vitamins					
Ascorbic Acid, mg	0.94	5.00	2.25	1.29	4.16
Thiamin, mg	0.038	0 .014	0 .052	0.048	0.065
Riboflavin, mg	0.162	0.036	0.135	0.138	0.355
Niacin, mg	0.084	0.177	0.091	0.277	0.417
Pantothenic Acid, mg	0.314	0.223	0.192	0.310	0.407
Vitamin B_6, mg	0.042	0.011	0.023	0.046	-
Folate, μg	5	5	6	1	-
Vitamin B_{12}, μg	0.357	0.045	0.363	0.065	0.711
Vitamin A,RE	31	64	53	56	42
Cholesterol, mg	14	14	19	11	-

*Adopted from: Milk and dairy product technology: Spreer, E., 1995; Handbook of Dairy Foods and Nutrition: Gregory D Miller; Judith K. Jarvis & Lois D. McBean., 2000 and Buffalo milk – chemistry and processing technology: Sahai. D., 1996.

1.2. Quality evaluation of milk

Raw milk of good quality is the basis for the production of high quality dairy products. Raw milk should comply with the standards laid by the law regulating agencies. In most of the countries, the following chemical quality

characteristics have been set for raw milk; (a) fat percentage, (b) total solids or solids-not-fat (SNF) percentage (c) protein content and (d) freezing point in °C. Based upon the chemical analysis results, raw milk is graded. The initial good quality milk is allocated high price. The purpose of evaluation of chemical quality of milk is to accept or reject the milk, to check if general hygienic standards in production at farm level are met, classification of milk as a basis of payment and further utilization and selection of milk for culture preparation and production of specific products. All over the world, quality evaluation is done on the basis of organoleptic, chemical, physical and microbiological parameters. Further, the menace of adulteration in milk can be checked by performing chemicals tests. Therefore, in the present scenario the importance of chemical testing of milk has gained impetus momentum so that in finished product prepared should meet international standards.

1.3. Preparation of milk sample for analysis

Before withdrawing portions for analytical determinations, bring the sample to a temperature of 26 to 28°C and mix thoroughly by pouring gently into a clean dry receptacle and back, until a homogeneous mixture is assured.

If lumps of cream do not completely disappear, warm the sample to about 40°C, mix thoroughly, then cool to 26 to 28°C. In case a measured volume is required in a determination, bring the temperature of the sample to about 27°C before pipetting.

All samples should be allowed to stand for 3 to 4 min after mixing to allow air bubbles to rise; the sample bottle shall then be inverted 3 or 4 times immediately prior to taking the milk for analysis.

Note: *If a sample gets curdled, excessively churned or decomposed, in such cases the sample should be rejected.*

2. Detection of Formalin in Milk

2.1. Introduction:

Formalin (37 to 40% solution of formaldehyde) is generally used by Public Health Departments to preserve the milk samples for chemical analysis purpose. Formaldehyde is very poisonous chemical. Though, it can preserve the milk for very long time, it should never be added to milk meant for processing due to its poisonous property. Moreover, it affects the quality of the milk products as it binds with proteins and affecting their functionality. If milk kept at room temperature (25 to 35°C) for longer time, does not sour, then that milk must be tested for formaldehyde by the following simple method(s):

2.2. Method I: Leach Test

2.2.1. Reagent(s) required:

i. Concentrated HCl
ii. Ferric Chloride

2.2.2. Reagent(s) preparation:

i. Ferric Chloride (10%, w/v): Weigh 10 g of ferric chloride and dissolve it in distilled water to obtain 100 ml 10% ferric chloride solution.
ii. Take 1 ml of 10% ferric chloride solution in a 500 ml volumetric flask and make up the volume using concentrated hydrochloric acid.

2.2.3. Methodology:

A. Take about 5 ml of milk in a test tube.
B. Add to it equal volume of detecting reagent.

C. Keep the tube in boiling water bath for about 3-4 min.

D. Observe the colour of the solution in the tube.

2.2.4. Interpretation:

Appearance of brownish pink colour confirms the presence of formalin in the sample, whereas; control sample will remain white **(Fig. 2.1)**.

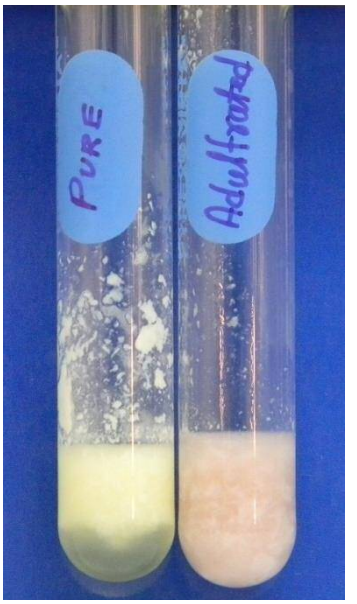

Fig. 2.1: Comparison of developed colour in presence and absence of formalin in milk using

2.2.5. Limits of detection:

Milk samples with formalin concentration more than 0.1% in milk, develops colour which is clearly distinguishable from colour of pure milk. Thus, limit of detection of the test is 0.1% formalin in milk **(Fig. 2.2).**

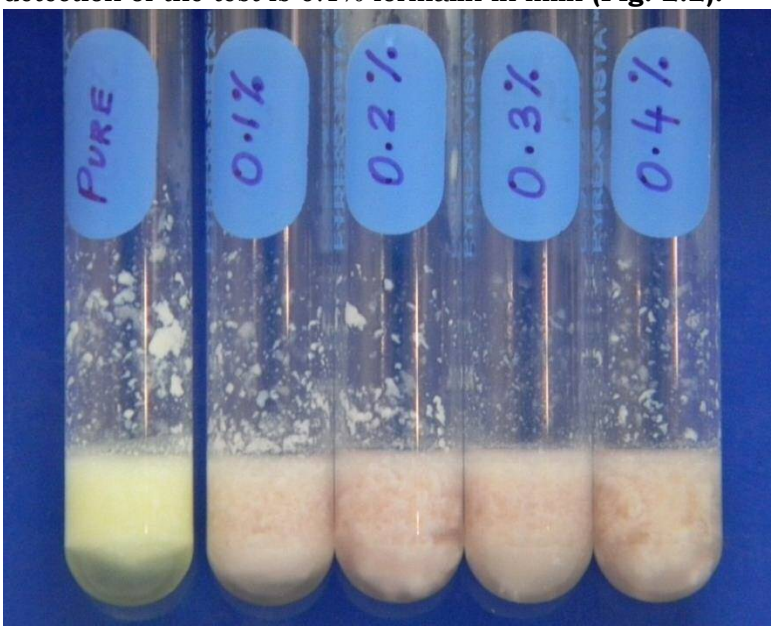

Fig. 2.2: Limit of detection of formalin in milk using Leach test.

2.3. Method II: Chromotropic Acid Test

2.3.1. Reagent(s) required:

i. 1,8-Dihydroxynaphthalene-3,6-disulphonic acid (Chromotropic acid)

ii. Sulphuric acid

2.3.2. Reagent(s) preparation:

i. Saturated solution of 1, 8-dihydroxynaphthalene-3, 6-disulphonic acid in about 72% sulphuric acid (about 500 mg/100 ml). Light straw-coloured solution should result.

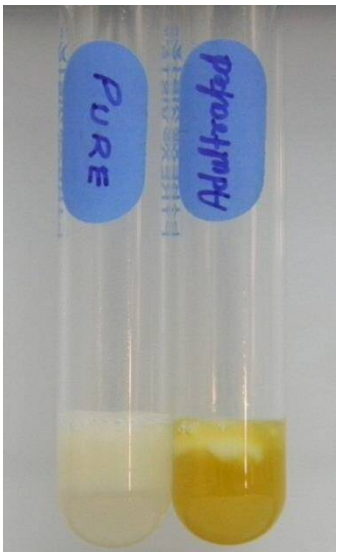

Fig. 2.3: Comparison of developed colour in presence and absence of formalin in milk using chromotropic acid test.

2.3.3. Methodology:

A. Take 1 ml of milk sample in a test tube.

B. Add 1 ml of the chromotropic acid reagent and mix well.

2.3.4. Interpretation:

Appearance of yellow colour confirms the presence

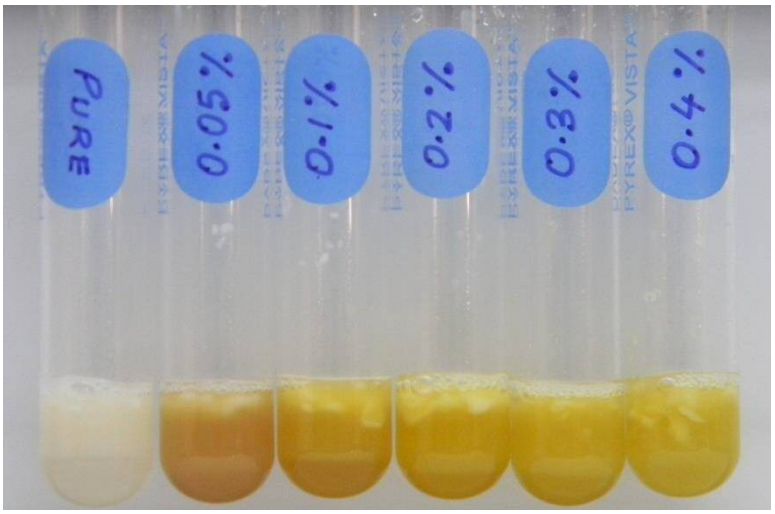

Fig. 2.4: Limit of detection of formalin in milk using chromotropic acid test.

of formalin in the sample, whereas; control sample will remain white (translucent) **(Fig. 2.3)**.

2.3.5. Limits of detection:

Development of slight brownish yellow colour at low concentration of formalin and thereafter increase in the intensity of yellow colour in presence of higher concentration of formalin in milk can clearly be observed from the **Fig. 2.4**. Distinguishable brownish yellow was observed when milk samples were adulterated with 0.05% formalin. Hence the limit of detection in milk for this method is 0.05% of formalin **(Fig. 2.4)**.

2.4. References:

BIS: (1981). Handbook of Food Analysis. Part XI Dairy Products. Bureau of Indian Standards, Manak Bhawan, New Delhi.

IS: 1479. (1960). Methods of test for dairy industry. Part I. Rapid examination of milk. Bureau of Indian Standards, New Delhi.

Roy, N.K. and Sen, D.C. (1991). Rapid analysis of milk. In: Textbook of Practical Dairy Chemistry. Vol. I. Chemical analysis of fluid milk. Kalyani Publishers, New Delhi, India.

Manual in Dairy Chemistry (1964, Revised in 1979). ICAR (Indian Council of Agricultural Research) Sub–Committee on Dairy Education, ICAR, New Delhi.

3. Detection of Hydrogen Peroxide in Milk

3.1. Introduction:

Hydrogen Peroxide is a preservative, but as per FSSAI Rules, it is not permitted to be added in milk. Hence if it is found, then milk is said to be adulterated.

3.2. Method I: Using Para-phenylenediamine Reagent

3.2.1. Reagent required:

i. Para-phenylenediamine

3.2.2. Reagent preparation:

i. Weigh 2.0 g of Para-phenylenediamine and dissolve it in distilled water to obtain 100 ml solution i.e. 2% aqueous solution, w/v. Dissolution of Para-phenylenediamine in water is difficult and require thorough mixing. The solution will appear pale yellow.

3.2.3. Methodology:

A. Add about 2 ml of milk in a test tube.
B. Add equal volume of raw milk.
C. Add two drops of 2% of Para-phenylenediamine reagent.
D. Mix well.
E. Observe the colour of the solution in the tube.

3.2.4. Interpretation:

Blue colour is developed in the presence of H_2O_2. Whereas pure milk sample remain white in colour **(Fig. 3.1)**.

Fig. 3.1: Comparison of developed colour in presence and absence of H_2O_2 in milk using para-phenylenediamine test.

3.2.5. Limits of detection:

Milk adulterated with 0.025% of H_2O_2 shows development of intense blue colour whereas pure milk

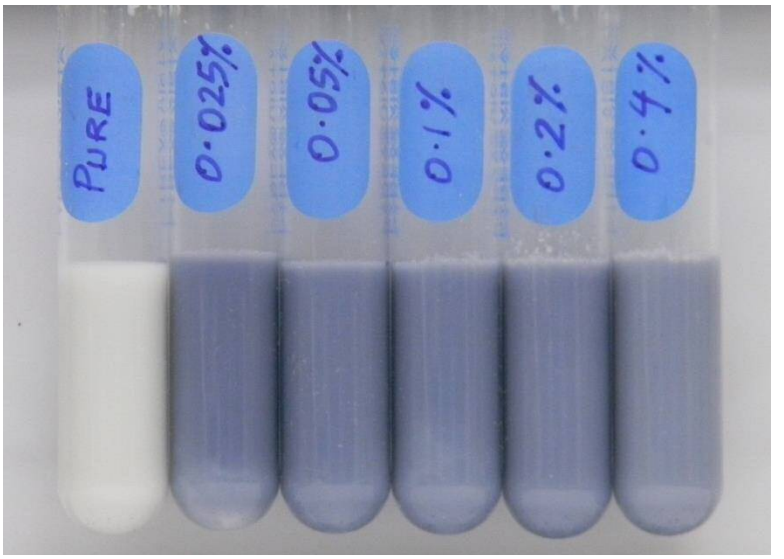

Fig. 3.2: Limit of detection of H_2O_2 in milk using para-phenylenediamine test.

sample remains white in colour. The limit of detection for this method is 0.025% (v/v) H_2O_2 in milk **(Fig. 3.2)**.

Note: *It is unlikely that the addition of less than 0.1% of H_2O_2 to milk could be detected after 24 h, owing to the action of peroxidase and catalase which stimulate its conversion into water. If more than 0.2% H_2O_2 is added, some will persist for considerable long time. Owing to the fact that larger amount of H_2O_2 are known to destroy peroxidase, it is always advisable to add to the sample an equal volume of raw unpreserved milk and to follow with addition of a few drops of a 0.2% solution of Para-phenylenediamine. Under these circumstances a blue colour will develop immediately if H_2O_2 has been added to the milk sample.*

3.3. Method II: Using Potassium Iodide and Starch Reagent

3.3.1. Reagent(s) required:

i. Potassium Iodide

ii. Starch

3.3.2. Reagent(s) preparation:

i. Weigh 20 g of potassium iodide and dissolve it in distilled water to obtain a 100 ml solution.

ii. Take 1 g starch powder and dissolve it in distilled water by heating and make up the volume to 100 ml.

iii. Potassium iodide-starch reagent: Mix equal volumes of 20% potassium iodide solution and 1% starch solution.

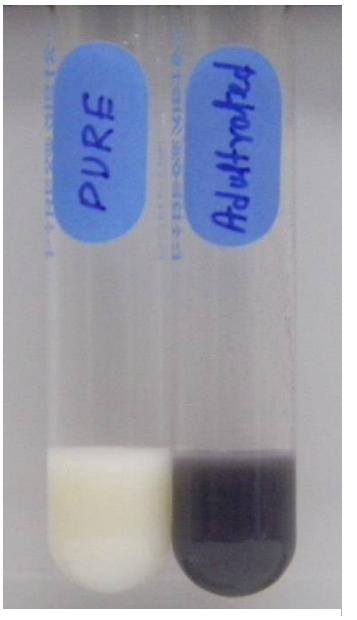

Fig. 3.3: Comparison of developed colour in presence and absence of H_2O_2 in milk using KI-starch reagent.

3.3.3. Methodology:

A. Take 1 ml of milk sample in a test tube.

B. Add 1 ml of the potassium iodide-starch reagent and mix well.

C. Observe the colour of the solution in the tube.

3.3.4. Interpretation:

Appearance of blue colour indicates the presence of hydrogen peroxide in the milk sample whereas control milk sample remain colourless **(Fig. 3.3)**.

3.3.5. Limits of detection:

Appearance of blue colour in the adulterated milk samples with increasing concentration of H_2O_2 is clearly distinguishable at 0.004% (v/v) level of adulteration.

Hence the limit of detection for this method is 0.004% (v/v) (**Fig. 3.4**).

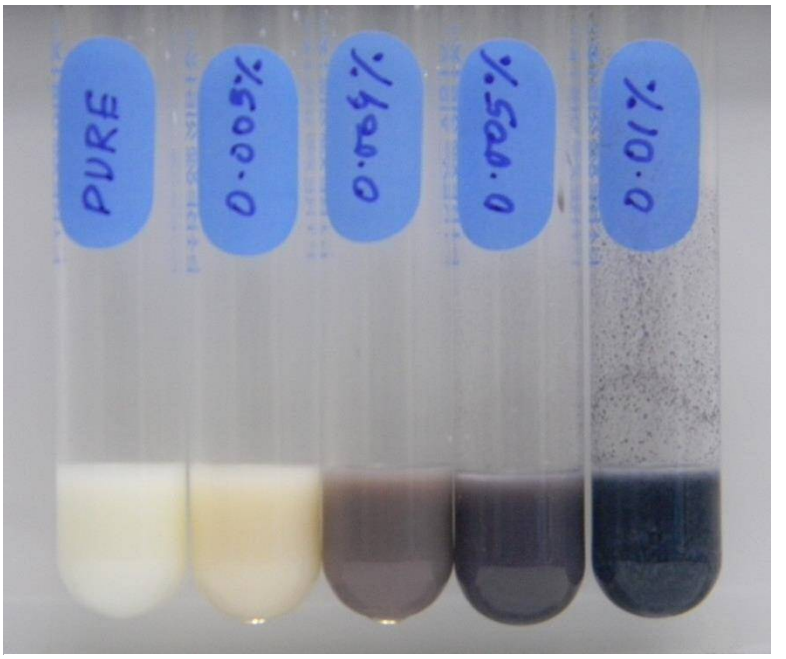

Fig. 3.4: Limit of detection of H_2O_2 in milk using KI-starch reagent.

3.4. References:

IS: 1479. (1960). Methods of test for dairy industry. Part I. Rapid examination of milk. Bureau of Indian Standards, New Delhi.

Roy, N.K. and Sen, D.C. (1991). Rapid analysis of milk. In: Textbook of Practical Dairy Chemistry. Vol. I. Chemical analysis of fluid milk. Kalyani Publishers, New Delhi, India.

Manual in Dairy Chemistry (1964, Revised in 1979). ICAR (Indian Council of Agricultural Research) Sub–Committee on Dairy Education, ICAR, New Delhi.

4. Detection of Cane Sugar in Milk

4.1. Introduction:

Unscrupulous milk producers were found to adulterate milk using cane sugar to increase the solids-not-fat (SNF) content of milk. As milk solids are quite costly compared to the cane sugar therefore, adulterating milk with cane sugar will earn the unscrupulous milk producer more profit. On the other hand, the consumers of such adulterated milk are economically cheated. As per the regulatory standards of milk anything adding to the milk is adulteration and is a punishable offence, therefore detecting cane sugars presence in the milk samples is very essential.

4.2. Method I: Using Seliwanoff's Reagent

To make the test easier, one can use Seliwanoff's reagent which consists of resorcinol and hydrochloric acid.

4.2.1. Reagent(s) required:

i. Resorcinol

ii. Concentrated Hydrochloric Acid (HCl)

4.2.2. Reagent(s) preparation:

i. Resorcinol Solution (0.5%): Weigh 0.5 g of resorcinol in about 40 ml of distilled water. Add 35 ml of concentrated HCl (12 N) to it and make up the volume to 100 ml using distilled water.

Fig. 4.1: Comparison of developed colour in presence and absence of cane sugar in milk using Seliwanoff's reagent.

4.2.3. Methodology:

A. Take 1 ml of milk in a test tube.

B. Add 1 ml of detecting reagent and mix.

C. Place the tube in boiling water bath for 5 min.

4.2.4. Interpretation:

In the presence of cane sugar in the milk sample, red colour is produced. Whereas in pure milk samples, no such red colour is developed and sample remains white in nature with visible coagulum **(Fig. 4.1)**.

4.2.5. Limits of detection:

Development of red colour in milk sample adulterated with cane sugar can be observed after heating it in the boiling water bath for 5 min. The intensity of the red colour increases with the increasing concentration of cane sugar in the milk samples. Distinct red colour can be

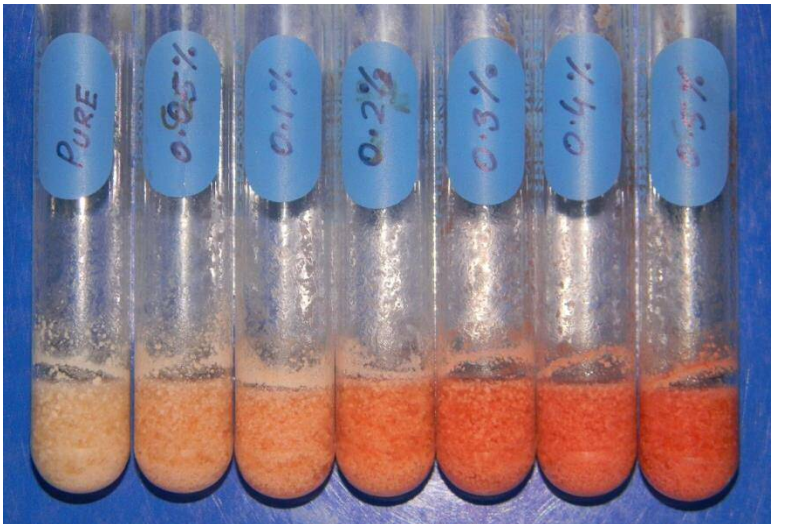

Fig. 4.2: Limit of detection of cane sugar in milk using resorcinol test.

observed in milk sample adulterated at the rate of 0.1% (w/v) with sucrose. Therefore the limit of detection for this method is 0.1% (w/v) of sucrose in milk (**Fig. 4.2**).

4.3. Method II: Resorcinol Test

4.3.1. Reagent(s) required:

i. Resorcinol

ii. Concentrated Hydrochloric Acid (HCl)

4.3.2. Methodology:

A. Take about 5 ml of milk in a test tube.
B. Add 1 ml of concentrated HCl.
C. Add 0.1 g of resorcinol and mix.
D. Place the tube in boiling water bath for 5 min.

Fig. 4.3: Comparison of developed colour in presence and absence of cane sugar in milk using resorcinol test.

4.3.3. Interpretation:

In the presence of cane sugar in the milk sample, red colour is produced. Whereas in pure milk samples no such red colour is develop and sample remains white with visible coagulum in it **(Fig. 4.3)**.

4.3.4. Limits of detection:

Presence of sucrose in milk samples can be determined by observing the red colour development in the experiment. The intensity of red colour increases with increase in the sucrose content in the milk. When the

Fig. 4.4: Limit of detection of cane sugar in milk using Seliwanoff's reagent.

concentration of sucrose in the sample is 0.2% (w/v), sufficient red colour develops so that it can easily be distinguished from pure or control milk sample. Hence the limit of detection for this method is 0.2% (w/v) (**Fig. 4.4**).

