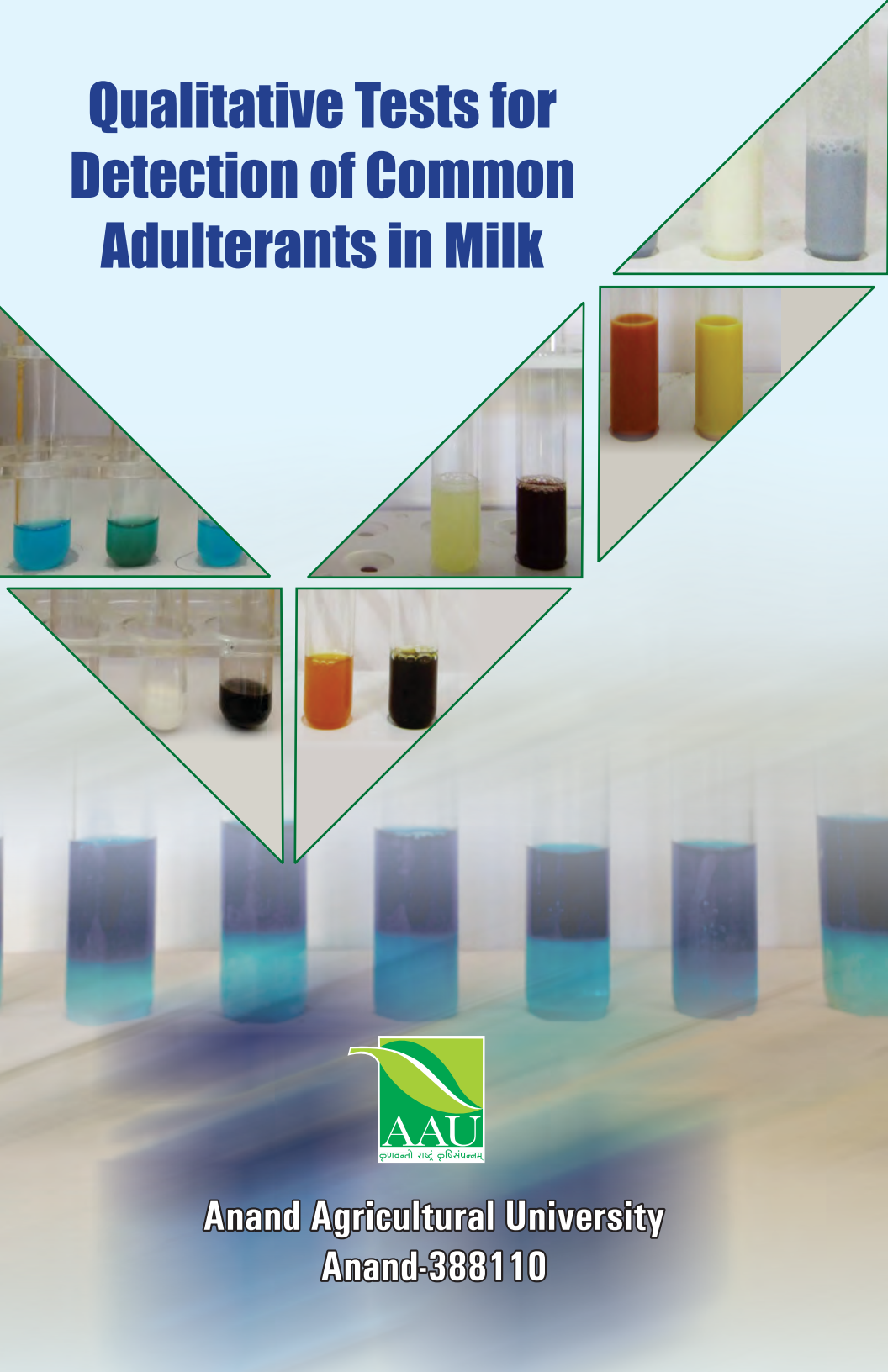# Qualitative Tests for Detection of Common Adulterants in Milk

Anand Agricultural University
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Qualitative Tests for Detection of
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Dr. R.V. Vyas
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MESSAGE

India is world's largest milk producing country with per capita milk availability of 394 g/day. This is highly significant from the point of view of health and nutrition security of citizens. However, a lot more is required to be done in the area of quality and safety of milk and milk products. The concerns for human health and environment are getting national and international attention owing to increased awareness in consumers. Milk being costly and daily used essential item, it is vulnerable to adulteration. Adulteration of milk is posing serious threat to human health, concerted efforts are needed to prevent it, including checking and ensuring safe food to the consumers.