4.4. References:

IS: 1479. (1960). Methods of test for dairy industry. Part I. Rapid examination of milk. Bureau of Indian Standards, New Delhi.

Roy, N.K. and Sen, D.C. (1991). Rapid analysis of milk. In: Textbook of Practical Dairy Chemistry. Vol. I. Chemical analysis of fluid milk. Kalyani Publishers, New Delhi, India.

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5. Detection of Starch or Cereal Flours in Milk

5.1. Introduction:

Starch is cheaply available in various forms such as wheat flour, corn flour and commercially manufactured starch. It is sometimes added in the milk by adulterators to raise the SNF.

5.2. Method I: Using Iodine (Without heated milk sample)

5.2.1. Reagent(s) required:

i. Potassium iodide

ii. Iodine crystal

5.2.2. Reagent(s) preparation:

i. Iodine solution (1%): Dissolve 2.5 g potassium iodide in 100 ml water, add to it 1 g pure iodine crystal, shake well to give a clear solution.

5.2.3. Methodology:

A. Take 1 ml milk in a test tube.

B. Add 2 drops of iodine solution.

5.2.4. Interpretation:

Appearance of blue colour indicates the presence of starch in the milk sample whereas a control milk sample develops slight yellowish colour **(Fig. 5.1)**.

Fig. 5.1: Comparison of developed colour in presence and absence of starch in milk using iodine reagent (without heated milk sample).

5.2.5. Limits of detection:

Appearance of blue colour in the milk samples indicates the presence of starch in the milk samples. Blue

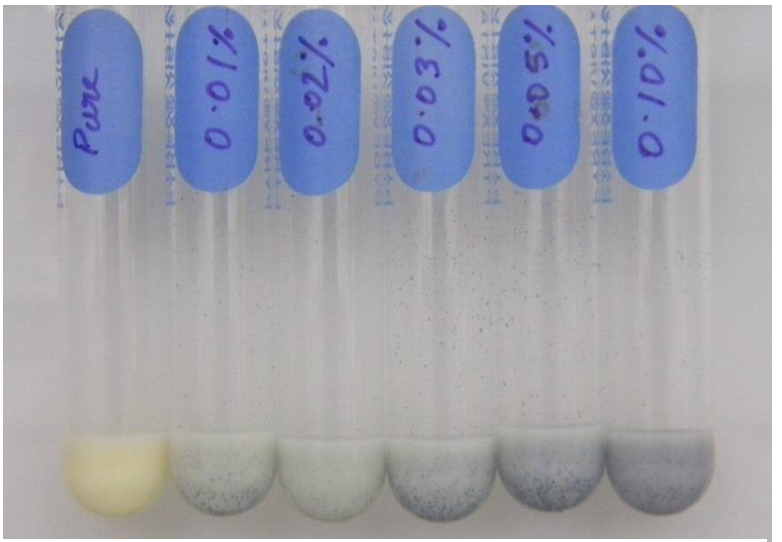

Fig. 5.2: Limit of detection of starch in milk using iodine reagent (without heated milk sample).

colour intensity increases with the increase in the starch content of the milk samples. Distinguishable blue colour develops in samples when it is adulterated with 0.01% (w/v) of starch (**Fig. 5.2)**. Therefore the limit of detection for this method is 0.01% (w/v).

5.3. Method II: Using Iodine (With boiled milk sample)

5.3.1. Reagent(s) required:

i. Potassium iodide
ii. Iodine crystal

5.3.2. Reagent(s) preparation:

i. Iodine solution (1%): Dissolve 2.5 g potassium iodide in 100 ml water and add to it 1 g pure iodine crystals, shake well to give a clear solution.

5.3.3. Methodology:

A. Take about 3 ml of well-mixed milk sample in a test tube.

B. Heat the milk to just boiling by holding the tube over flame, and thereafter cool to room time.
C. Add 1-2 drops of 1% iodine solution.
D. Mix well.
E. Observe the development of colour.

Fig. 5.3: Comparison of developed colour in presence and absence of starch in milk using iodine reagent (with boiled milk sample).

5.3.4. Interpretation:

Development of blue colour in the tube indicates the presence of starch in the milk sample whereas control milk sample develops slight yellowish colour due to the dark brown colour of the iodine reagent **(Fig. 5.3)**.

5.3.5. Limits of detection:

Increase in the intensity of developed blue colour

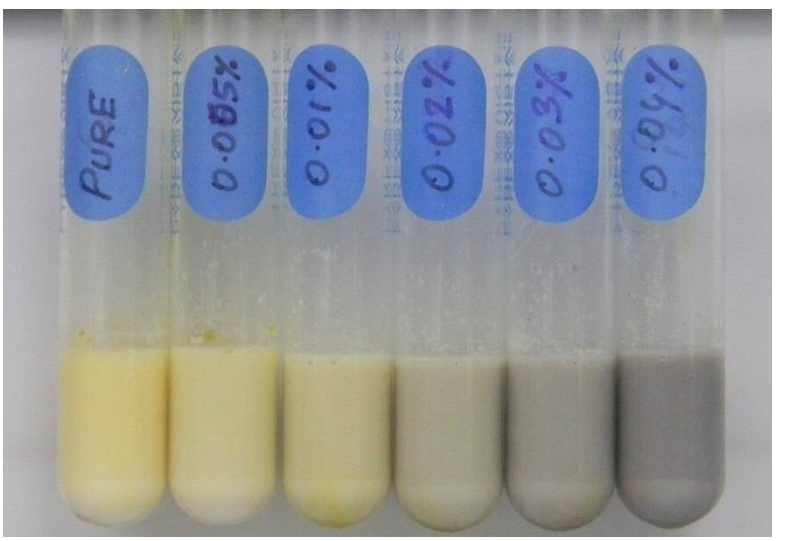

Fig. 5.4: Limit of detection of starch in milk using iodine reagent (with boiled milk sample).

in the presence of starch in milk sample has been observed after the milk samples were analyzed by this method. Milk samples containing 0.02% (w/v) starch gave distinguishable blue colour which can be differentiated from control or pure milk samples (**Fig.5.4**). Hence the limit of detection for this method is 0.02% (w/v) starch in milk.

Note: *The samples were boiled over flame to dissolve/gelatinize any possible starch presence in milk samples.*

5.4. References:

IS: 1479. (1960). Methods of test for dairy industry. Part I. Rapid examination of milk. Bureau of Indian Standards, New Delhi.

Roy, N.K. and Sen, D.C. (1991). Rapid analysis of milk. In: Textbook of Practical Dairy Chemistry. Vol. I. Chemical analysis of fluid milk. Kalyani Publishers, New Delhi, India.

Manual in Dairy Chemistry (1964, Revised in 1979). ICAR (Indian Council of Agricultural Research) Sub–Committee on Dairy Education, ICAR, New Delhi.

6. Detection of Sulfate Salts in Milk

6.1. Introduction:

Presence of sulfate salts, which may be added to milk to raise its SNF level in milk. This can be detected by using barium chloride.

6.2. Method: Using barium chloride

6.2.1. Reagent(s) required:

i. Barium chloride ($BaCl_2.2H_2O$)

ii. Trichloroacetic acid (TCA)

6.2.2. Reagent(s) preparation:

i. Barium chloride ($BaCl_2.2H_2O$) 5% (w/v) aqueous solution: Dissolve 5.0 g barium chloride in distilled water and make the final volume to 100 ml.

ii. Trichloroacetic acid (TCA), 24% (w/v, aq.): Dissolve 24 g of TCA into distilled water and make the final volume to 100 to obtain 24% TCA.

Fig. 6.1: Comparison of developed colour in presence and absence of sulfate salt(s) in milk.

6.2.3. Methodology:

A. Take 10 ml of milk in a 50 ml stoppered test tube.

B. Add 10 ml of TCA solution.

C. Filter the coagulated milk through Whatman filter paper Grade 42.

D. Take 5 ml of clear filtrate.

E. Add few drops of barium chloride solution.

F. Observe for any visible precipitates in the tube.

6.2.4. Interpretation:

Formation of milky-white precipitates indicates the presence of added sulfates like ammonium sulfate, sodium sulfate, zinc sulfate and magnesium sulfate etc. to milk **(Fig. 6.1)**.

6.2.5. Limits of detection:

Formation of milky white precipitates in milk sample filtrate is the indication that the milk sample has been adulterated with sulfate salt(s). The quantity of precipitates increases with the increasing level of adulteration with sulfate salt(s). When the milk sample was adulterated with 0.05% (w/v) of sulfate salt, sufficient

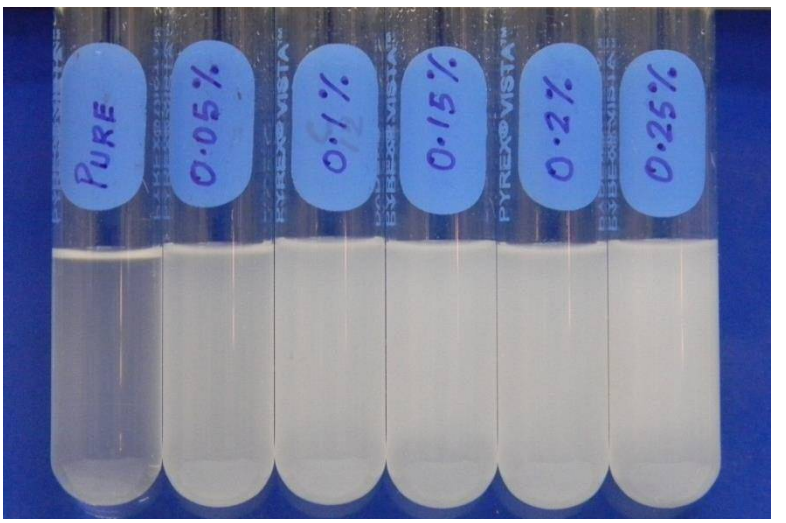

Fig. 6.2: Limit of detection of sulfate salt(s) in milk.

amount of precipitates are formed which are distinguishable from pure milk sample. Therefore, the limit of detection for this method is 0.05% (w/v) of sulfate salt in milk sample **(Fig.6.2)**.

6.3. Reference:

Sharma, R; Seth, R. and Barui A.K. (2011). Rapid methods for the detection of Adulterants in milk. In: Chemical analysis of value added dairy products and their quality assurance. Compendium of lectures, Winter School. Dairy Chemistry Division, NDRI, Karnal, India. pp 184-188.

7. Detection of Urea in Milk

7.1. Introduction:

Urea is a natural constituent of milk and it forms a major part of the non-protein nitrogen of milk. Urea concentration in milk is variable within herd. Urea is one of the ingredients of synthetic milk along with caustic soda, detergent, sugar and foreign fats. Adulteration of natural milk with synthetic milk increases the level of urea to such an extent that on consumption of this adulterated milk causes toxicological hazards. Estimation of urea concentration in milk may serve as a tool for checking the menace of adulteration of natural milk with synthetic milk. The average urea content in milk of Karan Swiss, Karan Fries and Sahiwal cows was reported to be 28.57, 28.79 and 25.39 mg/100 ml respectively (range 20 to 35 mg/100 ml). In buffalo milk, the average urea content was found to be 35.10 mg (range 25 to 40 mg/100 ml), (Kavita, 2000). As per FSSAI Act 2006, the urea content in milk should not be more than 70 mg/100 ml. If urea content in milk is more than 70 mg/100 ml, the milk may be considered adulterated with urea. The addition of urea to milk can be detected by using p-dimethylaminobenzaldehyde (DMAB). This method is based on the principle that urea forms a yellow complex with DMAB in a low acidic solution at room temperature. The intensity of yellow colour can be measured at 425 nm and hence method can be quantitative.

7.2. Method I (Qualitative method): Using Dimethylaminobenzaldehyde & Trichloroacetic Acid Method

7.2.1. Reagent(s) required:

i. Dimethylaminobenzaldehyde (DMAB)

ii. Ethyl alcohol

iii. Concentrated HCl

iv. Trichloroacetic acid (TCA)

7.2.2. Reagent(s) preparation:

i. 1.6% (w/v) DMAB reagent: Dissolve 1.6 g DMAB in 100 ml ethyl alcohol and add 10 ml concentrated HCl.

ii. Trichloroacetic acid (TCA), 24% (w/v, aq.): Dissolve 24 g of TCA into distilled water and make the final volume to 100 to obtain 24% TCA.

7.2.3. Methodology:

A. Take equal quantity of milk and equal quantity of 24% TCA in a glass stoppered test tube. Mix and filter it with Whatman No. 42 filter paper.

B. Take 3 ml of filtrate in another test tube

C. Add 3 ml of 1.6% DMAB reagent.

D. Note the colour obtained.

Fig. 7.1: Comparison of developed colour in presence and absence of added urea in milk using DMAB and TCA reagent.

7.2.4. Interpretation:

The occurrence of distinct yellow colour indicates the presence of added urea in milk sample whereas slight yellowish colour develops in control milk sample as pure milk itself contains 25-40 mg/100 ml urea **(Fig. 7.1)**.

7.2.5. Limits of detection:

Development of intensive yellow colour represents the adulteration of milk with urea. Light yellow colour develops in case of pure/control milk samples due to the reason that pure milk itself contains 25-40 mg per 100 ml.

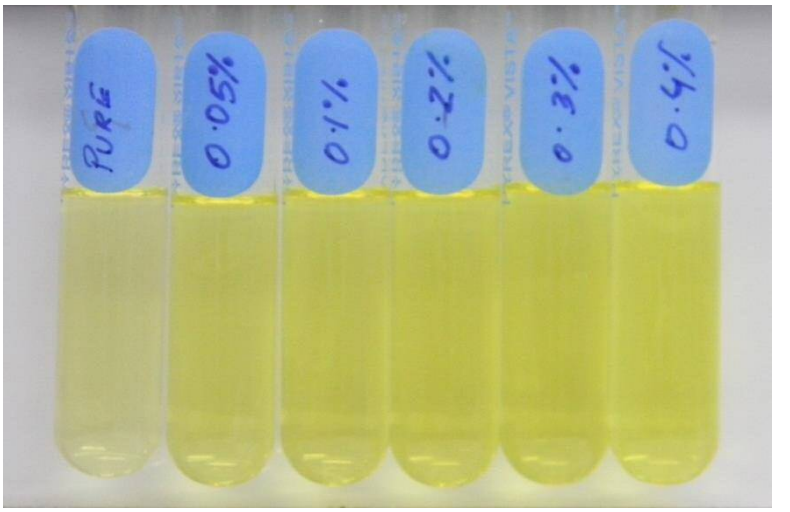

Fig. 7.2: Limit of detection of added urea in milk using DMAB and TCA reagent.

Distinct yellow colour develops when milk samples were adulterated with 0.2% (w/v) urea. Therefore, the limit of detection for this method is 0.25 (w/v) urea in milk **(Fig.7.2).**

Note: *The control (milk sample containing no added urea) showed a slight yellow colour due to the presence of natural urea in milk.*

7.3. Method II (Qualitative method): Using Dimethylaminobenzaldehyde Reagent

7.3.1. Reagent(s) required:

i. Dimethylaminobenzaldehyde (DMAB)
ii. Ethyl alcohol
iii. Concentrated HCl

7.3.2. Reagent(s) preparation:

i. 1.6% (w/v) DMAB reagent: Dissolve 1.6 g DMAB in 100 ml ethyl alcohol and add 10 ml concentrated HCl.

7.3.3. Methodology:

A. Take 1 ml of milk in a test tube.
B. Add 1 ml of 1.6% (w/v) DMAB reagent and mix well.
C. Note the colour obtained.

7.3.4. Interpretation:

The occurrence of distinct yellow colour indicates the presence of added urea in milk sample whereas slight yellowish colour develops in control milk sample as pure milk itself contains 25-40 mg/100 ml urea **(Fig. 7.3)**.

Fig. 7.3: Comparison of developed colour in presence and absence of added urea in milk using DMAB reagent.

7.3.5. Limits of detection:

Distinct yellow colour in adulterated milk sample obtained when the milk sample is adulterated with minimum of 0.2% (w/v) of added urea. Therefore the limit of detection for this method employing DMAB as reagent is 0.2% (w/v) **(Fig. 7.4).**

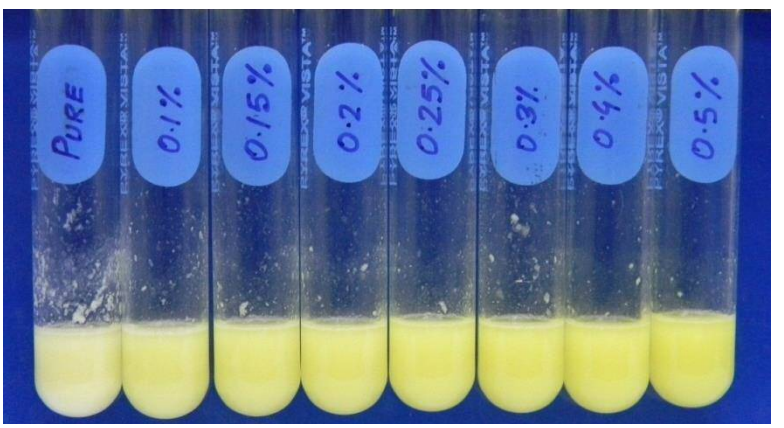

Fig. 7.4: Limit of detection of added urea in milk using DMAB reagent.

Note: *The control (milk sample containing no added urea) showed a slight yellow colour due to the presence of natural urea in milk.*

7.4. Method III (Quantitative method): Using Dimethylaminobenzaldehyde Reagent

7.4.1. Reagent(s) required:

i. Dimethylaminobenzaldehyde (DMAB)
ii. Concentrated HCl
iii. Trichloroacetic acid (TCA)
iv. Anhydrous potassium di-hydrogen orthophosphate (KH_2PO_4) and anhydrous di-potassium mono-hydrogen orthophosphate (K_2HPO_4)
v. Urea

7.4.2. Reagent(s) preparation:

i. 1.6% (w/v) DMAB reagent: Dissolve 1.6 g DMAB in 100 ml ethyl alcohol and add 10 ml concentrated HCl.
ii. Trichloroacetic acid (TCA): 24% (w/v, aq.).
iii. Phosphate buffer (pH 7.0): Dissolve 3.403 g anhydrous potassium di-hydrogen orthophosphate (KH_2PO_4) and 4.355 g anhydrous di-potassium mono-hydrogen orthophosphate (K_2HPO_4) in distilled water and make the volume to one liter with distilled water.
iv. Diluting reagent: Equal volumes of 24% (w/v) TCA and phosphate buffer (pH 7.0) are mixed to make the diluting reagent.
v. Standard urea solution (1 mg/ml): Weigh 100 mg of AR Grade quality urea and dissolve in phosphate buffer (pH 7.0). Make up the volume to 100 ml with the above phosphate buffer.

7.4.3. Methodology:

A. Take 10 ml of milk sample and add 10 ml of 24% TCA in 50 ml glass stoppered test tube. Mix the content and filter through Whatman filter paper grade 42.
B. Take 5 ml of the above filtrate in a test tube and add 5 ml of 1.6% DMAB reagent.

C. Take the absorbance of the yellow colour so obtained at 425 nm in a spectrophotometer against reagent blank.

D. For reagent blank take 5 ml of diluting reagent and add 5 ml of 1.6% DMAB reagent.

E. For standard curve preparation, take different concentration of urea solution (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 mg) separately in different test tubes and make the total volume to 5 ml in each case with diluting reagent. Then add 5 ml of 1.6% DMAB reagent to each test tube to develop yellow colour. Take the absorbance at 425 nm against the reagent blank.

F. Draw a standard curve by plotting the absorbance along Y-axis and urea concentration (in mg) along X-axis.

7.4.4. Calculation:

Read from the graph the concentration of urea (mg) corresponding to absorbance of the sample.

Say the absorbance for the sample be X and the corresponding concentration from the standard curve for urea is = Y mg

Therefore, 5 ml of filtrate from sample has = Y mg urea

7.5. References:

IS: 1479. (1960). Methods of test for dairy industry. Part I. Rapid examination of milk. Bureau of Indian Standards, New Delhi.

Bector, B.S., Ram, M. and Singhal, O.P. (1998). Rapid platform test for the detection/determination of added urea in milk. Indian Dairyman, 50(4): 59-62.

Kavita, P. (2000). Studies on the levels of urea in milk. M. Sc. Thesis NDRI Karnal.

8. Detection of Dextrin / Maltodextrin

8.1. Introduction:

Maltodextrins are hydrolysis products of starches with dextrose equivalent (DE) value lower than 20. They represent a mixture of saccharides with a broad molecular weight distribution between polysaccharides and oligosaccharides and are available as white powders mostly or concentrated solutions. Maltodextrin consists of D-glucose units connected in chains of variable length. The glucose units are primarily linked with α(1-4) glycosidic bonds. Maltodextrin is available with different DE value which depend on the chain length. In contrast to native starches, the maltodextrins are soluble in water and do not give characteristic blue colour with iodine solution (Chronakis, 1998). Maltodextrins have been used as food additive, and some of its important functional properties include bulking, gelling, crystallization prevention, promotion of dispensability, freezing control and as fat replacer (Blanchard and Katz, 1995). Maltodextrins are also being used in the preparation of various dairy products such as yoghurt (Domagala *et al.*, 2006), ice-cream (Hyvonen *et al.*, 2003), various types of milk powders (Schoonman *et al.*, 2001; Hua *et al.*, 2004), various types of cheeses (Bhaskaracharya and Shah, 2001) and in indigenous milk products such as burfi (Ramakrishna *et al.*, 2005). In recent time, maltodextrin has been reported to be added as adulterant to milk, mainly to increase its lactometer reading and also to increase the yield of the product prepared from it such as khoa and burfi. Here, two methods for the detection of maltodextrin in milk have been given. The qualitative method involves the addition of dilute iodine solution to milk sample which gives chocolate brown in the adulterated milk sample. In the quantitative method, amyloglucosidase is used

for hydrolysis of maltodextrin present in milk sample followed by quantitative estimation of released glucose. Amyloglucosidase [α-(1,4)-D-glucanglucohydrolase, EC 3.2.1.3] liberates glucose from non-reducing end of α-(1,4) and α-(1,6) glycosidic linkages (Brunt *et al.*, 1998; Reilly, 2003) and therefore, the enzyme is capable of releasing glucose from maltodextrin and starch. Using this concept, a simple method has been described for the quantification of maltodextrin in milk and milk products (Sharma *et al.*, 2010).

8.2. Method I: Using Iodine Reagent

8.2.1. Reagent(s) required:

i. Iodine

8.2.2. Reagent(s) preparation:

i. Iodine solution (0.05 N): Weigh 317 mg iodine crystal in 200 ml beaker and add 50 ml water. Add KI till all the iodine crystals are dissolved.

8.2.3. Methodology:

A. Take about 5 ml milk sample in a test tube.
B. Add 2 ml of detecting reagent to the tube.
C. Mix well.
D. Observe for the change in colour.