Dairy Chemistry Department at SMC College of Dairy Science, Anand Agricultural University, Anand rightly undertook the research work on evaluation and modification/improvement in certain qualitative tests used for detection of common adulterants in milk. The work led to screening and improvement in several reported qualitative tests and some of which are also adopted at national level by FSSAI. As Indian dairy industry is still characterized by marginal farmers and unorganized sector, these tests assumes immense significance for detection of common adulterants encountered in milk and milk products. I believe this to be a step in right direction for pure and safe dairy products.

I heartily congratulate the team involved in the research work for doing broad survey of literature, exhaustive research and bringing out this booklet "Qualitative Tests for Detection of Common Adulterants in Milks" at the opportune time. I am hopeful that the information compiled will be of great use to dairy industry personnel and regulatory authorities.

Date : 20/07/2020   **(R.V.Vyas)**

Dr. J.B. Prajapati
Principal & Dean

FOREWORD

Milk is considered nutritionally inseparable part of human diet since time immemorial. Human beings consume milk throughout their life span in order to build and sustain a strong and healthy body. Milk contains several vital nutrients, including proteins, carbohydrates, fats, minerals, vitamins and several biologically active substances. Considering healthy image of milk products in the society and rising prosperity, consumption of milk and milk products is growing steadily in India. However at the same time for people, safety of milk and milk products also becomes of paramount importance. One of the serious problems of safety with milk and milk products is menace of adulteration.

Several measures including regulatory control, increasing awareness and extensive testing/analysis of milk by dairy industry personnel are in place to control adulteration. However, still it is going on and hence qualitative and quantitative tests for analysis of milk, which are precise and effective are most desired. Qualitative tests used for detection of common adulterants in milk possesses several advantages like better specificity, better sensitivity, better reliability and affordability in comparison to some of the instrumental methods employed at field/plant level. There are several tests in practice, but there were large variations in methodology and clear detection was difficult. Looking to these difficulties and a strong need for optimized methods, our Dairy Chemistry department took initiative to conduct detailed survey of such methods and took up research and development work to optimize several tests for most of the adulterants. The work of some of the tests was validated by NDDB and has been recommended to FSSAI to include in their manual.

This book entitled "Qualitative Tests for Detection of Common Adulterants in Milk" is the culmination of the extensive research work undertaken. This book will be of immense help to the industry in ensuring safe and adulteration free milk and milk products to consumers.

(J. B. Prajapati)

PREFACE

Milk is considered nature's most complete food; which is liked by all age group population. As people are becoming health conscious, the demand of milk and milk products is steeply rising. However, still people show concern for quality and safety of milk available in various part of our country. Thus, adulteration is one of the major hurdles against the progress of dairying, since it has serious detrimental effect on human health. The most important reason for widespread adulteration of milk is its highly heterogeneous and very complex physicochemical nature, because of which it can hide many things when added to it. Thus, milk can be adulterated easily and in many ways that affect quality and safety of the milk and milk products.

Considering the seriousness of the issue of milk adulteration tests for extensive work was undertaken on various aspects of qualitative tests used for detection of common adulterants in milk, at Dairy Chemistry Department, SMC College of Dairy Science, Anand Agricultural University, Anand. By intensive research work remarkable success is achieved in overcoming several limitations faced in application of the qualitative tests. The study has resulted in modification/development of several qualitative tests with improved clarity in result, better sensitivity, and replacement of some hazards/prohibited/costly/unstable chemicals and simplification of some procedures.

We are hopeful that the information compiled herein will greatly benefit the dairy industry personnel and regulatory agencies for ensuring safety of milk and milk products. This is an effort in the direction of improvement of the quality of milk and milk products by providing reliable, sensitive and improved methods for detection of adulteration of milk.

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Mr. S. I. Patel

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Contents

1. Introduction

Milk is considered one of the most complete food consumed by humans since time immemorial. Milk and dairy products have become a major part of the human diet and a substantial amount of our food expenditures goes on milk and dairy products. Quality of milk and milk products has thus attracted considerable attention in the recent years (Harding, 1995). India is the largest milk producer in the world with an output of 187.7 million tonnes (MT) recorded in 2018–19 (Department of Animal Husbandry & Dairying, 2019). Though, to ensure quality milk and milk products for Indian consumers is a challenge owing to various factors. Poor raw milk quality, largely unorganized dairy sector, poor infrastructure and adulteration are considered some of the major hurdles for Indian dairy sector. Adulteration in market milk implies addition of any substance to normal milk or removal of any of its constituents with an intent to deceive the consuming public and derive extra profit from a given volume of milk (Singhal *et al.*, 1997). Adulteration is one of the most predominant phenomenon endangering safety of dairy products in the developing world in general and India in particular.

Under the Food Safety Regulation (2011), milk is defined as the normal mammary secretion derived from complete milking of healthy milch animal without either addition thereto or extraction therefrom. Thus, no extraneous addition of any substance in milk is permitted under legal provisions. Further as per FSS regulations an adulterant is defined as "Any material which is or which could be employed for making food unsafe or substandard or misbranded or containing extraneous matter".

Reasons for adulteration of milk and milk products

Several factors are responsible for prevalence of milk adulteration including demand and supply gap, physical nature of milk, degraded moral of the society, spoiled socio-economic structure, perishable nature of milk, low purchasing power of customer, unorganized condition of dairy industry, lack of strict and effective regulatory system and lack of suitable, rapid and sure tests (Srivastava, 2010). The important reason for wide spread adulteration of milk is its physical and chemical nature, due to which it can hide many things when added to it. Thus milk can be adulterated easily and in many ways that affect its quality and safety of dairy products manufactured

(Kamthania *et al*., 2014). Some reasons for prevalence of milk and milk products adulteration are discussed hereunder;

- To make more profit: Since ancient times, the tendency of human has remained to earn more money out of his business/profession. However, when people use unscrupulous means to earn more money it leads to societal harm.

- Degraded moral of the society: Degradation in the morality of the society is an observed phenomenon. There is an increase in greed, selfishness, indifference and exploitation of the society that is feverishly acceptable these days.

- Gap between demand and supply in some pockets of the country: At times there is a mismatch between supply and demand. In summer there is a dip in the milk production; however, demand of milk increases or remains constant. This opens up the space for fraudsters for adulterating the milk.

- Low purchasing power of the people: Sizeable Indian population still do not possess adequate money to buy good quality milk and milk products. This condition is exploited by various local milk handlers.

- Competition to capture more market: During the periods when milk production is limited, some milk sellers are trying to reach more customers by way of adulterating available milk.

- Physical nature of milk: Milk can hide many things due to its inherent nature. It becomes visibly difficult to identify genuineness of milk.

- Less share of organized dairy sector in dairy business: Nearly 80% of the Indian dairy sector is dominated by unorganized sector. This leads to inadequate infrastructure for systematic handling of milk and milk products.

  Where there is lack of cooling facilities, hot weather makes it difficult to deliver milk (a perishable commodity) without deterioration in quality. There is often a temptation on the part of farmers, middlemen and retailers to increase the milk available to them when supplies are short, either by water or skimmed milk addition.

- Lack of suitable, rapid and sure tests: Indian dairy industry

is still characterized by scattered milk production and lack of infrastructure. The problems are compounded by unavailability of rapid, sure and convenient test for testing adulteration of milk.

- Existence of several species and breed of milk animals: This leads to enormous compositional variations in the milk available. Formulation of breed wise standards is practically difficult. Thus, compositional differences are used to deceive consumers and authorities for adulteration purposes.

- Difficulties in enforcement of the available regulatory standards: Existence of several breeds but no individual standards, lack of sufficient staff, complicated legal process are also some important reasons for prevalence of adulteration in milk and milk products.

Types of adulterants:

(1) Addition of cheaper ingredients like water, skimmed milk, synthetic milk, vegetable oils, etc.

(2) Separation of costly ingredient *i.e.* fat.

(3) To improve keeping quality, addition of preservatives and neutralizers.

(4) To improve physical characteristics

- Addition of thickening agents to increase viscosity and specific gravity.

- Colouring agents to mimic natural colour of milk.

(5) Interspecies adulteration: Addition of buffalo milk in cow milk followed by addition of some masking agent.