8.2.4. Interpretation:

Appearance of chocolate-red brown colour indicates the presence of maltodextrin in the milk sample whereas in pure milk sample no such coloration will be observed and it will be very slight yellowish in colour **(Fig. 8.1)**.

Fig. 8.1: Comparison of developed colour in presence and absence of added maltodextrin in milk using iodine reagent.

8.2.5. Limits of detection:

Increase in the intensity of chocolate-red brown colour was observed with increasing concentration of maltodextrin in milk samples. Distinguishable chocolate brown colour was obtained when milk samples were adulterated with 0.3% (w/v) maltodextrin. Hence, the limit of detection for this method is 0.3% (w/v) of maltodextrin in milk (**Fig. 8.2**).

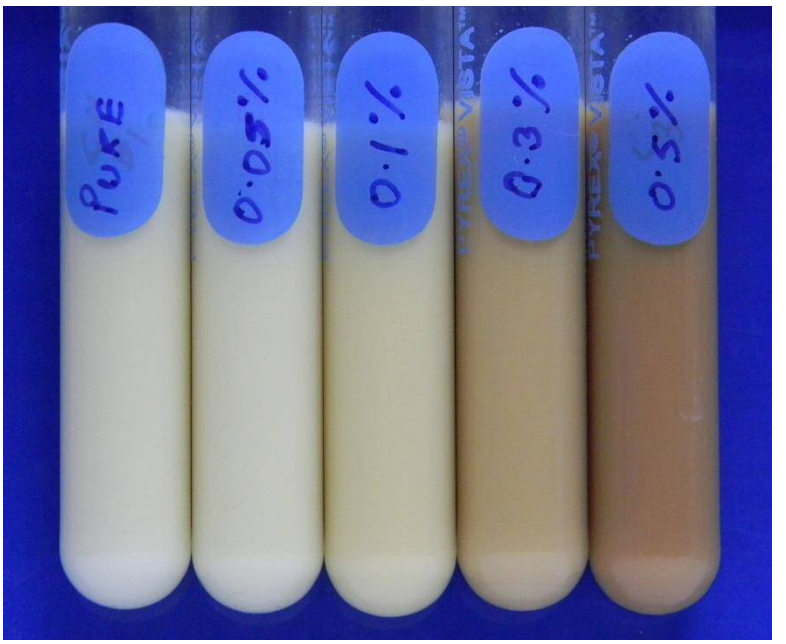

Fig. 8.2: Limit of detection of added maltodextrin in milk using iodine reagent.

8.3. Method II: Using Citric Acid and Iodine Reagents

In this test, milk is first coagulated with citric acid and clear filtrate is obtained which is then treated with iodine solution. The reaction is due to the formation of carbohydrate-iodine complex (brownish) in low pH condition.

8.3.1. Reagent(s) required:

i. Iodine

ii. Citric acid

8.3.2. Reagent(s) preparation:

i. Iodine solution (1%): Dissolve 2.5 g potassium iodide (KI) in 100 ml distilled water, add to it 1 g pure iodine crystals, shake well until the iodine crystals dissolve.

ii. Citric acid (10% solution): Dissolve 10 g citric acid in distilled water and make up the volume to 100 ml.

8.3.3. Methodology

1. Take 20 ml milk in a small beaker. Heat it to boil and cool up to 70°C.
2. Add 0.5 ml of citric acid solution (10%) drop wise, while swirling the contents slowly so as to coagulate the milk. Allow the contents to cool at room temperature.
3. Filter the coagulated milk through a filter paper Whatman no.1 filter paper. Alternatively, centrifuge the contents at 5000 g for 10 min and collect the supernatant.
4. Collect about 2 ml of the filtrate in the test tube.
5. Add 3 drops of iodine solution with the help of 10 ml pipette. Observe the colour.

8.3.4. Interpretation:

Appearance of orange - brown shade or any darker shade indicates presence of maltodextrin **(Fig. 8.3)**.

8.3.5. Limit of detection:

Increase in the intensity of brown shade was observed with increasing concentration of maltodextrin in milk samples. Distinguishable brown colour was obtained when milk samples were adulterated with 0.1% (w/v) maltodextrin. Hence, the limit of detection for this method is 0.1% (w/v) in milk **(Fig. 8.3)**.

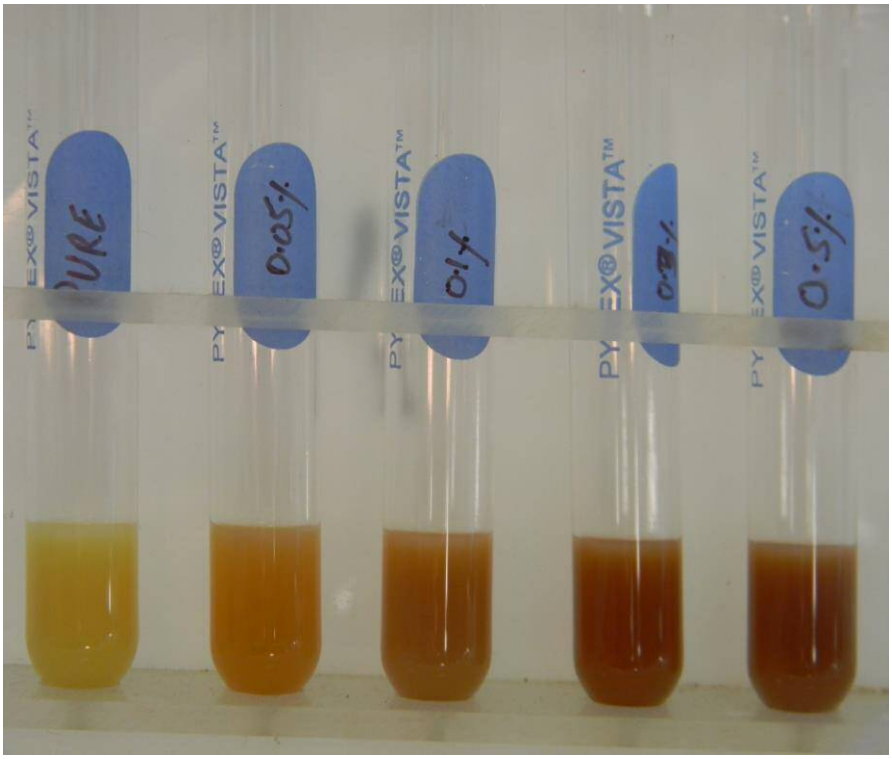

Fig. 8.3: Limit of detection of added maltodextrin in milk using citric acid and iodine reagents.

8.4. Method II: Quantitative estimation of maltodextrin in milk and milk products

The method involves the following steps:

- Estimation of native (or added) glucose content in milk by glucose-assay-kit.
- Hydrolysis of maltodextrin to glucose by amyloglucosidase at pH 4.5.
- Estimation of total glucose content after hydrolysis of maltodextrin in milk by glucose-assay-kit.

- Estimation of maltodextrin content in milk. The difference between glucose levels (before and after hydrolysis) measures glucose released from maltodextrin and is multiplied by 0.91 for obtaining maltodextrin levels.

8.4.1. Materials Required:

i. Maltodextrin – Hi-Media, India.
ii. Citrate buffer (500 mM, pH 4.5).
iii. Amyloglucosidase [α-(1,4)-D-glucanglucohydrolase, EC 3.2.1.3) (67 units/mg)] from Fluka Analytical, USA (product no. 10115 or any other equivalent type).
iv. Amyloglucosidase solution: 2.5 mg, 167 units prepared in 20 mM citrate buffer, pH 4.5.
v. Glucose-assay-kit from Span Diagnostic Ltd. India.
vi. Carrez I (500 mM potassium ferrocyanide) solution.
vii. Carrez II (500 mM zinc acetate) solution.
viii. NaOH: 0.1 N.

8.4.2. Methodology:

A. Preparation of standard curve of glucose and estimation of native (or added) glucose content in milk by glucose-assay-kit.

a) Preparation of standard curve of glucose

i. Take 10 µl of aqueous solution of glucose (2.5 µg to 40 µg per 10 µl) in 5 ml test tube. Add 1000 µl of glucose-assay-reagent containing glucose oxidase, peroxidase, phenol and aminoantipyrine. Incubate the content at 37°C for 30 min record the absorbance at 505 nm. Prepare the standard curve by plotting glucose concentration against absorbance at 505 nm. Glucose oxidase oxidizes glucose to gluconic acid with simultaneous release of hydrogen peroxide. Hydrogen peroxide, phenol and aminoantipyrine in presence of peroxidase form coloured quinoneimine dye. A

standard curve prepared in authors' laboratory is shown in **Fig 8.4**.

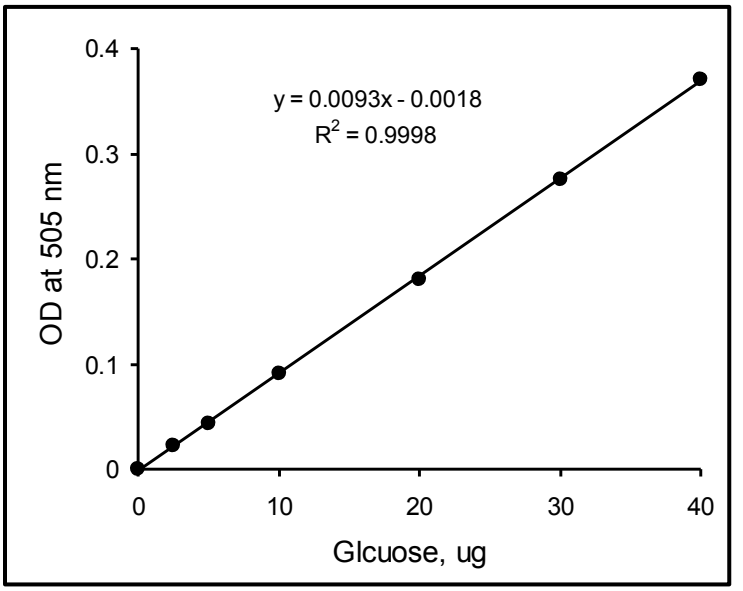

Fig. 8.4: Standard curve of glucose.

b) Estimation of native (or added) glucose content in milk by glucose-assay-kit

i. Take 2 ml milk sample in a 15 ml centrifuge tube and add 1 ml citrate buffer (500 mM, pH 4.5) and 0.5 ml amyloglucosidase (2.5 mg, 167 units prepared in 20 mM citrate buffer, pH 4.5). Mix the content by swirling and incubate the test tube at 60°C for 30 min.

ii. Bring the temperature of the test tube to room temperature by immersing in tap water. Then add 0.25 ml Carrez I (500 mM potassium ferrocyanide) and 0.25 ml Carrez II (500 mM zinc acetate) solutions. Mix the contents and add 0.5 ml 0.1 N NaOH. Mix the content and make up the final volume to 5 ml with distilled water. Centrifuge the tubes (8000 x g, 10 min, 10°C).

iii. Take 10 µl of supernatant and estimate glucose content as explained above (in Step A.a.i) by adding 1000 µl of glucose-assay-reagent.

Note: *The conditions for estimation of native glucose have been kept essentially same as that have been used for hydrolysis of maltodextrin.*

B. Hydrolysis of maltodextrin to glucose by amyloglucosidase at pH 4.5

a) Take 2 ml milk sample in a 15 ml centrifuge tube and add 1 ml citrate buffer (500 mM, pH 4.5) and 0.5 ml amyloglucosidase (2.5 mg, 167 units prepared in 20 mM citrate buffer, pH 4.5). Mix the content by swirling and incubate the test tube at 60°C for 30 min.

b) Bring the temperature of the test tube to room temperature by immersing in tap water. Then add 0.25 ml Carrez I (500 mM potassium ferrocyanide) and 0.25 ml Carrez II (500 mM zinc acetate) solutions. Mix the contents and add 0.5 ml 0.1 N NaOH. Mix the content and make up the final volume to 5 ml with distilled water. Centrifuge the tubes (8000 x g, 10 min, 10°C). Save the supernatant for further estimation.

C. Estimation of total glucose content after hydrolysis in milk by glucose-assay-kit

a) Take 10 µl of supernatant and estimate glucose content as explained above (in Step 1) by adding 1000 µl of glucose-assay-reagent.

b) Estimate the glucose content in the milk sample using standard curve of glucose prepared.

D. Estimation of maltodextrin content in milk

a) Subtract the glucose content in milk samples obtained before and after hydroloysis step. The difference between glucose levels (before and after hydrolysis) measures glucose released from maltodextrin. Calculate the maltodextrin content in milk by multiplying glucose concentration with 0.91.

Note: *The hydrolysis of maltodextrin (2% in milk) by amyloglucosidase at pH 4.5 (citrate buffer) at 60°C is usually accomplished within 30 min* ***(Fig. 8.4)****. In one of the experiment done at authors' laboratory, the progress of maltodextrin degradation by amyloglucosidase was evaluated at pH 4.5 in citrate buffer by incubating enzyme (167 units) and substrate (maltodextrin, 2%) for different length of time. Hydrolysis of maltodextrin was completed in less than 30 min* ***(Fig. 8.5)****. However each analyst should ensure complete hydrolysis of maltodextrin in his/her own laboratory.*

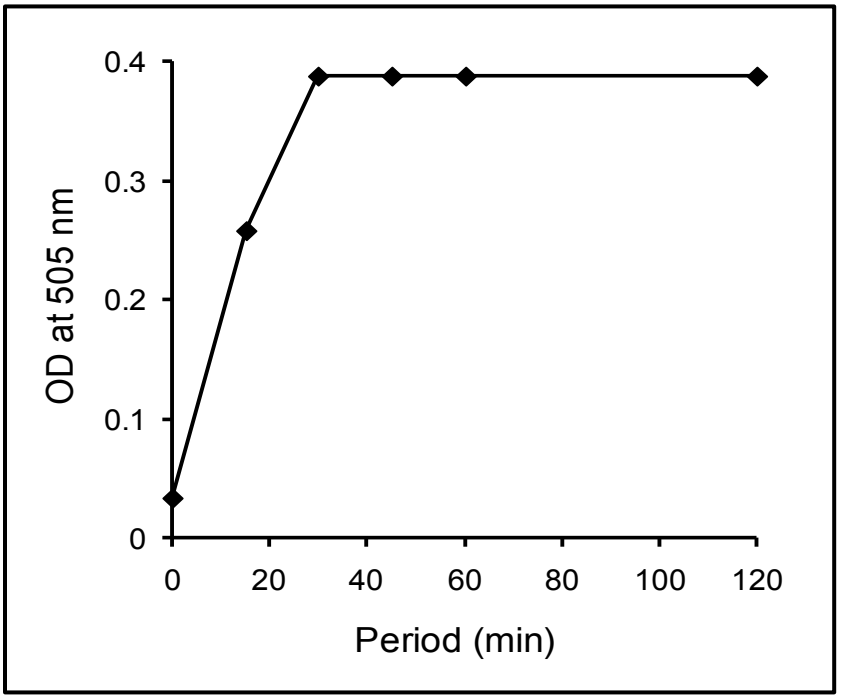

Fig. 8.5: Progress of maltodextrin hydrolysis. Milk spiked with 2% maltodextrin was treated with amyloglucosidase at 60°C for different period. Released glucose was assayed.

The performance of the method has also been evaluated in presence of other common adulterants and preservatives. The results (data not presented) indicated that presence of urea (0.3%), sucrose (2%), neutralizers (1%), formalin (0.4%), bronopol (0.05%) and sodium azide (0.2%) do not interfere in estimation of maltodextrin.

The method has also been validated by spiking milk samples with known concentrations of maltodextrin (0.5, 1 and 2%). Estimated values of maltodextrin in spiked samples and recovery calculations are shown in **Table 8.1**. At all the levels of added maltodextrin, the recovery of added maltodextrin was in the range of 91 to 93%. Unspiked milk contained 10.25 mg glucose/100 ml **(Table 8.1)**.

Table 8.1: Recovery of added maltodextrin in milk

Level of maltodextrin added in spiked sample (mg/100 ml)	Estimated glucose (mg/100 ml)		Recovery of spiked maltodextrin		
	Unspiked samples	Spiked samples	Difference in glucose concentration between spiked and unspiked samples	Calculated maltodextrin in samples (mg/100 ml)	In percent
(a)	(b)	(c)	$(d) = c - b$	$(e) = d \times 0.91$	$(f) = \frac{e}{a} \times 100$
500	10.25	512.39	502.14	456.95	91.4
1000	10.25	1025.60	1015.35	923.96	92.4
2000	10.25	2055.85	2045.60	1861.50	93.1

8.5. References:

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Blanchard, P.H. and Katz, F.R. (1995). Starch hydrolysates. In Food Polysaccharides and Their Applications (Stephen, A.M., Ed.) Marcel Dekker, New York, 99.

Brunt, K., Sanders, P. and Rozema, T. (1998). The enzymatic determination of starch in food, feed and raw materials of the starch industry. Starch, 50(10): 413 – 419.

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9. Detection of Neutralizers in Milk

9.1. Introduction:

The natural acidity of freshly drawn milk ranges between 0.12 - 0.16% (lactic acid) for cow as well as buffalo milk. It is mainly due to phosphate, casein, and to a less extent citrate and carbon dioxide. The true lactic acid level in freshly drawn milk is about 2 mg % (range 0.4 to 2.8 mg %). As the milk ages, the milk bacteria proliferate and produce lactic acid. Highly acidic milk is not suitable and thus not acceptable for manufacturing processes. It may result into final products of inferior quality. In order to prevent the rejection of poor quality milk, the producer or the middlemen attempt to add neutralizers in the form of NaOH, $NaHCO_3$, Na_2CO_3 and $Ca(OH)_2$ etc. But such a practice of adding neutralizers to milk is not permissible under FSSAI Rules, (2006).

Rosalic acid test, alkalinity of ash and pH determination are the methods recommended by BIS (1981), for the detection of neutralizers in milk. However, these methods have their limitations. Rosalic acid is an indicator which shows a change in the colour on addition to alkaline milk. This test will work only when neutralizers are added in excess quantities and milk is alkaline in nature. If the sour milk is under-neutralized below the normal pH of the milk, then this test will fail to detect added neutralizers. The other test for the detection of neutralizers in milk i.e. test for alkalinity of ash is based on the fact that the neutralization of milk invariably increases the ash content and also the total alkalinity of the ash from a fixed quantity of milk. However, because of the natural variations in the alkalinity of the ash and also their differences in cow and buffalo milks, this test also cannot be used reliably to detect and determine the

neutralizers when added in low concentrations (Sharma and Narayanan, 1975).

The pH of the normal milk is generally below 7.0 (average-cow 6.6; buffalo 6.8). The milk with higher pH should be suspected of either being abnormal milk such as mastitic or neutralized one. Similarly determination of titratable acidity can also be employed for the detection of neutralizers in milk. However, both these tests will enable the detection of only an over-neutralized milk.

Among the various methods available today, the determination of true lactic acid/lactate content coupled with the titratable acidity measurement seems to be the most reliable approach to detect the added neutralizers, as the addition of neutralizers to milk will reduce the titratable acidity but does not affect the lactic acid/lactates content. Therefore, a disturbed relationship between the lactic acid/lactates content and the titratable acidity can be regarded as an indication of the presence of neutralizers (IDF, 1989). Neutralizers can also be detected by estimating the sodium ions in suspected milk sample. The normal range of sodium ions in the raw fresh milk is between 350 to 600 ppm; if the content is higher than the normal, it shows added sodium.

The details of the above methods commonly employed for the detection of neutralizers in milk are given as follows:

9.2. Method I: Rosalic Acid Test

9.2.1. Reagent(s) required:

i. Rosalic acid
ii. Ethyl alcohol

9.2.2. Reagent(s) preparation:

i. Rosalic acid solution (0.1%, w/v): Weigh 100 mg of rosalic acid powder and dissolve it in the 30 ml ethyl alcohol and

make up the volume with distilled water to obtain final volume of 100 ml.

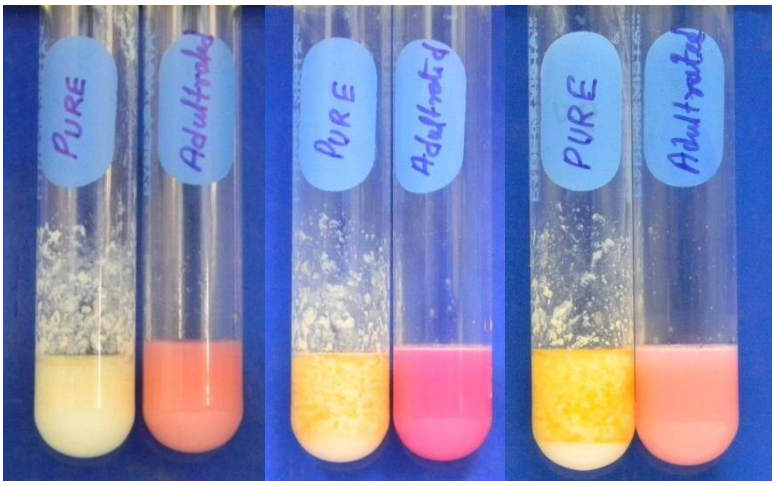
ii. Ethyl alcohol (95%): Take 95 ml of ethyl alcohol in a 100 ml volumetric flask and make the volume up to the mark with distilled water and mix well.

9.2.3. Methodology:

A. Take about 5 ml milk in a test tube.
B. Add 5 ml ethanol and mix well.
C. Add of 2-3 drops of Rosalic acid solution.
D. Note the developed colour.

9.2.4. Interpretation:

Formation of rose red colour indicates the presence of neutralizers in milk samples. Whereas pure milk samples produces brownish or brownish yellow colour only **(Fig. 9.1)**.



NaOH Na_2CO_3 $NaHCO_3$

Fig. 9.1: Comparison of developed colour in presence and absence of neutralizer in milk.

9.2.5. Limits of detection:

The colour characteristic varies in milk samples adulterated with different types of neutralizers' viz., NaOH, Na_2CO_3, $NaHCO_3$ etc. Accordingly, limit of detection is

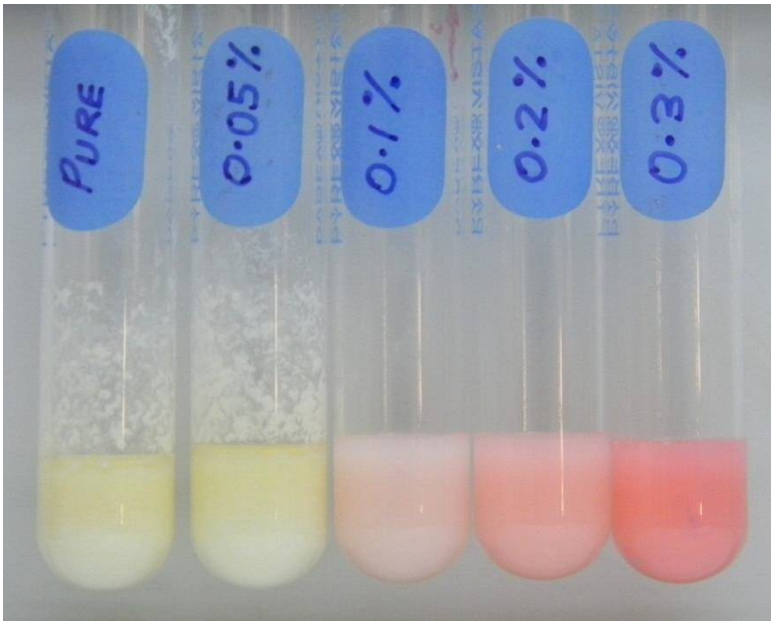


Fig. 9.2: Limit of detection of neutralizer (NaOH) in milk.