Adulterants in milk can be generally classified into two major groups of substances. The first group comprises of those substances whose purpose is to increase the economical yield and the second group comprises of those substances whose purpose is to increase/extend the storage of milk by delaying its spoilage (Cerdfin *et al.,* 1992). In economic adulteration of milk when water is mixed, other additional substances are also necessitated in order to conceal the watering. The selection of these substances is carried out in such a way that visual appearance, some of the common physical properties (e.g. density and viscosity) and gross chemical composition are simulated to that of the genuine milk. To make up the fat content, cheaper vegetable

oils often of dubious quality are added. To raise the SNF and mask dilution mixture of carbohydrates, non-protein-nitrogenous compounds and some selected salts are added. From carbohydrate glucose, sucrose, maltodextrin, starch and/or cellulose may find their way in to milk. For simulating the protein in terms of nitrogen content, use of ammonium sulphate, urea or such other compounds are encountered. In addition other miscellaneous adulterants like salt, detergent, gelatin, colouring matter, neutralizers, etc. are also mixed.

White coloured milk-like fluid (so called "synthetic milk") is produced by blending a well-designed assortment of vegetable oil, detergent, urea, sugar, neutralizers and water for adulteration of milk. This fluid is partially blended with pure milk to earn profit. Since, it is made with several chemicals of dubious nature it poses serious health implications to consumers.

Detergents

Detergent is considered one of the indispensable part of so called synthetic fluid added in milk for the purpose of emulsification of cheap foreign fat. Generally anionic detergents are used in such milk-like preparations owing to their low cost and easy availability. The detergent in adulterated milk can cause food poisoning and harm gastrointestinal tract in human body. Further, its alkaline nature can also damage certain body tissues and proteins.

Urea

The most widely followed practice is to adulterate milk with water and subsequently adding urea to raise solids-not-fat (SNF). Urea is also observed to be one of the major ingredients of so called synthetic milk. Additionally urea being nitrogenous compound will give false high level of protein if milk is analyzed by Kjeldahl method. Whereas FSSAI has set the maximum limit of urea content in milk at 70 mg per 100 ml; adulteration with urea can cross this limit increasing proneness to health issues.

Ammonium sulphate

The ammonium salts like ammonium chloride, ammonium sulphate, ammonium nitrate and ammonium dihydrogen orthophosphate are being added to milk to raise its SNF. Like urea, ammonium sulphate is a chemical fertilizer, which is added to milk adulterated with water

to raise the lactometer reading (Sharma *et al.,* 2012 and Kamthania *et al.*, 2014).

Glucose

Both glucose and glucose syrup of dubious quality is inexpensively available. As being cheap and able to blend easily with milk imparting sweet taste; its use is rising for the purpose of adulteration. Glucose is used for increasing solids content of milk. It is difficult to detect glucose adulteration in milk apparently due to high solubility and sweet taste.

Sucrose

Sucrose has long been used to practice double deception as it is added to raise solid not fat (SNF) after watering of milk and also contributes to sweet taste of milk. Even, recently sucrose was found to be one of the most popular adulterant in milk received at milk collection centers. As milk solids are quite costly compared to the cane sugar; adulterating milk with cane sugar will earn the unscrupulous milk producer more profit.

Maltodextrin

Maltodextrins are nutritive carbohydrates polymers having Dextrose Equivalent (DE) value less than 20. It is obtained by either chemical or enzymatic hydrolysis of starches. Maltodextrins are classified based on the amount reducing sugar relative to total carbohydrates; which range from 3 to 20 percent (Hofman *et al.*, 2016). Maltodextrin is highly soluble in water with the solubility of about 1.2 kg per litre; it is used primarily in foods and beverages as a thickener, sweetener, and/ or stabilizer (Anon., 2018). As maltodextrin imparts certain important functional properties like bulking, gelling, binding, crystallization prevention, promotion of dispensability, freezing control, it is used in various dairy products such as yoghurt, ice-cream, milk powders, cheeses and in indigenous milk products such as burfi (Chronakis, 1998). Maltodextrin has been reported to be added as an adulterant in milk, mainly to increase its lactometer reading and also to increase the yield of the product prepared from it such as khoa and burfi.