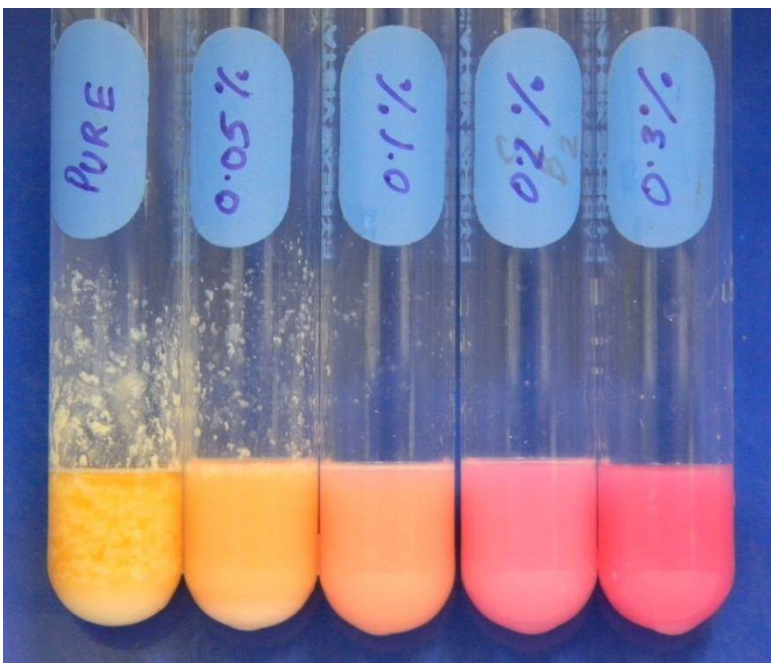


Fig. 9.3: Limit of detection of neutralizer (Na_2CO_3) in milk.

different for different types of neutralizers. The limit of detection of NaOH, Na_2CO_3, $NaHCO_3$ in milk is 0.1, 0.1 and 0.2% (w/v) (**Fig. 9.2, 9.3** and **9.4**), respectively. In

these experiments done in authors' laboratory, fresh milk samples were used for ascertaining the limit of detection of various neutralizers. The limit of detection would be

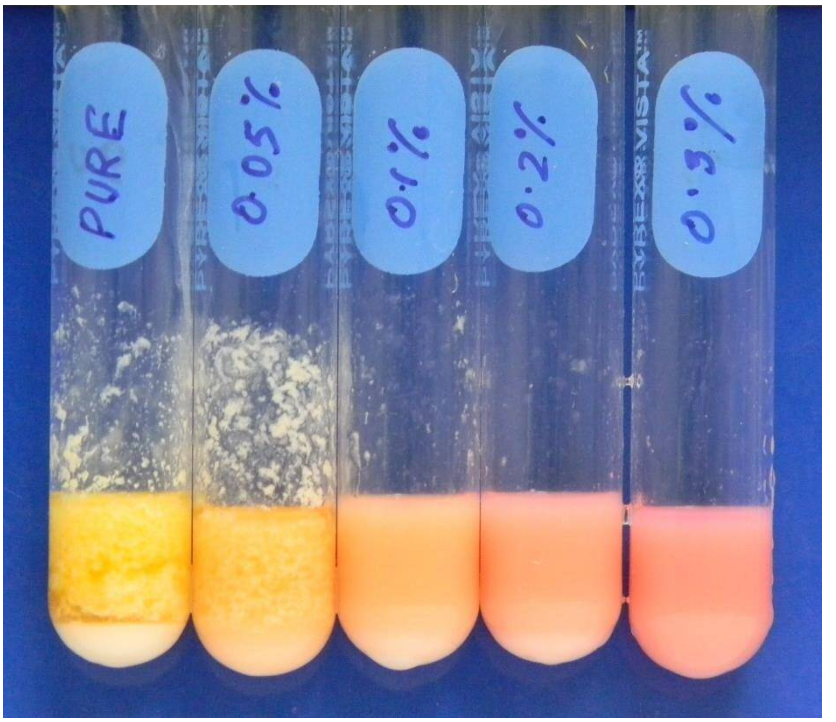


Fig. 9.4: Limit of detection of neutralizer ($NaHCO_3$) in milk.

different in stored or milk with developed acidity.

Note: *Rosalic acid is an organic dye, which is used as an indicator-changing colour at pH 7.0 to 8.0. Hence, milk made even faintly alkaline by addition of neutralizers can be detected due to formation of rose red colour with rosalic acid solution.*

9.3. Method II: Alkalinity of Ash Test

9.3.1. Reagent(s) required:

i. Concentrated Hydrochloric acid (HCl)
ii. Phenolphthalein

9.3.2. Reagent(s) preparation:

i. 0.1 N HCl
ii. Phenolphthalein [1% (w/v) in ethanol (95%, v/v)]: Weigh 1 g phenolphthalein in 200 ml beaker and add 40 ml

ethanol (95%, v/v) and finally make up the final volume to 100 ml.

9.3.3. Methodology:

A. Take 20 ml of milk sample into a silica dish and evaporate to dryness over boiling water bath.
B. Prepare the ash in Muffle Furnace at 550 ± 50^{0}C. This may take 4-6 hour.
C. Let the residue cool to room temperature.
D. Add to the residue 10 ml of water and disperse the ash in water by stirring with a glass rod.
E. Titrate the ash dispersate by standard HCl using phenolphthalein indicator.

9.3.4. Interpretation:

If the volume of 0.1N HCl required to neutralize the ash dispersate exceeds 1.20 ml; then the milk is suspected to contain neutralizers.

9.4. Method III: Determination of true lactic acid/lactate content in milk:

9.4.1. Principle:

The true lactic acid/lactate content in milk and milk products can be determined by the method described by IS:11202-1984. In this procedure, the interfering substances like fat, protein and lactose are simultaneously removed by the addition of copper sulfate and calcium hydroxide, followed by filtration. The lactic acid in the filtrate is then oxidized to acetaldehyde with sulphuric acid in the presence of copper sulfate. The acetaldehyde develops a purple colour with *p*-hydroxydiphenyl and its intensity can be measured at 570 nm (yellow-green filter) in a colorimeter. Amount of lactic acid/lactate in the sample can be calculated from the standard curve prepared under similar conditions.

9.4.2. Precautions:

Throughout the experiment, avoid contamination of the sample with impurities, especially with saliva and perspiration. Care should be taken not to touch the tips of the pipettes.

9.4.3. Apparatus:

For the successful operation of this test method, it is essential that all glassware to be perfectly clean. Use only glassware that has been reserved exclusively for this test method.

i. Spectrophotometer: For reading at 570 nm.
ii. Water bath at 30 ± 2^0C.
iii. Boiling water bath.

9.4.4. Reagents:

All reagents shall be of analytical reagent quality. The water used shall be distilled in glass apparatus or to be at least equivalent purity, free from organic matter.

i. Copper (II) sulfate solution: Dissolve 250 g of copper(II) sulfate ($CuSO_4.5H_2O$) in water and dilute to 1000 ml in volumetric flask.
ii. Calcium hydroxide suspension: Grind 300 g of calcium hydroxide [$Ca(OH)_2$] in a mortar with a total of 900 ml of water. Keep the suspension in a tightly stoppered bottle.
iii. Copper (II) sulfate-sulphuric acid solution: Add 0.5 ml of copper (II) sulfate solution (Reagent i) to 300 ml of sulphuric acid (95.5 to 97.0% (m/m) H_2SO_4).
iv. p-Hydroxydiphenyl ($C_6H_5C_6H_4OH$) solution: Dissolve by shaking and by heating slightly, 0.75 g of p-hydroxydiphenyl in 5 ml of an aqueous 50 g/L solution of sodium hydroxide and dilute to 50 ml with water in a volumetric flask. Keep the solution in a brown glass bottle in a dark and cool place. Do not use this solution if its colour changes or if turbidity occurs.

v. Lithium lactate ($CH_3CHOHCOOLi$) standard solution corresponding to 0.1 mg of lactic acid per ml. Dissolve, shortly before use, 0.1067 g of lithium lactate in water and dilute to 1000 ml in a volumetric flask.

9.4.5. Procedure:

A. Pipette 5 ml of milk sample into a 50 ml volumetric flask and dilute to 35 ml. For blank, directly take 35 ml of distilled water in another 50 ml volumetric flask. Treat both these flasks as described below:

B. Add slowly, while swirling, 5 ml of the copper (II) sulfate solution and allow to stand for 10 min.

C. Add slowly, while swirling, 5 ml of the calcium hydroxide suspension and allow to stand for 10 min.

D. Dilute to the mark with water, shake vigorously, allow to stand for 10 min and filter through Whatman No.1 filter paper. Discard the first running.

E. Using a pipette, introduce 1 ml of the filtrate into the bottom of a test tube.

F. Add to the test tube, by means of an automatic pipette or other suitable device 6.0 ml of the sulphuric acid-copper (II) sulfate solution. Mix it thoroughly.

G. Heat the test tubes in boiling water bath for 5 min. Cool to ambient temperature in running water.

H. Add 2 drops of the p-hydroxydiphenyl solution and shake vigorously to spread the reagent uniformly throughout the liquid.

I. Place the test tubes in the water bath at 30 ± 2^0C. Keep it for 15 min, shaking from time to time.

J. Place the test tubes in the boiling water bath for 90 sec. Cool to ambient temperature in running water.

K. Measure the absorbance against the blank within 3 h, using the spectrophotometer at wavelength of 570 nm.

9.4.6. Preparation of standard curve:

A. Pipette 5 ml of the freshly drawn milk having traces of lactic acid content (alternatively 5 ml of reconstituted milk having the lowest lactic acid content – at most 30 mg of lactic acid per 100 gm of SNF) into each of the six 50 ml volumetric flasks.

B. Pipette into these flasks 0, 1, 2, 3, 4 and 5 ml respectively of the standard lithium lactate solution.

C. Dilute the contents of each of these 50 ml flask to about 35 ml and proceed with each as done in case of sample.

D. Plot the measured absorbance against the masses of lactic acid.

E. Draw the best fitting straight line through the points and prepare the calibration curve by drawing a parallel line passing through the point of origin. You will get a standard curve corresponding to 0, 2, 4, 6, 8 and 10 μg per ml of filtrate containing lactic acid.

9.4.7. Calculation:

Read from the graph the concentration of lactic acid corresponding to the absorbance (O.D.) of the sample.

Say, the O.D. for sample be X and the corresponding concentration from graph = Y μg of lactic acid.

$$\therefore \text{ 1 ml of filtrate of sample has } = Y \text{ μg}$$

$$\text{50 ml of filtrate of sample has } = Y \times 50 \text{ μg}$$

$$\text{i.e. 5 ml of milk sample has } = Y \times 50 \text{ μg}$$

$$\text{100 ml of milk sample has } = \frac{Y \times 50}{5} \times 100 \text{ μg}$$

$$= \frac{Y \times 50 \times 100}{5 \times 1000} \text{ mg of lactic acid}$$

$$= Y \text{ mg of lactic acid per 100 ml of milk}$$

9.4.8. Interpretation:

If the acidity of milk is either within the normal range or below normal, and the lactic acid content is more

than 30 mg per 100 ml of milk, the sample may be suspected as neutralized one.

Note: *Since sodium and potassium salts are used as neutralizers e.g. NaOH, Na_2CO_3, $NaHCO_3$, their presence can also be detected by estimating the Na, K content in milk and milk products using flame photometer. Flame analysis is based on the fact that when a metallic salt solution is drawn into a non-luminous flame, it emits light of characteristic wave length. This emitted light, isolated to the characteristic wave band by an optical filter, is allowed to fall on a photocell whose output is measured by a suitable deflection for instance electronic amplifier and a meter or a galvanometer. Normal/Natural concentration of Na ranges from 45-55 mg/100 ml in buffalo milk and 50-60 mg/100 ml in cow milk. For K the range is 100-120 mg/100 ml in buffalo milk and 140-150 mg/100 ml in cow milk. Therefore, if the concentration of these metals is above this range then we presume that neutralizer has been added. Another technique to measure the concentration of such ions in milk is by using ion-selective electrode.*

9.5. References:

Arora, K.L. and Balachandran, R (1995). Tests for neutralizers in milk. Indian Dairyman, 47(1): 47.

IS:1479. (1960). Methods of test for dairy industry. Part I. Rapid examination of milk. Bureau of Indian Standards, New Delhi.

IDF:102A, (1989). Dried Milk-Guideline for the detection of neutralizers. Int. Dairy Fed.

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Lal, D., Sharma, B.B. and Narayanan, K.M. (1983). Determination of lactic acid/lactate contents in cow and buffalo milk to detect the added neutralizers. Indian J. Anim. Sci., 53:761-763.

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10. Detection of Nitrates (Pond Water) in Milk

10.1. Introduction:

Pond water is heavier than the tap water; some unscrupulous persons for adulteration in milk usually prefer it. However, it can be easily detected by the following method. This method actually detects nitrates present in the pond water. In the pond water nitrates may come from fertilizers used in the fields.

10.2. Method: Diphenylamine Test

10.2.1. Reagent(s) required:

i. Diphenylamine
ii. Concentrated sulphuric acid

10.2.2. Reagent(s) preparation:

i. Diphenylamine (2%, w/v, in sulphuric acid): Weigh 2 g of diphenylamine and dissolve it in sulphuric acid to obtain final volume of 100 ml.

10.2.3. Methodology:

A. Take 2 ml of milk in a test tube. Rinse the tube with the milk and drain the milk from the test tube.
B. Add two-three drops of the reagent along the side of the test tube.
C. Note the developed colour.

Fig. 10.1: Comparison of developed colour in presence and absence of nitrate in milk.

10.2.4. Interpretation:

Deep blue colour will be formed in presence of nitrate in the milk sample. Pure milk sample will not develop any colour **(Fig. 10.1)**.

10.2.5. Limits of detection:

Samples adulterated with nitrates shown to have development of blue colour on the walls of the tubes as well at bottom portion. The intensity of blue colour increases with increasing concentration of nitrates in milk. Milk samples containing 0.02% (w/v) KNO_3 shown to

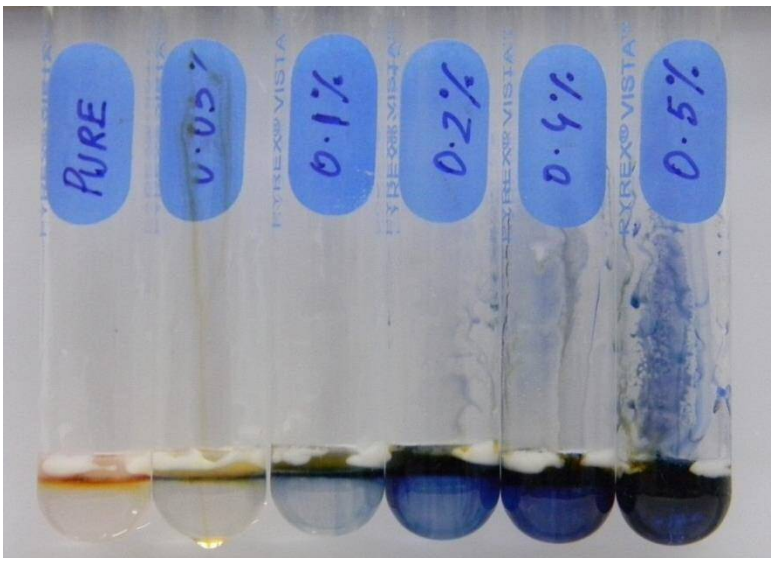

Fig. 10.2: Limit of detection of nitrate in milk.

have development of blue colour in the tube wall whereas when the sample is adulterated with 0.1% (w/v) KNO_3 shown to have development of blue colour at the bottom of the tube. As the method reagents contain concentrated H_2SO_4 the blue colour changes to dark brown/black in 1 minute which is then very hard to differentiate from that of pure or control milk as it also developed dark brown/black colour after reacting with H_2SO_4. Therefore the limit of detection for this is 0.2% of KNO_3 (**Fig. 10.2**).

10.3. Reference:

Roy, N.K. and Sen, D.C. (1991). Rapid analysis of milk. In: Textbook of Practical Dairy Chemistry. Vol. I. Chemical analysis of fluid milk. Kalyani Publishers, New Delhi, India.

11. Detection of Ammonium Salt in Milk

11.1. Introduction:

The added ammonium salts e. g. ammonium chloride, ammonium sulfate, ammonium nitrate and ammonium di-hydrogen orthophosphate can be detected in milk by two methods *i.e.* Nessler's reagent method and turmeric paper method. These salts are being added to milk to raise its SNF content fraudulently.

11.2. Method I: Nessler's Reagent Method

11.2.1. Reagent(s) required:

i. Mercuric chloride
ii. Sodium hydroxide
iii. Potassium iodide

11.2.2. Reagent(s) preparation:

i. Nessler's reagent: Dissolve the following chemicals separately.
   a. 8.0 g of mercuric chloride in 150 ml distilled water.
   b. 60.0 g of sodium hydroxide in 150 ml distilled water.
   c. 16.0 g of potassium iodide in 150 ml distilled water.
ii. Add reagent 'a' to reagent 'b' and mix well. To this mixture, add reagent 'c', mix and dilute the contents to 500 ml. Leave this solution undisturbed and decant the clear upper layer of the solution and store in a stoppered glass bottle.

11.2.3. Methodology:

A. Pipette 5 ml of suspected milk sample into a test tube.
B. Add 1 ml of Nessler's reagent.
C. Mix the contents of the tube thoroughly.
D. Observe and note the colour.

11.2.4. Interpretation:

Appearance of yellowish or grey colour confirms the presence of added ammonium salts in milk **(Fig. 11.1)**.

Fig. 11.1: Comparison of developed colour in presence and absence of ammonium salts in milk.

11.2.5. Limits of detection:

The control milk sample gives slight grayish colour. At low concentration of ammonium compound (using ammonium sulfate), brownish shade appears which is distinguishable at 0.15% followed by yellowish colour and then orange colour development at higher concentration (**Fig. 11.2**). Therefore the limit of detection for this method is 0.15% (w/v).

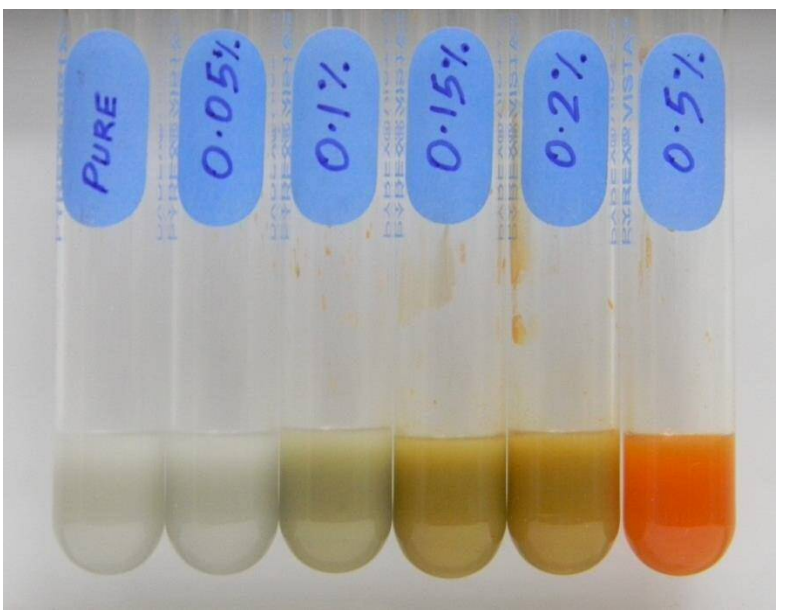

Fig. 11.2: Limit of detection of ammonium salt (ammonium sulfate) in milk.

11.3. Method II: Turmeric Paper Method

11.3.1. Reagent(s) required:

i. Turmeric powder
ii. Sodium hydroxide

11.3.2. Reagent(s) preparation:

i. Turmeric paper: Dissolve 10 g of pure turmeric powder in 100 ml distilled water and dip Whatman filter paper Grade 1 strips into it for 2 minute. Dry the paper at room temperature. The dried filter paper is wetted with distilled water before use.
ii. Sodium hydroxide solution (10%, w/v, aqueous): Weigh 10 g sodium hydroxide and dissolve in distilled water to obtain 100 ml solution.

11.3.3. Methodology:

A. Take 5 ml of milk sample in a test tube
B. Add 1 ml of 10% sodium hydroxide solution in such a manner that should not touch the rim of the test tube while adding.
C. Mix the contents of the tube.
D. Place a piece of wet turmeric paper on the rim of the test tube and keep the test tube undisturbed.
E. Observe for the change in the colour of the turmeric paper and note it.

11.3.4. Interpretation:

Appearance of pinkish red colour confirms the presence of ammonium salt in milk.

11.4. Reference:

Guleria, V. (1998). Detection of added ammonium salts in milk with and without the addition of formalin. M. Sc. Thesis. NDRI, Karnal, India.

12. Detection of Glucose in Milk

12.1. Introduction:

Glucose being a reducing sugar poses many problems in its detection as lactose, major carbohydrate in milk, is also a reducing sugar. Moreover, it is easily available in commercial form as concentrated syrup. These days adulteration of milk with glucose or such syrups is increasing. Now it has become possible to detect glucose in milk by the following method:

12.2. Method: Modified Barfoed's Method

12.2.1. Reagent(s) required:

i. Cupric acetate
ii. Lactic acid
iii. Ammonium molybdate
iv. Sodium tungstate
v. Sodium hydroxide
vi. Phosphoric acid

12.2.2. Reagent(s) preparation:

i. Barfoed's reagent: Dissolve 24 g cupric acetate in 450 ml boiling water and immediately add 25 ml of 8.5% lactic acid to the hot solution. Shake to dissolve almost all precipitate, cool and dilute with water to 500 ml. If necessary decant of filter to get a clear solution.
ii. Phosphomolybdic acid reagent: Take 35 g ammonium molybdate and 5 g sodium tungstate in a large beaker; add 200 ml of 10% NaOH solution and 200 ml water. Boil vigorously (20-60 min) so as to remove nearly whole of ammonia. Check removal of ammonia with the help of red litmus paper. Cool, dilute with water to about 350 ml. Add 125 ml concentrated H_3PO_4 (85%) and dilute further to 500 ml.

12.2.3. Methodology:

A. Take 1 ml of milk sample in a test tube.
B. Add 1 ml of modified Barfoed's reagent.
C. Heat the mixture for exact 3 min in a boiling water bath
D. Rapidly cool under tap water.
E. Add 1 ml of phosphomolybdic acid reagent to the turbid solution
F. Observe the colour.

12.2.4. Interpretation:

Immediate formation of deep blue colour after adding phosphomolybdic acid reagent indicates the presence of added glucose in the milk sample. In case of pure milk only faint bluish colour can be observed due to the dilution of Barfoed's reagent. The results are shown in the **Fig.12.1**.

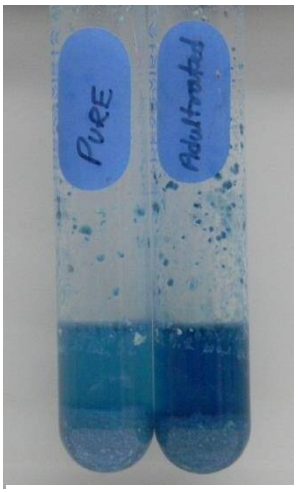

Fig. 12.1: Comparison of developed colour in presence and absence of added glucose in milk.

12.2.5. Limits of detection:

Presence of glucose in the milk samples can be detected after observing the more

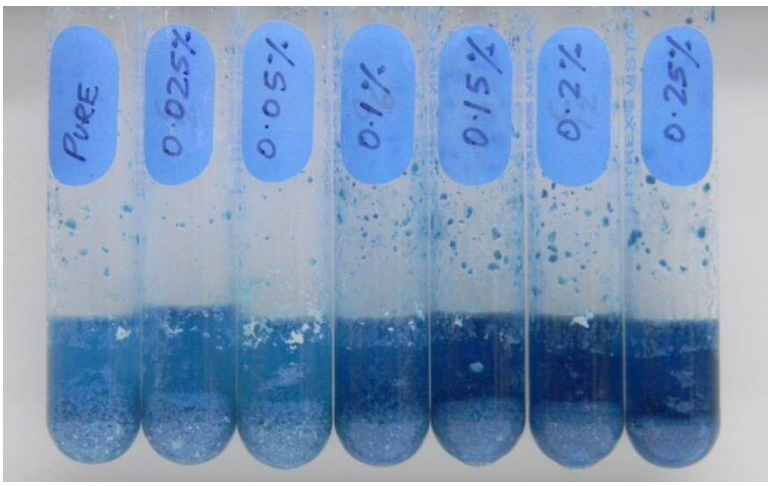

Fig. 12.2: Limit of detection of added glucose in milk.

intense blue colour in the adulterated milk samples compared to the control or pure milk samples. The intensity of the blue colour in 0.1% (w/v) glucose adulterated milk samples increases to such an extent that it can be clearly differentiated from pure milk samples. Therefore, the limit of detection for this method of glucose detection is 0.1% (w/v) glucose adulteration in milk **(Fig. 12.2)**.

12.3. Reference:

Roy, N.K. and Sen, D.C. (1991) Rapid analysis of milk. In: Textbook of Practical Dairy Chemistry. Vol. I-Chemical analysis of fluid milk. Kalyani Publishers, New Delhi, India.

13. Detection of Detergent in Milk

13.1. Introduction:

Chemical quality of milk suffers due to intentional adulteration of milk with various types of adulterants. Synthetic milk has been found to be the youngest entry among the list of adulterants of milk. Detergents are considered as the essential component of the formulation being used for the preparation of synthetic milk. Because of ease in availability of anionic detergent, these are being used for emulsification of added fat of non-milk origin. The other ingredients being used for synthetic milk formulation are urea, salt, soda, sucrose, vegetable oils, skim milk powder, water etc. The liquid thus formed has the appearance of genuine milk (i.e. colour, consistency) and it is reported to be used for the adulteration of dairy milk from 5 to 10%. The detection of detergent in milk is therefore essential for checking the adulteration of milk with synthetic milk.

A new qualitative test for detection of anionic detergent in milk has been developed at the authors' laboratory. The method is simple and fast. This method is based on the ionic interaction between the anionic detergent and cationic dye. Anionic detergents have a property to form a complex with cationic dyes. The solubility of dye and dye-detergent complex differs significantly as dye-detergent complex is relatively less polar in comparison to dye alone. Formation of dye-detergent complex between cationic dye and anionic detergents and subsequently its extraction into the hydrophobic solvent layer (lower) is the principle behind the method. The method is performed by addition of methylene blue dye solution and chloroform to milk, mixing of the content followed by centrifugation. This results in distribution of dye colour in upper layer and lower layers. Relative

intensity of the colour is noticed in these layers. Appearance of relatively more blue colour in lower layer indicates the presence of detergent in milk. (Rajput, Y.S.; Sharma, R. and Kaur, S. (2006) A kit for detection of detergent in milk. Indian Patent office file No. 1970/Del/2006, Indian Patent No. 264661).

The developed test is sensitive to detect detergent up to 0.0125% (12.5 mg/100 ml). This implies that addition of 2 to 3 liters of synthetic milk to 100 liters of normal milk can be easily detected. The developed method is colour based and colour differentiation between pure milk and milk adulterated with "synthetic milk" can be easily made. The method does not require expensive reagents and the result can be available in 5 to 10 min. The test can be used as platform test at Dairy Plant or milk collection centers.

13.2. Method: Methylene blue test

13.2.1. Reagent(s) required:

i. Methylene blue dye

ii. Chloroform

13.2.2. Reagent(s) preparation:

i. Methylene blue dye - 12.5 mg is dissolved in 100 ml of distilled water. Protect the solution against direct sunlight.

ii. Chloroform (Inflammable and toxic on inhalation. Mouth pipetting is not recommended).

13.2.3. Methodology:

A. Pipette 1 ml of suspected milk sample into a 15 ml test tube.

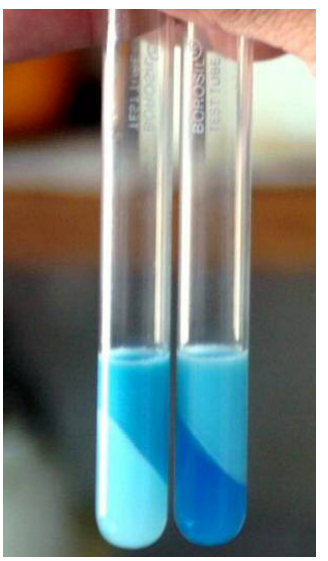

Fig. 13.1: Comparison of developed colour in presence and absence of detergent in milk. Relatively more blue colour in lower layer (right side tube) than upper layer indicates presence of detergent in milk.

B. Add 1 ml of dye solution followed by addition of 2 ml chloroform.
C. Vortex the contents for about 15 sec and centrifuge at about 1100 rpm for 3 min.
D. Note the intensity of blue colour in lower and upper layer.

13.2.4. Interpretation:

Relatively, more intense blue colour in lower layer indicates presence of detergent in milk. Relatively more intense blue colour in upper layer indicates absence of detergent in milk **(Fig. 13.1)**.

13.2.5. Limits of detection:

The method can detect presence of 12.5 mg of laboratory grade detergent (labolene) in 100 ml of milk sample. Thus limit of detection of method is 0.0125% labolene in milk.

Note: *The method is capable of detecting all the commonly available detergents in different brand names such as Ezee, Safe Wash, Super Nirma, Rin Advanced, Rin Shakti, Rin (Powder), Tide (Powder), Nip (Powder), Clinic Plus, Sunsilk, Pentene, Head & Shoulder etc. The efficacy of the method is not affected in presence of other additives viz., urea, sucrose, glucose, starch, formalin, hydrogen peroxide and neutralizers etc. In presence of high concentration of neutralizers, lower layer may appear pinkish. It has been observed that defatted milk provides better clarity in results. Defatted milk can be prepared by centrifuging milk at 15°C for 10 minute followed by removal of fat accumulated at top surface of milk present in centrifuge tube. In the literature, bromocresole purple test has been mentioned. However, the test has not worked in authors' laboratory even in the presence of 1% detergent in milk.*

13.3. Reference:

Rajput, Y.S.; Sharma, R. and Kaur, S. (2006) A kit for detection of detergent in milk. Indian Patent No. 264661 (Patent Grant Date: 14.01.2015).

14. Detection of Common Salt in Milk

14.1. Introduction:

Sodium chloride may be added to milk to increase the lactometer reading of milk. i.e. to increase the SNF content of milk. This can be detected by the use of silver nitrate and potassium chromate.

14.2. Method: Silver Nitrate Test

14.2.1. Reagent(s) required:

i. Silver nitrate

ii. Potassium dichromate

14.2.2. Reagent(s) preparation:

i. Silver nitrate ($AgNO_3$) solution: 0.1 N, aqueous.

ii. Potassium chromate (K_2CrO_4) solution: 10% (w/v) aqueous.

14.2.3. Methodology:

A. Pipette 5 ml of suspected milk sample into a test tube.

B. Add 1 ml of 0.1 N silver nitrate solution. Mix the content thoroughly and add 0.5 ml of 10% potassium chromate solution.

C. Observe the colour of the test tubes.

14.2.4. Interpretation:

Appearance of yellow colour indicates the presence of added salts, whereas, brick red colour indicates the milk free from added salt **(Fig. 14.1)**.

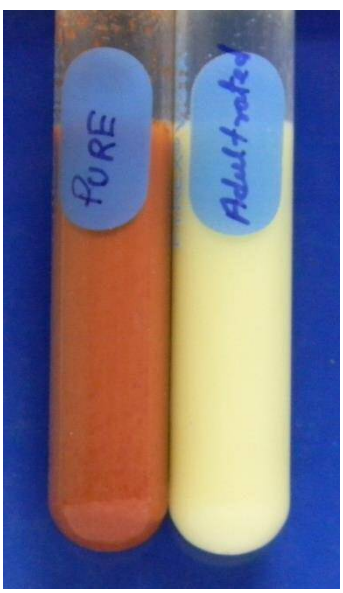

Fig. 14.1: Comparison of developed colour in presence and absence of common salt in milk

14.2.5. Limits of detection:

The method can detect presence of 0.02% (w/v) of NaCl in milk. The intensity of developed red-brown colour

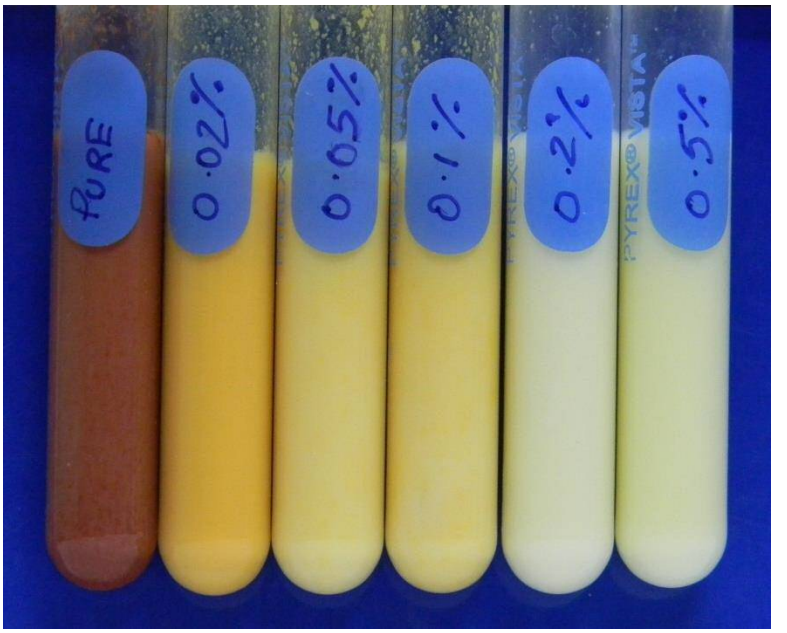

Fig. 14.2: Limit of detection of added NaCl in milk.

in pure milk samples decreases with increase in concentration of NaCl in milk samples. The limit of detection for this method is 0.02% (w/v) in milk as at this level of adulteration of milk the developed colour no longer remains red- brown but turns light yellow **(Fig. 14.2)**.

14.3. Reference:

Sharma, R; Seth, R. and Barui A.K. (2011). Rapid methods for the detection of Adulterants in milk. In: Chemical analysis of value added dairy products and their quality assurance. Compendium of lectures, Winter School. Dairy Chemistry Division, NDRI, Karnal, India. pp 184-188.

15. Detection of Vegetable Oil/Refined Oil in Milk

15.1. Introduction:

In Indian context, among the various milk constituents, milk fat is the costliest. Often, unscrupulous traders, remove the milk fat from milk and admix milk with vegetable oil/refined oil with help of detergent or by adding other emulsifiers. In the division of dairy chemistry at NDRI, Karnal, a rapid test has been developed to detect such adulteration (Arora *et al.*, 1996; Lal *et al.*, 1998). In this test, milk fat is isolated from given milk sample and is subject to butyro-refractometer reading (B.R.). Since, most of vegetable fat/oils have higher B.R. compared to milk fat, any increase in B.R. reading above reference value of milk fat indicate adulteration of milk with vegetable/refined oil. For isolation of milk fat from milk, above authors have suggested an innovative method wherein normal Gerber butyrometer **(Fig. 15.1)** has been modified **(Fig. 15.2)**. In the specially designed milk butyrometer have both end open. Stem side opening of the butyrometer (which is generally closed) is closed with a good quality removable silicon stopper. After the milk fat test, silicon stopper is removed and milk fat is removed with the help of a syringe and same is subjected to B.R. at 40°C. Since Gerber sulfuric acid causes some hydrolysis of fatty

Fig. 15.1: Normal Gerber butyrometer

acids/triglycerides, the B.R is multiplied by a factor to obtain corrected B.R. The fat in suspected milk sample can also be isolated by solvent extraction method in which case correction of B.R. is not required.

15.2. Method: Using Butyro-Refractometer

15.2.1. Reagent(s) required:

i. Gerber sulfuric acid (90% sulfuric acid)

ii. Amyl alcohol

15.2.2. Methodology:

A. Isolate the fat from milk by Gerber method using specially designed milk butyrometer, which is open at both ends. Close the stem side opening with a good quality acid resistant silicon stopper.

B. Add 10 ml of Gerber sulfuric acid, 10.75 ml milk and 1 ml iso-amyl alcohol. Close the neck side with lock stopper; mix the content and centrifuge at 1200 rpm, 5 min to get a clear fat column. Remove the silicon stopper from the stem side and take out the fat from the stem of the butyrometer using a capillary or a syringe.

C. For taking B.R. reading of the milk fat, clean the prism of the Butyro-Refractometer

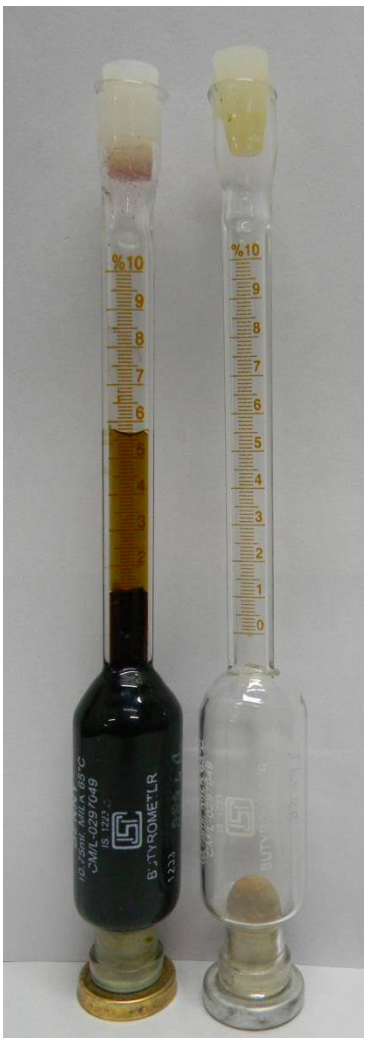

Fig. 15.2: Modified Gerber butyrometer

with diethyl ether. Allow the ether to evaporate to dryness. Maintain the temperature of the prism at 40°C by circulating water using a thermostatically controlled water-bath. Calibrate the Butyro-Refractometer by applying standard liquid solution of known B.R. reading. Again clean the prism with diethyl ether; apply 1-2 drops of clear, extracted fat between the prism. Wait for 2 min before taking the reading so that sample should attain temperature of 40°C. A correction of 0.55 is added to the observed B.R. reading for each degree above 40°C or subtracted for each degree below 40°C to get corrected B.R. reading of the sample.

D. Calculate the B.R. reading of isolated fat as follows:
Corrected B.R. = Observed B.R. + (0.08 x Observed B.R.)

15.2.3. ***Interpretation:***

B.R reading of pure milk fat isolated by any of the above method falls in the range of 40-43. Any deviation from the standard value indicates adulteration of milk with vegetable oil. However as per IS:1479 (1960), the milk sample may be considered adulterated with vegetable oil/refined oil if isolated fat has B.R. more than 45.

15.2.4. Limits of detection:

The method is capable of detecting 10 – 20% level of vegetable oil/refined oil (% of the original milk fat) in milk.

Note: *Fat column should be clear and transparent. If blackening occurs due to charring, repeat the experiment to get clear fat, as B.R. reading is affected due to blackening of the fat column.*

Fat from suspected milk sample can also be isolated by (i) Solvent extraction method or (ii) Heat-clarification method. Whenever these methods are used for isolation of fat, correction is not required and observed B.R can be directly considered for interpretation of results.

i. *Solvent extraction method: Take 100 ml of milk sample in a 500 ml flask. Add 15 ml of ammonium hydroxide, and shake thoroughly. Add 50 ml of ethanol; 100 ml diethyl ether and 100 ml petroleum ether and shake thoroughly after each addition. Allow to stand for half an hour. Decant the ethereal layer in another 250 ml conical flask. Add 50 g of anhydrous sodium sulfate (Na_2OS_4) to remove the traces of moisture from the ethereal layer. Transfer the ether layer in a clean, dry 250 ml conical flask containing 2-3 glass beads. Evaporate the ether extract to dryness on a boiling water-bath taking care to prevent bumping. After complete evaporation transfer the flask in an oven maintained at 100 ± 2°C.*

ii. *Heat clarification method: Obtain 50 g of cream by skimming the milk using in cream separator or in a laboratory centrifuge (4000 rpm, 10 min). Convert cream into anhydrous milk fat (ghee) by heat clarification.*

15.3. References:

Arora, K.L.; Lal, D.; Seth, R. and Ram, J. (1996). Platform test for detection of refined mustard oil adulteration in milk. Indian J. Dairy Sci., 49 (10): 721-723.

IS:1479. (1960). Methods of test for dairy industry. Part I. Rapid examination of milk. Bureau of Indian Standards, New Delhi

Lal, D.; Seth, R.; Arora, K.L. and Ram, J. (1998). Detection of vegetable oils in milk. Indian Dairyman, 50 (7): 17-18.

16. Detection of Presence of Soymilk in Milk

16.1. Introduction:

Chemical quality of milk suffers due to intentional adulteration of milk with various types of adulterants. With test developed for most of adulterants, unscrupulous people are finding more innovative ways to adulterate the milk with cheaper ingredients. One such adulterant of recent times is soymilk. Soymilk is a liquid extract from soybean grains with colour and composition similar to dairy milk. Due to this it is being used for adulteration of milk for economical reason. The production cost of this milk is around is 60-70% less than normal milk and the technology of its production is easy. Soymilk is admixed to bovine milk either to sale as fluid milk or to prepare skim milk powder and cheese for revenue maximization (Seker and Harper, 2004; Arvanitoyannis and Tzouros, 2005). Despite the good nutritional and functional properties of soybean proteins, addition of non-milk proteins as supplements to and substitutes for bovine milk is forbidden in many countries (Cattaneo *et al.*, 1994; Arvanitoyannis and Tzouros, 2005). Soybean protein is cheaper than bovine protein and thus presents a lucrative inducement to manufacturers to use soy protein in infant formulae and milk replacers (May *et al.* 1982; Dawson *et al.* 1988). However, in milk plants obtaining milk for processing as fluid milks or as milk products such as cheeses, ice-creams, fermented cheese preparations, etc., the determination of raw material quality is essential and the illegal addition of protein content enhancers such as soymilk to bovine milk by unscrupulous suppliers can be detrimental to such raw materials in the downstream process. In this section, a number of methods for detection of adulteration of milk with soymilk have been described. These methods have

been previously published also (Sharma *et al.*, 2009, 2010). These methods are based on the following concepts

a) Differences in optical activity of sugars present in bovine milk vis-à-vis soymilk (polarimetric method),
b) Differences in isoelectric point of major milk proteins vis-à-vis soy proteins (isoelectric precipitation method),
c) Differences in molecular weights of proteins present in bovine milk vis-à-vis soymilk (SDS-PAGE method),
d) The reactivity of soy protein antiserum towards adulterated milk (immunodiffusion method).
e) Difference in the sugar profile of soymilk and dairy milk (High Performance Liquid Chromatography method).

16.2. Method 1: Polarimeteric method

Carbohydrates present in soymilk and bovine or buffalo milk are different. Soymilk contains sucrose (glucose-fructose), raffinose (galactose-glucose-fructose) and stachyose (glucose-glucose-galactose-fructose) (Wang *et al.*, 2003). These are absent in bovine or buffalo milk. Lactose (glucose-galactose) is present in abundant amount in bovine or buffalo milk (Fox and McSweeney, 1986). These sugars are optically active and rotate plane polarized light and this concept has been exploited to develop a method for detection of adulteration of bovine or buffalo milk with soymilk. The method involves preparation of protein free filtrate using mercuric iodide followed by centrifugation and filtration. The protein free clear filtrate is analyzed by polarimeter.

16.2.1. Methodology:

A. Take 60 ml milk sample in 100 ml volumetric flask. Add 30 ml of mercuric iodide (prepared by dissolving 33.2 g potassium iodide and 13.5 g mercuric chloride in 200 ml glacial acetic acid and 640 ml of distilled water) and 10 ml of phosphotungstic acid (5%, aqueous). Mix the content by

inverting the volumetric flask twice and keep aside for 15 min at room temperature.

B. Transfer the contents of the flask to two 50 ml centrifuge tubes and centrifuge the contents at 6000 x g for 6 min. Filter the supernatant so obtained through Whatman No. 42 filter paper.

C. Maintain the clear filtrate at 20°C. Measure the optical rotation of filtrate in a polarimeter (e.g. Autopol II, Rudolph Research Analytical Corporation, USA) at 20°C using 200 mm polarimeter tube at wavelength of 589.3 nm (sodium D Line).

D. The average optical rotation of protein free filtrates from pure cow milk, pure buffalo milk, pure soymilk and cow or buffalo milk adulterated with 10% or 20% or 50% soymilk is shown in **Table 16.1**.