Starch

Starch is cheaply available in various forms such as wheat flour, corn flour and commercially manufactured starch. Starch or cereal flours,

may be added to makeup the density of diluted milk and interfere with the detection of water (BIS, 1960). Starch is seldom added in the pure form to adulterate the milk. Often poor quality starches as well as certain flours like arrowroot, wheat flours, mashed potato, etc. are used as adulterant. Due to ease with which starchy material can get mixed in milk and certain milk products like khoa, burfi, peda, etc., its adulteration is quite prevalent. Starch adds to the weight of the milk and milk products for deriving profit.

Gelatin

Gelatin, for many years has been used as a thickening agent in various dairy products including ice cream, where it is used to improve the texture and overall quality of the product. However, it is not permitted legally in India for use in dairy products.

Sodium chloride (common salt)

Sodium chloride (common salt) may be added to milk to increase the lactometer reading *i.e.* to show increased SNF content. Generally, it is added to milk in small quantities to hide watering.

Nitrate

Presence of nitrate is one of the indication of the pond water/surface water addition in milk. Pond water is heavier than the tap water; it is usually preferred by some unscrupulous persons for adulteration in milk. In the pond water nitrates may come from fertilizers used in the fields. Sodium and potassium nitrates are oxidizing agents and hence act as preservative.

Sulphate

Milk contains trace levels of sulphates naturally. However, sulphate is added to falsely increase the SNF.

Salicylic acid and benzoic acid

Salicylic acid and benzoic acid are colourless crystalline organic acids which are sometimes used as a preservative in milk. Consumptions of such acids leads to various harmful health implications.

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is commonly used as a food preservative in milk or as a sterilant in packaging materials due to its inherent sporicidal and bactericidal properties. Excess of hydrogen peroxide

can bring deleterious effects on the nutritional value of milk such as the degradation of folic acid, which is an essential vitamin to human body. Moreover, the ingestion of hydrogen peroxide at high levels can cause severe gastrointestinal problems. Hydrogen peroxide is not permitted in milk as per the Food Safety and Standards Regulations in India.

Formaldehyde

Formaldehyde is commercially available as a 37–40% (w/v) aqueous solution, commonly referred to as 'formalin'. The addition of formaldehyde to milk decreases the bacterial content and prolongs the keeping quality. Formalin is permitted for analytical sample preservation purposes as per FSS Regulations, 2011 at 0.4% maximum level. When added in such small amounts, formalin changes neither the odour nor the taste of milk. Formalin is a dangerous and poisonous chemical, which has serious implications on human health and is considered a carcinogen and hence it is prohibited as preservative in milk. Moreover, it affects the quality of the milk products as it binds with proteins and affecting their functionality.

Borax and boric acid

Boric acid or borax is used as preservative in milk and milk products, however it is not permitted as per FSSAI. In addition, regular intake of boric acid can adversely affect the stomach, intestines, liver, kidney, and brain.

Neutralizer

Milk sold in the cities is being transported often from remote villages covering long distances. India being tropical country, chances of spoilage of milk is very high if cooling facility is not available. In such circumstances unscrupulous people resort to mixing of neutralizer substances like sodium hydroxide, sodium carbonate and sodium bicarbonate. Neutralizer apparently delays the spoilage. Legally, neutralizers are not permitted as they poses several health hazards.

2. Analytical Techniques for Detection of Adulteration in Milk

Various measures are taken to contain the menace of adulteration; including regulatory monitoring, enhancing awareness in the communities, extensive testing/analysis of raw milk, etc. Analysis is one of the essential part of overall quality assurance system operated by dairy plants. Various analytical methods are used for the purpose of checking adulteration of milk including physical methods, instrumental methods and chemical methods.

Physical methods

Methods based on physical properties of milk are density (lactometer reading), freezing point, refractive index, etc, which are easy to perform, but can be very easily manipulated due to natural variations in milk composition. Physical methods are simple, fast, easy, cheap and convenient. However, sensitivity of these tests are less in comparison to chemical and instrumental methods.

Freezing point can be significantly affected by seasonal and regional factors. Thus, considering geographical vastness of India and consequent seasonal and regional variation it cannot be a reliable means of adulteration detection.

The density (or specific gravity) depends on composition, temperature and temperature history of milk. As Indian dairy sector is still predominantly unorganized in nature; it is difficult to control most parameters affecting density. Therefore, density measurement cannot be a useful tool for adulteration detection.