Table 16.1: Optical rotations of protein free filtrate from cow, buffalo, soya and adulterated milks

Sample*	Optical rotation in degree at 20°C
100% cow milk	2.89±0.03
100% buffalo milk	2.81±0.04
100% soymilk	0.90±0.02
90% cow milk+10% soymilk	2.63±0.01
80% cow milk+20% soymilk	2.51±0.01
50% cow milk+50% soymilk	1.82±0.02
90% buffalo milk+10% soymilk	2.71±0.03
80% buffalo milk+20% soymilk	2.56±0.02
50% buffalo milk+50% soymilk	1.80±0.01

*Samples collected/prepared on different days. Mean in column with different superscript were significantly different (LSD test, $P < 0.05$) from each other. Data is presented as mean ± SE (n = 6)

16.2.2. Interpretation:

Interpret the results based on the above reference readings (**Table 16.1**). In the experience of authors, milk adulterated with 10% soymilk can be detected by polarimetric method. The polarimetric method can't be

applied to milk adulterated with optically active sugars (e.g. glucose, sucrose etc.). Polarimetric method can provide results within 45 min and does not require expensive consumables.

16.3. Method II: Isoelectric precipitation method

Many of the soya proteins have iso-electric point around 5.7 and this property was utilized for developing a test for detection of adulteration of milk with soymilk. Iso-electric precipitation method was applied to (i) milk samples and (ii) rennet whey prepared from milk sample.

16.3.1. Methodology:

16.3.1.1. Method II a

A. Take 3 ml of milk sample in a glass tube. Adjust, pH of milk sample to 5.7 using 0.1 N HCl. Use pH meter for pH adjustment. The volume of 0.1 N HCl required for pH adjustment of 3 ml milk can be found prior to the start of the actual experiment.

B. Incubate the milk sample at 50°C in water-bath. Check the coagulation of milk at regular intervals by gently inverting the test tube. Interpret the results using **Table 16.2.**

16.3.1.2. Method II b

A. Prepare skim milk from the given milk sample by centrifugation (6000 x g, 5 min., 10°C).

B. Take 2.5 ml milk in a test tube and add 100 µl of rennet solution (0.25% aq). Incubate the contents at 35°C for 20 min. Centrifuge the contents at 6000 x g, 10 min., 10°C.

C. Take 0.5 ml of supernatant in another test tube and then add 1 ml piperazine-1,4 bis-2-ethanesulphonic acid (PIPES) buffer (0.5 M, pH 5.7). Place the test tube in a water-bath maintained at 50°C for 30 min. and observe for coagulation. Interpret the results using **Table 16.2.**

16.3.2. Interpretation:

Major soymilk proteins (7S and 11S) can be precipitated in the pH range 5.4 to 6.0 (Liu, 1997). In this pH range, major milk proteins casein (pI 4.6) and β-lactoglobulin (in spite of pI 5.2) remains soluble (Fox and McSweeney, 1986; Damodaran, 1996). The results of the experiment done in the authors' lab are depicted in **Table 16.2**. When pH adjusted (pH 5.7) soymilk, cow milk and cow milk adulterated with soymilk were incubated at 50°C, visible coagulation was noticed in soymilk and cow milk adulterated with 10% or 20% soymilk (**Table16.2**). At times it is difficult to notice coagulation in milk samples containing 10% soymilk. The coagulum was observed within 10 min. of incubation. pH adjustment of milk is to be carefully achieved. The coagulum could not be noticed in milk samples containing 5% soymilk even when incubation was extended up to 30 min. Further, when pH adjusted rennet whey samples prepared from pure milk and adulterated milk were incubated at 50°C, similar results were obtained during 10 min. incubation. During extended incubation up to 20 min. coagulum was also noticed in milk samples adulterated with 5% soymilk.

Table 16.2: Isoelectric precipitation of milk and rennet whey at pH 5.7

Sample	Time of incubation at 50°C		
	10 min	20 min	30 min
100% SM	+	+	+
100% CM	-	-	-
95% CM +5% SM	-	-	-
90% CM +10% SM	+	+	+
80% CM +20% SM	+	+	+
Whey (100% SM)*	NA	NA	NA
Whey (100% CM)	-	-	-
Whey (95% CM + 5% SM)	-	+	+
Whey (90% CM +10% SM)	+	+	+
Whey (80%CM +20% SM)	+	+	+

SM, soymilk; CM, cow milk; +, coagulation; -, no coagulation; NA, not applicable. *Rennet whey from 100% soymilk could not be obtained

Thus, the method has the capability of detecting presence of soymilk in milk at around 5-10% level.

16.4. Method III: SDS-PAGE method

This method is based on the identification of soya protein bands in adulterated bovine milk with soymilk. Proteins in soymilk and cow milk are of plant and animal origin respectively, and therefore these may differ in their molecular weights. When SDS-PAGE electrophoresis was carried out, four bands specific to soymilk were identified which were clearly distinguished from bovine milk proteins in adulterated milk.

16.4.1. Methodology:

Carry out the SDS-PAGE of milk protein as per the method of Laemmli (1970) using 12.5% acrylamide gel. Remove fat from milk sample by centrifugation (6000 x g, 5 min., 10°C). Mix 20 µl prepared skim milk sample with 180 µl sample buffer (0.125 M Tris, 4% SDS (sodium dodecyl sulfate) 20% Glycerol, 0.2 M β-mercaptoethanol, 0.02% bromophenol blue, pH-6.8) in a 1 ml micro-centrifuge tube. Keep the centrifuge tubes in boiling water-bath for 5 min using floaters. Cool the contents of the tube and then dispense 20 µl of prepared sample to individual wells. If any visible sediments are observed, centrifuge the sample and take the clear supernatant. Use broad range molecular weight markers (New England BioLabs Inc. USA) and treat molecular weight marker similarly. Run the electrophoresis. Visualize the protein bands on the gel with coomassie brilliant blue dye. The different steps required for running SDS-PAGE are given at the end of this document **(Appendix – 1)**. Interpret the results based on the appearance of protein band in the gel **(Fig. 16.1).**

16.4.2. Interpretation:

Proteins in soymilk and cow milk are of plant and animal origin respectively, and therefore these may differ in their molecular weights. The observations of similar experiment done in the author's laboratory are presented in **Fig. 16.1**. It shows the proteins from pure cow milk and cow milk adulterated with 5% or 10% or 20% or 50% soymilk separated on SDS-PAGE and stained with coomassie brilliant blue. Proteins from cow milk as well as soymilk were resolved on 12.5% acrylamide gel using Laemmli buffer (Laemmli, 1970). A comparison of protein bands from pure cow milk and pure soymilk (lane 2 and lane 7, **Fig. 16.1**) suggested that proteins of molecular weight about 20 KDa, 38 KDa, 50 KDa and 95 KDa were present in soymilk but absent in cow milk. These bands were also noticed in cow milk adulterated with 5% soymilk (lane 3, **Fig. 16.1**). Amongst these bands, proteins of molecular weight 20 kDa, 50 kDa and 95 kDa were more

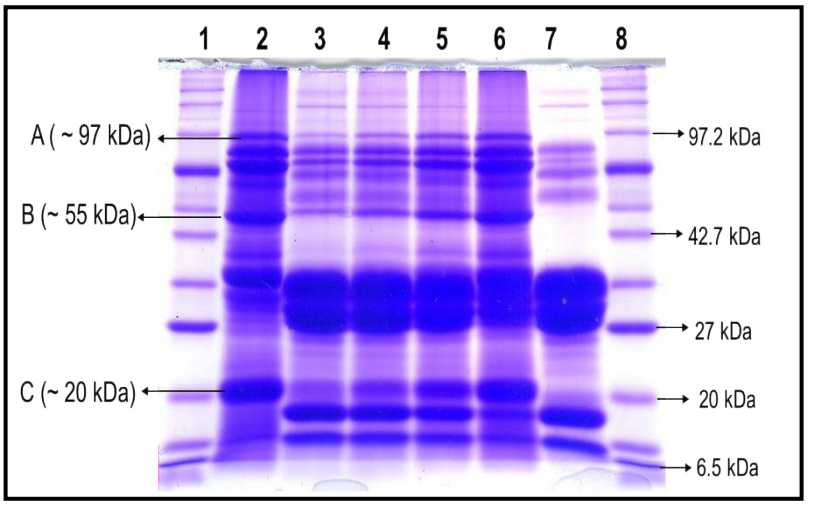

Fig. 16.1: SDS-PAGE pattern of proteins from bovine, soya and adulterated milk. Lane 1 & 8, protein marker; lane 2, 100% soymilk; lane 3, 5% soymilk + 95% cow milk; lane 4, 10% soymilk + 90% cow milk; lane 5, 20% soymilk + 80% cow milk; lane 6, 50% soymilk + 50% cow milk; lane 7, 100% cow milk.

pronounced in adulterated milk. The presence or absence of these bands indicates presence or absence of soymilk in milk. A similar approach has been used for detection of soya proteins in meat samples (Lee & Lee, 1988) and in milk products (Lopez-Tapia *et al.*, 1999; Manso *et al.*, 2002).

Since, SDS-PAGE method is based on molecular weight of proteins, other commonly used adulterants viz., sugars and neutralizers will not interfere in detection of adulteration in milk with soymilk. The results of SDS-PAGE method become available in 8 h.

16.5. Method IV: Immunodiffusion method

This method involves detection of adulteration of milk with soymilk on the basis of interaction of soya specific antisera with soya proteins present in adulterated milk. Immunodiffusion can be carried out as per the method described by Johnstone and Thorpe (1996).

16.5.1. Methodology:

A. Take a clean glass plate (size 10 cm x 10 cm) and first pre-coat the plate with 2% agarose (in water), followed by its coating with 1% agarose (in PBS, pH 7.2).
B. Punch wells of 4 mm diameter in agarose gel.
C. Prepare milk sample by mixing 20 µl of milk sample and 80 µl PBS containing 20 mM EDTA.
D. Load 30 µl of the milk sample and anti-soybean antiserum (r-biopharm, Germany) in different wells as indicated in **Fig. 16.1**. Incubate the plates overnight at room temperature in a humid atmosphere.
E. Stain the precipitated lines with coomassie brilliant blue (Johnstone and Thorpe, 1996).

16.5.2. Interpretation:

The antiserum specific to soybean proteins is commercially available. As indicated in an experiment, the antiserum did not cross-react with bovine milk proteins (**Fig.16.2**). The presence or absence of precipitating lines between the wells containing milk and antiserum sample indicated presence or absence of soymilk. Bovine milk adulterated with 1% or 5% or 10% soymilk was able to form precipitation lines with soya protein specific antiserum **(Fig.16.2).** Thus, immunodiffusion method can

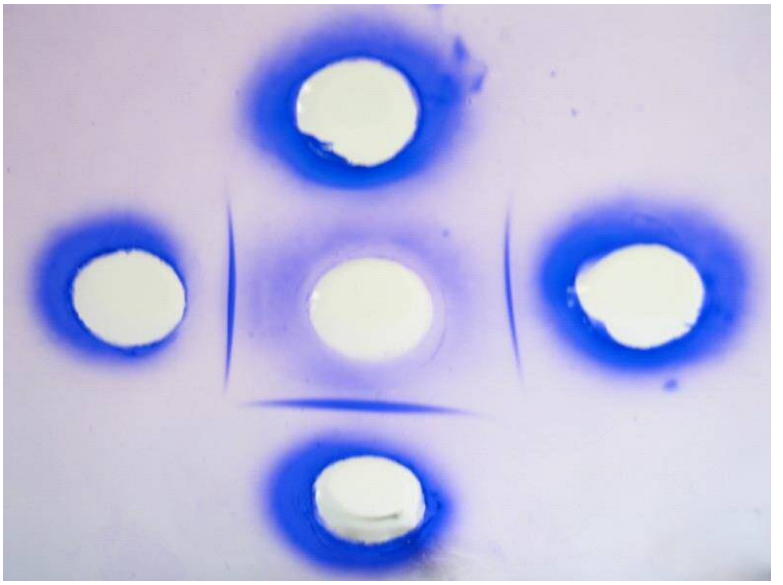

Fig. 16.2: Detection of soymilk in bovine milk by immunodiffusion method. Precipitation lines between antiserum (central well) and bovine milk adulterated with 1% (right well) or 5% (left well) or 10% (bottom well) soymilk. Precipitation line was absent between antiserum (central well) and pure bovine milk (top well).

detect presence of soymilk in milk at 1% level. The other common adulterants viz., sugars, neutralizers are not likely to interfere in detection. The results are available in 12 h.

16.6. Method V: HPLC based method

The major carbohydrates in soymilk are sucrose, raffinose and stachyose whereas lactose is abundantly present in bovine milk. The HPLC profile of milk sugars can be used for detection of adulteration of bovine milk with soymilk. Separation and estimation of sugars from bovine milk and soymilk by HPLC using bonded phase Luna NH_2 HPLC column (Phenomenex) is done and chromatographic profile has been used for ascertaining the presence of soymilk in milk. The presence of stachyose in the milk sample has been suggested as the marker sugar for the presence of soymilk in milk samples.

16.6.1. Methodology:

The method involves sample pre-treatment for extraction of sugars from milk and removal of other interfering substances. The pre-treated sample is then subjected to HPLC analysis.

A. Extraction of sugars from milk: Mix 2.0 ml of milk sample with 1.5 ml of distilled water and incubate the diluted milk sample at 60°C for 10 min. Then, add 0.25 ml Carrez I solution (500 mM aqueous potassium ferrocyanide), 0.25 ml Carrez II (500 mM aqueous zinc acetate) and 1 ml acetonitrile. Mix the contents gently and then keep it undisturbed for 1 h at room temperature. Centrifuge (10,000 g, 8 min, 20°C) the contents and remove the precipitate so obtained. Filter the resulting supernatant containing the extracted sugars through a 0.45 µm Nylon membrane.

B. Separation and quantification of sugars by HPLC: Inject 20 µl of clarified extract into the HPLC system for analysis. In the authors' laboratory, a Shimadzu HPLC equipped with a LC20AD pump, DGU20A5 on line degasser, 7725

Rheodyne manual injector with 20 µl loop, RIO10A RI detector, CTO20A column oven, Phenomenex Luna NH_2 column (5 µm particle size, 100Å pore size, 4.6 x 250 mm) and CBM20A system controller with Chromatography CLASS VP™ software has been used. However, HPLC system of any make meeting above mentioned hardware specifications can be used. Maintain the column and RI-cell temperatures at 40°C. Use isocratic solvent system; acetonitrile: water in a 70:30 ratio at a flow rate of 1ml/min. The standard sugars such as fructose, glucose, galactose, lactose, sucrose, raffinose and stachyose can be used to identify their retention times and relationship between sugar concentration and peak area. Use the concentrations of the standard sugar solutions (in water) in following range: 2 to 200 µg fructose, 2 to 80 µg glucose, 2 to 200 µg sucrose, 100 to 2000 µg lactose monohydrate, 1 to 20 µg raffinose pentahydrate and 2 to 200 µg stachyose tetrahydrate. Sonicate (in ultrasonic bath) the sugar standards and milk filtrates at 40W for 5 minutes in bursts of 2 minutes with a 1 minute hiatus to remove dissolved gases. Load these degassed prepared samples in a 20 µl volume into the manual injector of HPLC for analysis.

16.6.2. Interpretation:

Sugars can interact with amino column. The observations of similar experiment done in authors' laboratory are presented in **Fig. 16.3, 16.4** and **16.5.** When standard sugars viz. fructose, glucose, galactose, sucrose, lactose, raffinose and stachyose are separated on NH_2 column, all sugars except glucose and galactose elute with different retention time with 70:30 (v/v) acetonitrile-water mobile phase **(Fig. 16.3)**. Monosaccharides (fructose, glucose, galactose) eluted earlier to

disaccharides (sucrose, lactose), disaccharides elute earlier to trisaccharide (raffinose) and trisaccharide elute earlier to tetrasaccharide (stachyose). Further, fructose elutes earlier to glucose/galactose and the disaccharide containing fructose (sucrose) elutes earlier to the disaccharide containing galactose (lactose). In author's laboratory, the average (of six observations) retention times in minutes for these sugars were as follows: Fructose 5.007 ± 0.037; Glucose/Galactose 5.528 ± 0.034; Sucrose 6.536 ± 0.034; Lactose 6.851 ± 0.015; Raffinose 8.393 ± 0.06 and Stachyose 11.055 ± 0.092. The data indicates that the sugars with less number of hydroxyl groups eluted earlier to sugars having more number of hydroxyl groups.

Further, a linear relationship was also observed between the peak area of individual sugar solution and concentration of the solution, which was loaded to the amino-column (**Table 16.3**). The coefficient of correlation was greater than 0.99. The linearity was in the range of 2-200 µg for fructose, 2-80 µg for glucose, 2-200 µg for sucrose, 100-2000 µg for lactose monohydrate, 1-20 µg for raffinose pentahydrate and 2-200 µg for stachyose tetrahydrate.

Table 16.3: Linearity in sugars estimation by HPLC-RI

Compound	*Range (µg/20 µl)*	*R2*	*Equation*
Fructose	*2-200*	*0.9988*	*y = 76.532x + 25.682*
Glucose	*2-80*	*1*	*y = 72.95x - 2.6415*
Sucrose	*2-200*	*1*	*y = 79.813x - 1.1711*
Lactose monohydrate	*100-2000*	*0.997*	*y = 0.6804x - 3.8331*
Raffinose pentahydrate	*1-20*	*0.9974*	*y = 70.73x + 0.9742*
Stachyose tetrahydrate	*2-200*	*0.9999*	*y = 67.64x + 8.8466*

x: concentration (mg/ml); y: peak area

The HPLC profile of sugars extracted from bovine milk, buffalo milk and soymilk is shown in **Fig. 16.3**.

Bovine and buffalo milk exhibited major peak corresponding to lactose (peak no. 5 in **Fig. 16.4A** and **16.4B**) and this peak was well visualized in raw chromatogram. However, minor peaks (peak no. 2 and 3 and one peak between peak no. 2 and solvent peak) could be visualized only upon bringing the chromatogram into higher resolution (**Fig. 16.4A′** and **16.4B′**). The peak no. 2 and 3 corresponded to retention time of fructose and glucose/galactose, respectively. At higher resolution, lactose peak was very broad in bovine as well as buffalo milk (**Fig. 16.4A′** and **16.4B′**) and spread to retention time corresponding to sucrose and raffinose (**Fig. 16.4A′, 16.4B′** and **16.4C′**). The raw chromatogram obtained from pure soymilk extract (**Fig. 16.4C′**) did not show any major peaks (except solvent peak) and the sugar profile was visible only at higher resolution with five peaks (peak no. 2, 3, 4, 6 and 7 in **Fig. 16.4C′**) which corresponds to fructose, glucose/galactose, sucrose, raffinose and stachyose. There was no lactose peak in soymilk. The HPLC profile of sugars extracted from bovine/buffalo milk adulterated with soymilk (at 5% level) is shown in Figure 5. The lactose peak from bovine or buffalo milk masked the peaks of sucrose and raffinose from soymilk in adulterated milk. However, stachyose peak (peak no. 7 in **Fig. 16.5A′** and **16.5B′**) remained well separated. The levels of various sugars present in bovine milk, buffalo milk and soymilk are shown in **Table 16.4.** Raffinose and stachyose were absent in bovine and buffalo milk. There was one additional peak just after solvent peak in bovine as well as in buffalo milk (**Fig. 16.4A′** and **16.4B′**) and this peak could not be identified.

In order to check recovery of sugar during extraction and separation of sugar, 100 ml soymilk was

supplemented with 100 mg raffinose and recovery was 96.3%. This indicated that extraction of sugars from milk with 20% acetonitrile in presence of Carrez solutions was quantitative.

Since, raffinose and stachyose are present in soymilk and absent in bovine milk, this information can be utilized to detect presence of soymilk in bovine/buffalo milk. Sugars from bovine/buffalo milk adulterated with different levels of soymilk were separated on amino column. The stachyose peak (peak no. 7) was visible in bovine and buffalo milk adulterated with 5% soymilk (**Fig. 16.5A′** and **16.5B′**). This indicates that presence or absence of stachyose peak can be used for detection of adulteration of milk with soymilk. The method can detect upto 5% soymilk in milk.

Table 16.4: Sugar content in cow, buffalo and soymilk

Sugars	*Concentration in milk†*		
	Cow	*Buffalo*	*Soya*
Fructose(µg/ml)	*213 ± 16.7*	*216 ± 49.4*	*172 ± 10.9*
Glucose/Galactose (µg/ml)	*168 ± 12.3*	*124 ± 9.5*	*118 ± 15.9*
Sucrose(mg/100 ml)	‡	‡	*238 ± 3.2*
Lactose(g/100 ml)	*4.97 ± 0.11*	*4.92 ± 0.23*	‡
Raffinose (mg/100 ml)	‡	‡	*77 ± 3.8*
Stachyose(mg/100 ml)	‡	‡	*306 ± 8.06*

†Data are presented as means ± SE (n = 6)
‡ Peak was absent

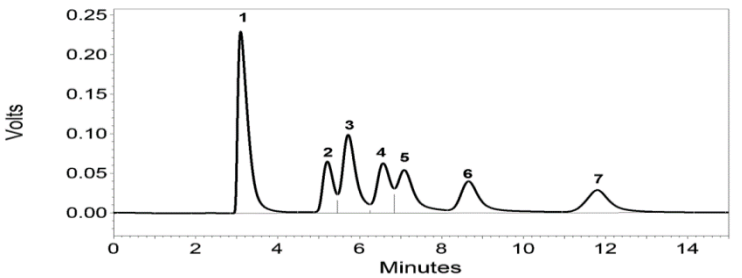

Fig. 16.3: A 20 µl of sugar mix containing 200 µg each of fructose, glucose, galactose, sucrose, lactose, raffinose and stachyose was subjected to isocratic separation using 70:30 acetonitrile : water mobile phase; peak 1, solvent; 2, fructose; 3, glucose/galactose; 4, sucrose; 5, lactose; 6, raffinose and 7, stachyose.

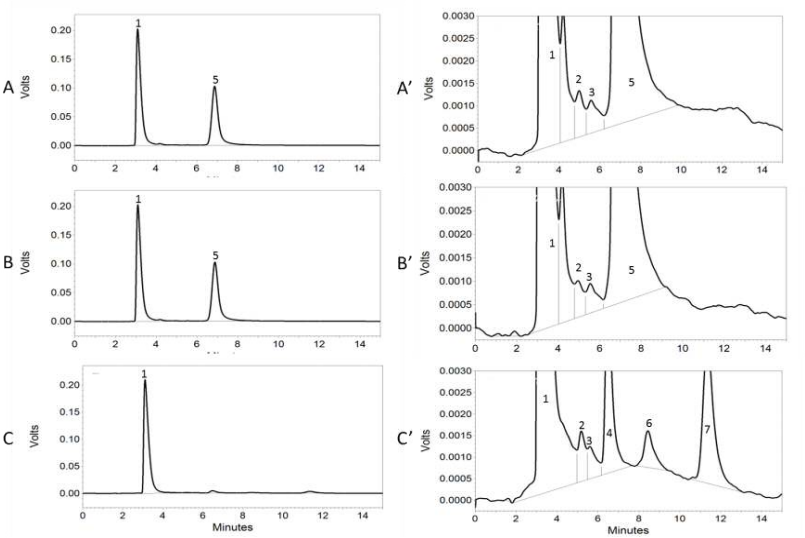

Fig. 16.4: Separation of sugars from bovine, buffalo and soya milk. A, bovine milk; B, buffalo milk; C, soya milk. A′, B′ and C′ correspond to high resolution chromatogram of bovine, buffalo and soya milk respectively. peak 1, solvent; 2, fructose; 3, glucose/galactose; 4, sucrose; 5, lactose; 6, raffinose and 7, stachyose.

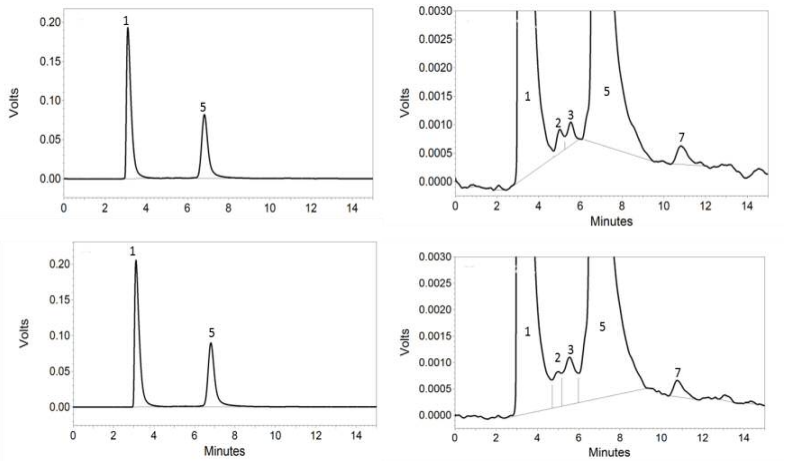

Fig. 16.5: Separation of sugars from adulterated milk. A, bovine milk adulterated with 5% soya milk; B, buffalo milk adulterated with 5% soya milk; A′ and B′ correspond to high resolution chromatogram of bovine milk adulterated with 5% soya milk and buffalo milk adulterated with 5% soya milk respectively. Peak 1, solvent; 2, fructose; 3, glucose/galactose; 5, lactose; and 7, stachyose.

16.7. Method VI: A rapid method for the detection of soymilk in milk

Soymilk contains enzyme trypsin inhibitor and will therefore inhibit trypsin activity. Inhibition of enzyme activity by adulterated milk is the basis of detection of presence of soymilk in milk. Pure milk will not inhibit enzyme activity. Trypsin acts on N-α- benzoyal DL-arginine p-nitroanilide (BAPNA) and coloured product (yellow) is formed. The difference in colour intensity between authenticated milk sample and adulterated milk after termination of enzyme activity is the basis of detection of adulteration of milk with soymilk. Sensitivity of detection is enhanced by diazotization of product obtained from enzymatic reaction. End Product is pink in colour. Pure milk sample is simultaneously tested.

The method involves addition of trypsin in the given milk sample as well as in pure milk sample and addition of trypsin substrate (BAPNA). If milk is adulterated with soymilk, the milk will have trypsin inhibitor which will inhibit trypsin activity and trypsin will not able to act on substrate. In case of pure milk sample, trypsin converts substrate to yellow coloured p-nitroaniline. Subsequently, the addition of sodium nitrite coverts p-nitroaniline into its diazonium compound which in presence of N-(1-Naphthyl) ethylene diamine dihydrochloride (NEDA) is converted to a pink coloured compound. Since activity of trypsin is inhibited in presence of soymilk, either substrate is not converted to p-nitoraniline or the product is formed in reduced amount.

Ammonium sulfamate is added to remove excess of sodium nitrite.

16.7.1. Reagent(s) required

i. Trypsin [1000 - 2000 benzoyl L-arginine ethyl ester (BAEE) units /mg solids]
ii. Hydrochloric acid
iii. N-α- benzoyl DL-arginine p-nitroanilide (BAPNA)
iv. Dimethylsulfoxide
v. Tris-HCl
vi. Calcium chloride
vii. Glacial Acetic acid
viii. Ammonium sulfamate
ix. Sodium nitrite
x. N-(1-Naphthyl) ethylene diamine dihydrochloride (NEDA)
xi. Authentic milk sample: The authentic milk sample can be skimmed milk powder or whole milk powder prepared by spray drying method. 10 gm powder is dissolved in water and volume is made upto 100 ml. Pure milk can also be used as authentic milk sample.

16.7.2. Reagent(s) Preparation

i. Enzyme solution: Dissolve 3 – 5 mg of trypsin in 100 ml of 1mM HCl.
ii. Substrate solution: Dissolve 50 mg of BAPNA in 1 ml dimethylsulfoxide. Before use, dilute substrate to 50 ml with 50 mM Tris-HCl buffer pH 8.2 containing 20 mM $CaCl_2$.
iii. Glacial acetic acid solution (30% v/v): Dilute 30 ml of glacial acetic acid to 100 ml with distilled water.
iv. Sodium nitrite solution (0.3%, w/v): Dissolve 300 mg sodium nitrite in distilled water and make up the volume to 100 ml with distilled water. Store the solution at refrigerated temperature.
v. Ammonium sulphamate solution (3%, w/v): Dissolve 3 gm ammonium sulphamate solution in distilled water and make up the volume to 100 ml with distilled water.

vi. NEDA Solution (0.20%): Prepare NEDA solution in distilled water.

16.7.3. Methodology

The method requires comparison of colour produced with pure milk *vis-a-vis* adulterated milk and thus pure milk sample is also subjected to identical treatment.

1. Mix 500 µl of reconstituted authentic milk or milk sample and add 100 µl of trypsin solution in a glass test tube (15 ml).
2. Place the tube in water-bath for incubation at 37°C for 15 min. and mix the contents intermittently.
3. Add 3 ml of BAPNA solution and incubate the tube further at 37°C for 30 min.
4. Stop the trypsin activity reaction by addition of 1 ml of 30% glacial acetic acid.
5. Take 1 ml of the above mixture in another glass test tube and add 0.5 ml of 0.3% sodium nitrite solution. Mix the contents gently and incubate the tube at 37°C for 3 min.
6. Add 0.5 ml of 3% ammonium sulphamate solution and again incubate the contents at room temperature for 3 min.
7. Add 0.5 ml of NEDA solution, mix gently and note colour of contents in the test tube.

16.7.4. Interpretations

1. The intensity of pink colour obtained in the test sample with that of authentic milk sample is compared (**Fig.16.6**).
2. The decrease in the pink colour intensity of test sample compared to authentic milk sample indicates adulteration of milk with soymilk.

16.7.5. Detection limit

The test is capable of detection of presence of 2.5 % soymilk in milk.

Fig. 16.6: Color of sample after reaction. P – Pure milk and A – Adulterated milk.

16.8. References:

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16.9 Appendix – I

16.9.1. SDS-PAGE

The purpose of sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) is to separate proteins according to their size. SDS-PAGE is the most widely used method for analyzing protein mixture qualitatively. It is particularly useful for monitoring protein purification and, because the method is based on the separation of proteins according to size, it can be used to determine the relative molecular mass of proteins. SDS (CH_3-$(CH_2)_{10}$-CH_2OSO_3-Na^+) is an anionic detergent and when proteins are treated with SDS in presence of a reducing agent like β-mercaptoethanol or dithiothreitol, SDS binds to hydrophobic regions of protein molecule and provides net negative charge on protein molecule. The binding of SDS to per-unit-length of protein molecules is almost constant for large number of different proteins and this brings charge-to–mass ratio almost constant for most proteins. The electrophoretic movement of protein in acrylamide gel is determined by molecular weight of proteins. Lower molecular weight proteins move faster than high molecular weight proteins. The method described by Laemmli (1970) is widely used. In this method, discontinuous buffer system is employed. A continuous buffer system has only single separating gel and uses same buffer in the tanks and gel. In discontinuous buffer system, large pore gel (stacking gel) is layered over small pore gel (separating or running gel). For preparation of stacking gel and separating gel, different buffers are used and also tank buffers are different from gel buffers. When electrophoresis is started; ions from stacking gel (leading ion), ions from buffer tank (trailing ion) and proteins start moving into stacking gel. In stacking gel, protein moves between leading ion and trailing ion and this leads to concentration of protein in a thin zone referred as stack. The protein molecules continue to move in the stack until they reach the separating gel.

16.9.1.1. Procedure:

The below mentioned procedure is for the separation of proteins using glycine-SDS-PAGE.

16.9.1.2. Equipment and Chemicals:

Mini-vertical gel electrophoresis dual model with glass plates, spacer, comb and power-supply; orbital shaker; 1 ml glass syringe with 2"22G needle; acrylamide, N,N^1 methylene bisacrylamide; ammonium persulfate; β-mercaptoethanol; sodium dodecyl sulfate; molecular weight markers; coomassie brilliant blue R-250; TEMED; tris; glycine; dithiothreitol.

16.9.1.3. Stock Solutions:

A. Acrylamide/Bisacrylamide (30%): 29.2 g acrylamide and 0.8 g bisacrylamide are dissolved in distilled water and total volume was made to 100 ml. The solution is filtered and filtered solution can be stored at 4^{0}C in dark bottle up to 3 months.

B. 4 x Running Gel Buffer (1.5 M Tris-HCl, pH 8.8): 18.15 g Tris is dissolved in about 80 ml distilled water. pH is adjusted to 8.8 with 1 N HCl and total volume is made to 100 ml with distilled water. Prepared buffer can be stored up to 3 months at 4^{0}C in dark bottle.

C. 4 x Stacking Gel Buffer (0.5 M Tris-HCl, pH 6.8): 3.0 g Tris is dissolved in about 40 ml distilled water. pH is adjusted to 6.8 with 1 N HCl and total volume is made to 50 ml with distilled water. Prepared buffer can be stored up to 3 months at 4^{0}C in dark bottle.

D. 10% SDS: 10 g sodium dodecyl (lauryl) sulfate is dissolved in distilled water and total volume is made to 100 ml with distilled water. Prepared solution can be stored at room temperature.

E. 5 x Electrode Buffer (125 mM Tris, 960 mM Glycine, 0.5 % SDS, pH 8.3: 15 g Tris, 72 g glycine and 5 g SDS are dissolved in distilled water and total volume is made to 1 liter with distilled water. The pH of buffer should be 8.3 ± 0.2. Stock electrode buffer is diluted five times with distilled water before use. The stock buffer can be stored at room

temperature up to 1 month. The diluted stock buffer is 25 mMtris, 192 mM glycine, and 0.1% SDS.

F. 10% Ammonium Persulfate: 100 mg ammonium persulfate is dissolved in 1.0 ml distilled water. The solution is always prepared fresh.

G. 2 x Sample Buffer (0.125 M Tris, 4% SDS, 20% glycenol 0.2 M DTT, 0.02% bromophenol blue, pH 6.8: 2 x sample buffer is prepared by mixing following solutions/chemical.

H. 2 x Sample buffer can be stored in small aliquots at - 20^{0}C up to 6 months. Instead of DTT, 1.0 ml of β-mercaptoethanol can be used but the volume of water is reduced to 0.5 ml.

Solutions		Volume
4 x stacking gel buffer	-	2.5 ml
glycerol	-	2.0 ml
10% SDS	-	4.0 ml
Bromophenol blue	-	2.0 mg
Dithiothreitol (DTT)	-	0.31 g
Distilled water	-	1.5 ml

I. Overlay Buffer (0.375 M Tris, 0.1%, SDS, pH 8.8): Overlay buffer is prepared by mixing 25 ml running gel buffer, 1 ml 10% SDS and 74 ml distilled water. This buffer can be stored up to 3 months at 4^{0}C in the dark bottle.

16.9.1.4. Procedure:

A. Glass Sandwich: One notched glass plate is placed on a flat surface. One spacer (1.0 mm) each is then placed along the each of two edges so that spacer aligns with the notch. Subsequently, rectangular glass-plate is placed over it. The sandwich is held firmly between thumb and fingers. Side-ways of both spacers were sealed with appropriate tape to overcome any possible gel-leak during gel plate preparation. There is always the possibility of leakage at the bottom of the plate. This is taken care by placing molted agar (1% in water) up to 5 mm height in trough of gel-casting unit. The plate in

Solutions	Final Gel Concentrations			
	7.5%	10%	12.5%	15%
Acrylamide / bisacrylamide (30%)	5.0 ml	6.7 ml	8.3 ml	10.0 ml
4 x Running gel buffer	5.0 ml	5.0 ml	5.0 ml	5.0 ml
10% SDS	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Distilled water	9.7 ml	8.0 ml	6.4 ml	4.7 ml
10% Ammonium persulfate	0.1 ml	0.1 ml	0.1 ml	0.1 ml
TEMED	6.7 µl	6.7 µl	6.7 µl	6.7 µl

standing position is then quickly placed in casting unit and screws are finger tightened.

B. Preparation of Running Gel: Running gel of desired concentration is prepared by mixing appropriate volumes of solutions as shown below. Acrylamide/bisacrylamide, running gel buffer, SDS and distilled water are added to conical flask and degassed. Then ammonium persulfate and TEMED are added and contents mixed gently. With the help of glass pipette, the running gel solution is delivered to sandwich to a level about 3 cm below the top of rectangular plate. Air should not be trapped while filling sandwich with running gel solution. A small volume of water or overlay-buffer (~ 200 µl) is layered over gel solution with the help of glass syringe with 2"22 G needle. This prevents exposure to oxygen.

C. Preparation of Stacking Gel: Stacking gel of 4% concentration is prepared by mixing appropriate volumes of solutions as shown below. The preparation of stacking-gel solution is similar to preparation of running-gel solution. After removal of water or overlay buffer, stacking-gel solution is layered over running gel. Appropriate comb is inserted into the stacking gel to make wells for sample application. Comb is removed after polymerization of gel.

Solutions	Volume
Acrylamide / bisacrylamide (30%)	1.3 ml
4 x Stacking gel buffer	2.5 ml
10% SDS	0.1 ml
Distilled water	6.1 ml
10% Ammonium persulfate	50 µl
TEMED	10 µl

D. Sample Preparation: Protein samples (1 mg/ml) are centrifuged (10,000 g, 5 min.) to remove any insoluble material and are mixed with equal volume of 2 X sample buffer. The resultant solution is boiled for 3 to 5 min. Molecular weight markers are also prepared in a similar way.

E. Electrophoresis: Gel plates are then tightly attached to electrophoresis unit. Stock electrode buffer is five times diluted with cold water. Anode and cathode chambers are filled with buffer. 5 to 20 µl of sample is applied to each well.

Electrophoresis is carried out at constant voltage of 50V till sample crosses stacking gel. When sample enters running gel, voltage is increased to 100V. Complete electrophoretic run takes around 2.5 to 3.0 h. During electrophoresis, temperature is kept low by circulating water in electrophoretic assembly. After electrophoretic run, stacking gel is removed. Small cut on top left side in running gel is made to remember the orientation of the gel.

F. Staining of proteins in gel: The gel is placed in glass tray containing coomassie brilliant blue solution (0.25%) prepared in methanol : acetic acid : water (40:7:53) mixture. Glass tray is then placed on orbital shaker for 4 h at room temperature. After staining for 4 h, the gel is transferred to the destaining solution I (methanol, acetic acid and water mixture in ratio of (40:7:53) for 30 min. Subsequently gel is placed in destaining solution II (methanol, acetic acid and water mixture in ratio of 7:5:88) till bands become visible against light background. During staining and destaining, gel should float free in glass tray.

16.9.1.5. Helpful-Hints

- ✓ A particular concentration of acrylamide gel is used for separating proteins of particular range of molecular weights. Whereas low acrylamide gel concentration is used for separating high molecular weight proteins, low molecular weight proteins are resolved in high gel concentration. Use following table in deciding gel concentration in separating gel.

Per Cent gel	Molecular weight of proteins to be separated (KD)
7.5	24 – 205
10.0	14 – 205
12.5	14 – 66
15.0	14 – 45

- ✓ Spacers can absorb heat and thus lowers the temperature of gel at edges. If the gel is hotter in the middle than at the edges, the mobility of dye front at edges will be lower as compared to mobility in the middle. This can be avoided by (i) using cooled electrode buffer and (ii) not allowing buffer to warm up during run. Thus, during electrophoretic run either use cooling device or use low current.

- ✓ If gel is not polymerized properly at edges, current can leak down the edges resulting in more mobility at edges. Air- bubbles at the bottom of glass plates can block current flow resulting abnormal dye front.
- ✓ While placing comb in stacking gel, care should be taken not to allow air-trap. Air inhibits polymerization.
- ✓ All stock solutions required for gel preparation are stored at refrigerated temperature and these should be brought to room temperature. At low temperature, polymerization is inhibited. Oxygen also inhibits polymerization of acrylamide and these solutions should be degassed before use.
- ✓ Sometimes boiling of sample in sample buffer may lead to irreversible precipitation and such samples remain at the top of separating gel. For such samples one can try incubating sample in sample buffer at 70^0C instead of 100^0C.

17. Detection of Cheese Whey Adulteration in Milk

17.1. Introduction

Addition of rennet whey from cheese production is one of the many frauds in liquid milk and milk products such as dried milk (Chavez *et al.*, 2008; Martin-Hernandez *et al.*, 2009). Adulteration of liquid or dehydrated milk by adding cheese whey is economically attractive because the cheese whey cost is four to five times lower than milk and does not adversely affect the sensory perception of the product by consumers. Due to its lower cost and assumed comparable functional and nutritional properties and freedom from objectionable flavor, cheese whey is an appealing alternative ingredient for adulteration of milk. However, this adulteration may give rise to important quality problems in finished products and incurs financial losses (Martin-Hernandez *et al.*, 2009) and has nutritional and legal implications (Alcazar *et al.*, 2000). Another reason for the adulteration of milk and milk products with cheese whey could be to its high BOD load and thus posing problems in its disposal. Milk processing industries and distributors of many countries have reported the problem of milk adulteration with cheese whey.

In this section, a method has been given for the detection of presence of cheese whey in milk. In this method, glycomacropeptide (GMP) is selectively precipitated from the adulterated milk samples followed by measurement of GMP-bound sialic acid by acidic ninhydrin reaction. Although, colour differentiation can be made visually among control and positive samples, spectrophotometric reading of the developed colour helps in better interpretation.

17.2. Method: Ninhydrin based spectrophotometric method

17.2.1. Methodology:

The method essentially involves two steps. In the first step, GMP from milk sample is selectively precipitated followed by the spectrophotometric estimation of GMP bound sialic acid (Rao *et al.*, 2012).

A. Selective precipitation of GMP from milk sample
   a) Take 10 ml of milk sample in 15 ml poly-propylene centrifuge tube and mix it with 24% trichloroacetic acid (TCA) solution to a final TCA concentration of 8%. Let the content stand at room temperature for 30 min.
   b) Centrifuges the contents at 8000 g for 10 min at room temperature.
   c) Take 10 ml of supernatant in another 15 poly-propylene centrifuge tube and add 50% TCA to a final TCA concentration of 14%. Again, let the content stand at room temperature for 30 min.
   d) Centrifuges the contents at 4000 g for 10 min at room temperature. Discard the supernatants gently.
   e) Disperse the sediments in 6 ml of 95% ethyl alcohol.
   f) Centrifuges the contents at 4000 g for 10 min at room temperature. Discard the supernatants gently. Save the pellet for sialic acid analysis.

   ***Note**: The pellet formation depends on the extent of adulteration of milk with cheese whey. More the extent of adulteration, more pellet formation.*

B. Spectrophotometric assay of sialic acid in GMP precipitates using acidic ninhydrin method [Fukuda *et al.* (2004)]:
   a) Add 2 ml of glacial acetic acid and 1 ml of acidic ninhydrin reagent (1 g of ninhydrin, 16 ml of hydrochloric acid and

24 ml of glacial acetic acid) to the pellet obtained after TCA precipitation.

b) Vortex the contents and place the tubes in a boiling water-bath for exactly 10 min. There will be colour development varying from yellow to brown-yellowish. Remove the tubes from the water-bath and transfer the tubes to chilled water. Measure the OD of the content within 10 min at 470 nm, against distilled water.

17.2.2. Interpretation

Sialic acid under acidic conditions reacts with ninhydrin reagent and by heating a sample solution containing sialoglycoprotein with the reagent at 100°C, a stable yellow-brown colour with absorption maxima at 470 nm is produced. The intensity of yellowish-brown colour increased with the increase in the level of adulteration. The

Table 17.1: Absorbance values at 470 nm of pure milk, adulterated milk samples and whey by acidic ninhydrin method

Sample	Reagent blank	Control Milk	Adulterated milk samples			Pure cheese whey
			10% Whey	20% Whey	50% Whey	
A_{470}	0.004 ± 0.002 [a]	0.032 ± 0.01 [b]	0.10 ± 0.01 [c]	0.16 ± 0.04 [d]	0.24 ± 0.04 [e]	0.41 ± 0.03 [f]

Data are presented as means ± S.D. (n=14). Means with different superscript letters are significantly different (P≤0.05) from each other

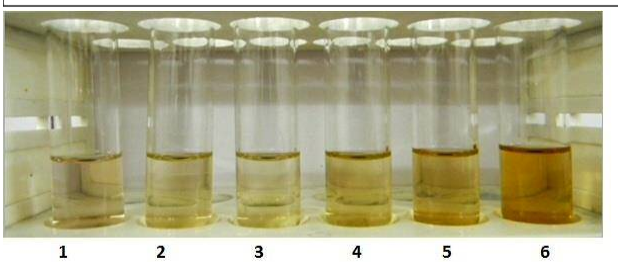

Fig. 17.1: Colour development in pure milk and adulterated milk samples by acidic ninhydrin reaction method

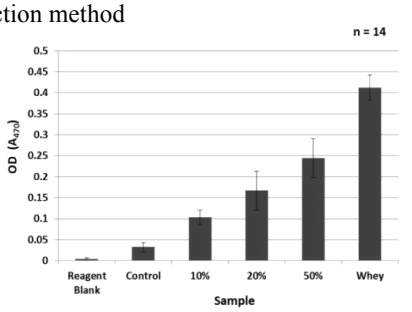

Fig. 17.2: Plot of pure and adulterated milk samples OD at 470 nm

reagent blank as well as control milk sample (unadulterated milk) also gave faint yellow colour. The increase in the OD value on adulteration of milk sample compared to control milk sample can be used as an index for the detection of adulteration of milk. The results indicate that the method has the potential to detect 10% adulteration of milk with cheese whey.

The results of similar experiment done in author's laboratory are presented in **Table 17.1** and **Fig. 17.1**. Colour development and OD (optical density) values of various samples are presented in **Fig. 17.1** and **Table 17.1**, respectively. The results presented in **Fig. 17.1** indicate the development of yellowish-brown colour in adulterated milk samples as well as in pure cheese whey samples. The intensity of yellowish-brown colour increases with the increase in the level of adulteration and is also evident from the readings of the OD values at 470 nm are presented in the **Table 17.1**. The reagent blank as well as control milk sample (unadulterated milk) also gave faint yellow colour with OD values of 0.004 ± 0.002 and 0.032 ± 0.01. The OD value of 10% adulterated milk sample was about 3.1 times higher (0.10 ± 0.01) than the OD value of control milks sample (0.032 ± 0.01). The OD value increases as the level of adulteration increases **(Fig. 17.2)**. This increase in the OD value on adulteration of milk sample can be used as an index for the detection of adulteration of milk. Since ninhydrin reacts with number of other compounds including free amino acids (Friedman, 2004), the OD value of 0.032 ± 0.01 in the control milk sample could not be avoided.

17.3. References

Alcazar, M.C.; Rosas, J.; Jaramillo, A.C. and Pena, S. (2000). Detection of glycomacropeptide as an indicator of adulteration with cheese whey in dehydrated milk. Vet. Mex., 37: 217-222.

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18 Test for Detection of Skimmed Milk Powder in Natural Milk

18.1 Introduction:

As per the law, use of skimmed milk powder (SMP) is not allowed for adjustment of SNF in case of sale of cow/buffalo or mixed milk. However, SMP can be used for the adjustment of milk solids in case of toned, double toned and recombined milk. A method has been developed for the detection of presence of SMP in liquid milk (Murthi, 1985).

The method is based on the fact that the coagulum obtained from reconstituted skim milk powder by addition of acetic acid, gives intense blue colour on boiling with phosphomolybdic acid due to certain reducing groups present in the proteins of milk powder which are able to cause reduction of molybdenum blue resulting in formation of blue colour.

18.2. Method: Phosphomolybdic acid

18.2.1. Reagent(s) required:

i. Acetic acid
ii. Phosphomolybdic acid

18.2.2. Reagent(s) preparation:

i. Acetic acid: 4%, aqueous
ii. Phosphomolybdic acid: 1% solution in water.

18.2.3. Methodology:

A. Take 50 ml of milk in a centrifuge tube. Place the tube in the centrifuge and balance it properly.
B. Centrifuge at 5000 rpm for 15 minutes. Remove the cream layer and take out 5 ml of skim milk, carefully in a test tube.

C. Add 2 ml of 4% acetic acid for coagulation and wash the curd using distilled water. To the curd then add 2 ml of 1% phosphomolybdic acid.

D. Mix the contents thoroughly and heat in a water bath at boiling temperature for 5 minutes and then cool.

E. Observe the colour of the solution in the tube.

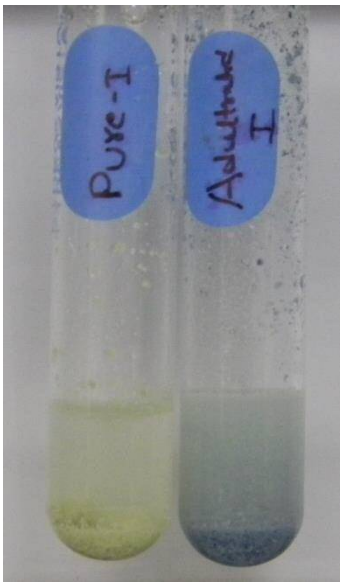

Fig. 18.1: Comparison of developed colour in presence and absence of skim milk powder in milk.

18.2.4. Interpretation:

The curd obtained from pure milk shall be greenish in colour whereas the curd of sample containing skimmed milk powder shall be bluish in colour. The intensity of bluish colour depends on the amount of the skim milk powder present in the sample **(Fig. 18.1)**.

18.2.5. Limit of detection:

Development of blue colour in the curd portion is an

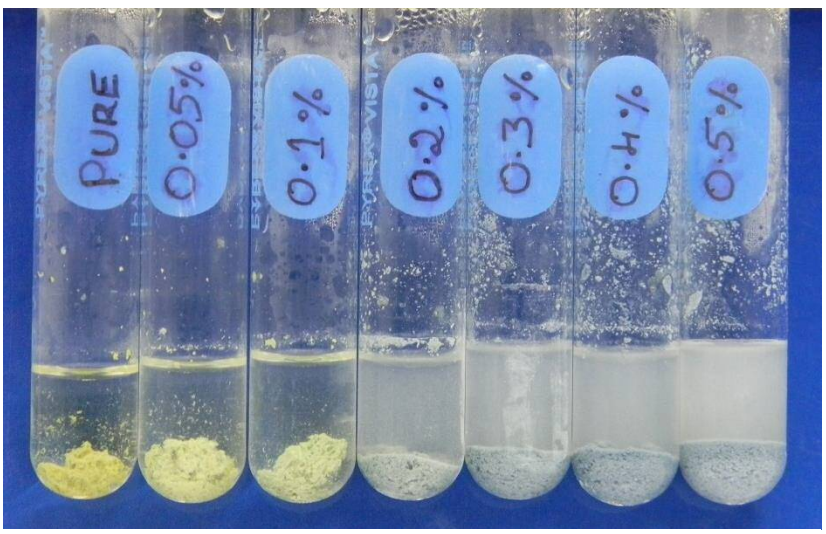

Fig. 18.2: Limit of detection of skim milk powder in milk.

indication of the presence of skim milk powder in the milk samples. The intensity of blue colour increases with the increase in the level of adulteration of milk with skim milk powder. In the present experiment done in authors' laboratory it is found that adulterating milk at the level of 0.2% (w/v) develops distinguishable blue colour curd compared with the light greenish colour in pure milk samples. Therefore the limit of detection for this method is 0.2% (w/v) of skim milk adulteration in milk **(Fig. 18.2)**.

18.3. Reference:

Murthi, T. N. (1985). Colorimetric determination of skim milk powder in normal milk. Journal of Food Science and Technology, 22: 207-208.

19. Determination of Fat, SNF and Total Solids in Milk

19.1. Introduction:

In this manual, methods for the detection of adulteration of milk with water have not been given as at present, no rapid and easy tests are available. However, presence of added water in milk can be judged by compositional analysis or just by estimation of fat and SNF content. Depression in freezing point of milk can be used for ascertaining the presence of water and now-a-days, commercial equipments are available for such measurement, though expensive. Similarly, rapid compositional analysis can be done by various kinds of milk analyzer available in the market. In this section, conventional methods for estimation of milk fat, SNF and total solids content has been elaborated. By the estimation of these parameters, quality of milk can be ascertained.

19.2. Method: Determination of Fat in Milk

Fat in milk exist in the form of small spherical droplets surrounded by a stabilizing membrane known as milk fat globule membrane. It is necessary to destroy this membrane and liberate the fat in liquid form so that it can be measured volumetrically. In the Gerber method, this is achieved by treating milk with sulphuric acid of known specific gravity, which dissolves milk proteins and carbohydrates and releases fat. The released fat is separated from the digested material by adding small amount of iso-amyl alcohol which acts as surfactant. The mixture is then centrifuged at 1100 rpm for 4 min and the volume of separated fat is then read on the graduated stem of the butyrometer at 65°C. This is a quick, routine method in common use and gives results within accuracy of ± 0.10% when compared with gravimetric method.

Dr. N. Gerber, a Swiss chemist in 1894 introduced this test. When Gerber method was first introduced, the use of 11.0 ml of milk was recommended. This is because graduation in the Gerber butyrometer is such that each 1% corresponds to an internal volume of 0.125 ml. Thus weight of fat in stem of butyrometer corresponding 1% is equal to 0.125 × D gm (D = M/V; or M = V × D), where D is the density of fat at temperature of reading (65°C). This is usually 0.9 for fat. Hence, the weight of fat would be 0.125 × 0.9 = 0.1125 gm. Since 0.1125 gm fat represent 1% sample of fat, it is obvious that the weight of sample to be taken for the fat must be around 11.25 gm and for milk with normal specific gravity, this weight corresponds to 10.9 ml and volume of milk delivered by 11.0 ml pipette is 10.9 ml. However, by taking 10.75 ml of milk in the Gerber method, the value of fat percentage agrees with the result obtained using the reference method (Röse-Gottlieb method).

19.2.1. Apparatus required:

i. Butyrometer: 0 to 10% scale.
ii. Pipette: 10 ± 0.25 ml or automatic measure or tilt measure for sulphuric acid.
iii. Pipette: 10.75 ± 0.03 ml for milk.
iv. Pipette: 1 ± 0.05 ml or automatic measure or tilt measure for iso-amyl alcohol.
v. Lock stoppers for butyrometer.
vi. Lock stopper key.
vii. Water-bath: The water-bath shall be made of a suitable material (e.g. stainless steel). It shall be capable of being maintained at 65 ± 2°C and shall be of sufficient depth as to support the butyrometer in vertical position with their scale completely immersed. The bath shall be fitted with horizontal perforated plates to hold the butyrometer and shall also carry a suitable thermometer.

viii. Gerber Centrifuge. The centrifuge may be hand-driven or electric driven. The centrifuge shall be capable of producing within 2 min when fully loaded, a relative centrifugal acceleration of 350 ± 50 g at the outer end of the butyrometer stopper. This acceleration is produced by centrifuges with the following effective radius (horizontal distance between the centre of the centrifuge spindle and the outer end of the butyrometer stopper) if operated at the speed indicated against each:

Effective Radius (mm)	Revolution Per Min (± 70)
240	1140
245	1130
250	1120
255	1110
260	1100
265	1090
270	1080
275	1070
300	1020
325	980

Note: *The relative centrifugal acceleration (g_n) produced in a centrifuge is given by the following formula:*

$$1.12 \times 10^{-6}\ r\ n^2$$

Where *r = effective horizontal radius in mm, and*
n = speed in revolutions per min

19.2.2. Reagent(s) preparation:

i. Gerber sulfuric acid: Sulfuric acid shall have a density of 1.807 to 1.812 g/ml at 27°C corresponding to a concentration of sulfuric acid from 90 to 91% by mass.

ii. Iso-amyl alcohol ($C_5H_{11}OH$): The iso-amyl alcohol shall have density of 0.803 to 0.805 g/ml at 27°C.

19.2.3. Methodology:

A. Mark the number of sample to be tested legibly on the bulb of the butyrometer.

B. Transfer 10 ml of Gerber sulfuric acid into the butyrometer by means of a 10 ml automatic measure for sulfuric acid,

taking care not to wet the neck of the butyrometer with sulfuric acid.

C. If the sample is fresh, warm it to approximately 27°C and mix thoroughly but do not shake it so vigorously as to cause frothing or churning of the fat. Pour the sample into another clean dry vessel and back to the original. Repeat this process of pouring back and forth until a homogeneous mixture is obtained. Allow the sample to stand for 3 to 4 min after mixing to allow air bubble to rise; invert the sample bottle 3 or 4 times immediately prior to taking milk for the test. (If the sample has aged and there is difficulty in dispersing the cream layer by the above method, warming to 30°C may be necessary for adequate mixing. If the sample shows evidence of slight churning, shown by the presence of white flakes, it should be slowly warmed to 34 to 38°C before mixing as described above. If after this treatment a sample does not appear to be homogeneous, it shall be rejected).

D. Transfer 10.75 ml of well mixed milk sample into the butyrometer by mean of 10.75 ml pipette. The temperature of sample should be brought to approximately 27°C when it is measured. Transfer 1 ml of iso-amyl alcohol into the butyrometer by means of 1 ml automatic measure for iso-amyl alcohol. Do not wet the neck of the butyrometer with alcohol.

E. Close the neck of the butyrometer firmly with the lock stopper without disturbing the contents using lock stopper key.

F. Shake the butyrometer carefully without inverting it until the contents are thoroughly mixed, the curd is dissolved and no white particles or flakes are visible in the liquid. Then invert the butyrometer a few times to mix the contents thoroughly.

G. Transfer the butyrometer quickly, with the bulb uppermost, into a water-bath having a temperature of 65 ± 2°C and leave it there for not less than 5 min. Take care to have the water level in the bath above the top of the fat column in the butyrometer.

H. Take out the butyrometer from water bath, wipe it with a cloth and transfer it to the centrifuge, placing two butyrometers diametrically opposite so as to balance the rotating disc.

I. Centrifuge the butyrometers at a speed of 1100 rpm for 4 minute. Bring the centrifuge to stop gradually. Transfer the butyrometers, stopper downwards, into a water bath having a temperature of 65 ± 2°C and allow the butyrometer to stand in the water bath for not less than 3 min and not more than 10 min.

J. Before taking a reading, adjust the position of the fat column to bring the lower end of the column on to a main graduation mark. Note the scale readings corresponding to the lowest point of the fat meniscus and the surface of separation of the fat and the acid; the difference between the two readings gives the percentage by mass of the fat in the milk.

19.3. Method: Determination of SNF (solids-not-fat) and Total Solids in Milk Using Lactometer

Measurement of specific gravity or density by a lactometer is based on the Archimedes principle. A floating object sinks till it has displaced a weight of fluid equal to its own weight. The greater the volume of displaced fluid, smaller is the density of the fluid and lower is the lactometer reading. The total solids and the SNF content of milk are related to its fat percentage and specific gravity by the Richmond's formula. The specific gravity of normal whole milk is 1.029 to 1.032 while for skim milk it is 1.036.

Milk drawn from the udder contains a large volume of air bubbles and the milk fat undergoes a gradual solidification. Due to these factors a gradual contraction in the volume of milk takes place with a slow increase in specific gravity to a maximum (Racknagal phenomenon). The specific gravity of milk will, therefore, vary with the duration and temperature of storage. This variation may be overcome by ensuring that the fat is completely in the liquid state before the specific gravity reading is taken. This is achieved by pre-warming the milk.

19.3.1. Apparatus

i. Lactometer (ISI lactometer): The lactometer should have the following specification. Dimensions, divisions and tolerances on lactometers. See also **Fig. 19.1.**

S No.	Characteristic	Dimensions/ Divisions/ Tolerances
1	Range covered by scale, specific gravity	1.020 to 1.035
2	Specific gravity equivalent for each subdivision	0.0005
3	Permissible error at any point	±0.0005
4	Number of sub-divisions on scale	30
5	Number of sub-divisions beyond nominal scale of the top graduation	One or nil
6	Length in mm of stem above top graduation mark	20 ± 5
7.	Scale length in mm	41 ± 4
8.	Distance in mm below the lowest graduation mark, where the stem has to remain uniform in diameter, Min	10
9.	External diameter in mm of stem containing scale (approx)	4.0
10.	External diameter in mm of bulb	22 ± 1
11.	Length in mm of uniform stem, Max	80
12.	Volume in ml below bottom graduation line:	
	not more than	37
	not less than	31
13.	Length in mm of the bulb	105 ± 5

ii. Thermometer: A thermometer having the following characteristics is suitable:

a) Overall length 255 ± 10 mm

b) Scale length 105 ± 10 mm

c) Distance between lowest graduation line and bottom bulb 110 ± 5 mm

d) Scale range 10 to 45°C

e) Graduation interval 0.5°C

f) Scale accuracy ± 0.2°C

iii. Lactometer Jars: Cylindrical vessels made of glass or metal having the top finished off square and without a spout, and having the internal diameter of 32 ± 2 mm and internal depth of 185 ± 5 mm.

iiii. Apparatus for the determination of milk fat: As already described for Gerber method.

19.3.2. Reagents:

As required for the determination of milk fat by Gerber method.

19.3.3. Methodology:

A. Preparation of milk sample for lactometer reading

Warm the sample of milk to a temperature of 40 to 45°C and maintain within this range for 5 min during which time the contents of the bottle are adequately mixed. Care shall be taken to avoid the formation of air bubbles or churning of fat when mixing the sample. After this, cool the sample to 27 ± 2°C and held within this range until the specific gravity reading is taken. The sample shall not be held for more than 1 h. The lactometer reading is then taken according to the following procedure.

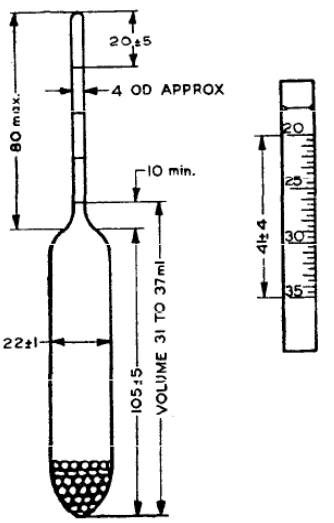

Fig. 19.1: BIS lactometer

B. Determination

a) Invert the sample bottle gently two or three times and then pour down milk in the lactometer jar along its side so as to avoid formation of air bubbles. Sufficient milk should be poured into the jar to ensure that some of it overflows when the lactometer is inserted.

b) The lactometer, held by the stem, is inserted in the sample and released when it is approximately in its position of equilibrium thus avoiding wetting more than a very short length of the stem above the milk surface. As soon as the lactometer is at rest, the scale reading corresponding to the top of the meniscus of milk is noted. The lactometer jar shall be vertical and the bulb of lactometer shall not touch the side. It is advisable to repeat the reading after depressing the lactometer about 3 mm and allowing it to come to rest. Note temperature of milk immediately after taking the lactometer reading with the help of the thermometer. It is generally preferred to take the lactometer reading at 27°C.

c) Determine the fat content of milk by the Gerber method as described previously. Convert the observed lactometer reading to the corrected lactometer reading at 27°C as per the fat percentage and temperature of milk with the help of **Table 19.1.** The % of SNF and total solids in milk is then calculated using the following formula:

Where,

SNF = Solids-not-fat in milk,
CLR = Corrected lactometer reading at 27°C,
F = Fat percentage of milk.

Table 19.1: Correction to be applied to lactometer readings taken at temperature other than 27°C to obtain corrected lactometer reading of milk at 27°C.

Temperature	Fat Percentage of Milk sample				
	0	**2**	**4**	**6**	**8**
19.0	-2.2	-2.4	-2.6	-2.7	-2.9
19.5	-2.1	-2.3	-2.4	-2.6	-2.7
20.0	-2.0	-2.1	-2.2	-2.4	-2.5
20.5	-1.8	-2.0	-2.1	-2.2	-2.3
21.0	-1.7	-1.8	-1.9	-2.0	-2.2
21.5	-1.5	-1.7	-1.7	-1.9	-2.0
22.0	-1.4	-1.5	-1.6	-1.7	-1.8
22.5	-1.3	-1.4	-1.4	-1.5	-1.6
23.0	-1.1	-1.2	-1.3	-1.4	-1.4
23.5	-1.0	-1.1	-1.1	-1.2	-1.3
24.0	-0.8	-0.9	-1.0	-1.0	-1.1
24.5	-0.7	-0.8	-0.8	-0.9	-0.9
25.0	-0.6	-0.6	-0.6	-0.7	-0.7
25.5	-0.4	-0.5	-0.5	-0.5	-0.5
26.0	-0.3	-0.3	-0.3	-0.3	-0.4
26.5	-0.1	-0.2	-0.2	-0.2	-0.2
27.0	**0**	**0**	**0**	**0**	**0**
27.5	+0.1	+0.2	+0.2	+0.2	+0.2
28.0	+0.3	+0.3	+0.3	+0.3	+0.4
28.5	+0.4	+0.5	+0.5	+0.5	+0.5
29.0	+0.6	+0.6	+0.6	+0.7	+0.7
29.5	+0.7	+0.8	+0.8	+0.9	+0.9
30.0	+0.8	+0.9	+1.0	+1.0	+1.1
30.5	+1.0	+1.1	+1.1	+1.2	+1.3
31.0	+1.1	+1.2	+1.3	+1.4	+1.4
31.5	+1.3	+1.4	+1.4	+1.5	+1.6
32.0	+1.4	+1.5	+1.6	+1.7	+1.8
32.5	+1.5	+1.7	+1.7	+1.9	+2.0
33.0	+1.7	+1.8	+1.9	+2.0	+2.2
33.5	+1.8	+2.0	+2.1	+2.2	+2.3
34.0	+2.0	+2.1	+2.2	+2.4	+2.5
34.5	+2.1	+2.3	+2.4	+2.6	+2.7
35.0	+2.2	+2.4	+2.6	+2.7	+2.9

19.4. Method: Determination of Total Solids Content in Milk by Gravimetric Method.

In this procedure (reference method; IS 12333: 1997), a known quantity of milk is dried and from the weight of the residue, the total solids content in milk is determined.

19.4.1. Apparatus

i. Flat-bottomed dishes: Made of aluminum alloy, nickel and stainless steel, 50 to 75 mm diameter, about 20 to 25 mm in height and provided with easily removable but closely fitting lids.
ii. Boling water-bath.
iii. Pipettes: Capacity 10 ml.
iv. Hot air oven: Maintained at 102 ± 2°C, well ventilated.
v. Desiccator with efficient desiccant (e.g. freshly dried silica gel with hygrometric indicators).

19.4.2. Methodology:

A. Heat a dish, with its lids alongside in the oven maintained at 102 ± 2°C for at least 1 h. Place the lid on the dish and immediately transfer to the desiccator. Allow to cool to room temperature (at least 30 min) and weigh it to the nearest 0.1 mg.
B. Pipette into the dish about 5 ml of the prepared sample of milk and weigh quickly, with the lid on the dish.
C. Place the dish without lid on the vigorously boiling water-bath in such a way that the bottom of the dish is maximally exposed to and directly heated by the steam. Leave the dish on the boiling water-bath for 30 min.
D. Remove the dish from the water-bath and then heat it, with its lid alongside in the oven (maintained at 102 ± 2°C) for 2 h. After 2 h, cover the dish with lid and

immediately transfer it to a desiccator. Allow to cool to room temperature for about 30 min and weigh to the nearest 0.1 mg.

E. Repeat the operations of heating (for 1 h), cooling and weighing until the difference in mass between two consecutive weighing does not exceed 1 mg. Record the lowest mass.

19.4.3. Calculation

Total solids content, % by mass = ———

Where,

M = mass in g, of dish and lid;

M1 = mass in g, of the dish, lid and test portion of liquid milk taken for the test; and

M2 = mass in g, of the dish, lid and dried test portion.

Round the value obtained to the nearest 0.01% (m/m).

19.5. References:

IS: 1479. (1960). Methods of test for dairy industry. Part I. Rapid examination of milk. Bureau of Indian Standards, New Delhi.

IS: 12333. (1997). Milk, Cream and Evaporated milk – determination of total solid content. Reference method (First revision). Bureau of Indian Standards, New Delhi.

IS: 9385. (1980). Indian Standard, Specification for lactometers. Bureau of Indian Standards, New Delhi.

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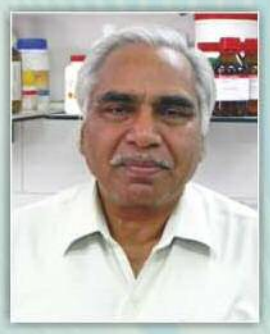

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