Thus, physical methods suffer from some of the general limitations due to large natural variations, lower sensitivity, poor specificity, proneness to manipulation, etc.

Instrumental methods

Instrumental methods are one of the good option for quality control of milk and milk products. Though, it possesses several advantages like higher sensitivity, high specificity and reliability, it also suffers from several limitations as described below.

- Very limited adoptability for practical applications.
- Requires high initial investment, operational cost and expensive maintenance.
- Most methods are time consuming as it necessitates isolation, purification, concentration &/or derivatization of the target analyte.

- Impractical for routine analysis and field applications.

As these methods require skilled manpower it is difficult to use as routine methods. Indian dairy industry is still characterised by small scale farming and small cooperative societies which may not be able to afford capital requirements for sophisticated instruments as well as its maintenance.

Chemical methods

The chemical methods are simple, fast, easy, cheap, convenient and have better specificity for adulterant/ chemical compounds being tested. However numerous qualitative tests are reported for detection of adulterants in milk with wide variation in procedure for a given test. There is lack of information regarding sensitivity between various reported qualitative tests. To overcome these limitations various reported tests and procedural variations were evaluated. The list of various reported tests with procedural variations is given in the Table 1. The test which was found most suitable is also mentioned.

Table 1: List of qualitative tests reported for detection of common adulterants in milk

Sr. No.	Adulterant and test(s) used for its detection	Reference (procedural variations in the tests for the same adulterant)
1	**Detergent**	
	(1) Methylene blue test	(i) Paradkar *et al.* (2000b)
		(ii) Sharma *et al.* (2012)
		(iii) FSSAI (2016)
	(2) Azure A dye test	Barui *et al.* (2012)
	(3) Bromocresol purple test	Anon. (2006), Singh *et al.* (2012), Kamthania *et al.* (2014) and Dixit (2012)
	(4) Lather test	FSSAI (2014)
	Note: Methylene blue test reported by Paradkar *et al.* (2000b) was found to be the best.	
2	**Urea**	
	(1) DMAB test	(i) Bector (1998), BIS (2006), Anon. (2009), Draaiyer *et al.*(2009), Sharma *et al.*(2012), Kamthania *et al.*(2014), FSSAI (2016)
		(ii) Sharma *et al.* (2012)

	(2) Urease test	(i) Paradkar *et al.*(2000)	
		(ii) DGHS (2005), Anon.(2009), Srivastava (2010)	
		(iii) Singh *et al.* (2012), Dixit (2012) Kamthania *et al.,* (2014)	
	(3) Phenol test	(i) Vishweshwar and Krishnaiah (2005)	
		(ii) Kamthania *et al.*(2014)	
	Note: DMAB test reported by FSSAI (2016) was found to be the best.		
3	**Ammonium compound**		
	(1) Phenol Test	(i) Mittal and Roy (1976), Srivastava (2010), FSSAI (2016)	
		(ii) Dixit (2012)	
		(iii) Kamthania *et al.*(2014)	
	(2) Nessler's test	Sharma *et al.*(2012), FSSAI (2016)	
	(3) Turmeric paper test	Sharma *et al.*, 2012	
	Note: Nessler's test reported by Sharma *et al.* (2012) was found to be the best.		
4	**Glucose**		
	(1) Barfoed's test	(i)Roy & Mittal (1977), Vishweshwar & Krishnaiah (2005) Anon. (2006), Singh *et al.* (2012), Sharma *et al.*(2012), Dixit (2012), Kamthania *et al.*(2014), FSSAI (2016)	
		(ii) Anon. (2009), Srivastava (2010)	
		(iii) Shaikh *et al.* (2011)	
	(2) Diastix strip	Anon. (2006), Srivastava (2010), Singh *et al.* (2012)	
	Note: Barfoed test reported by FSSAI (2016) was found to be the best.		
5	**Sucrose**		
	(1) Seliwanoff test (Resorcinol powder)	(i) Anon. (2006), Dixit (2012), Singh *et al.* (2012)	
		(ii) BIS (1960), BIS (1981), Draaiyer *et al.*(2009)	
		(iii) Sharma *et al.*(2012)	
		(iv) Kamthania *et al.*(2014)	
	(2) Seliwanoff test (Resorcinol solution)	(i) Anon. (2009)	
		(ii) Srivastava (2010)	
		(iii) Sharma *et al.*(2012)	
	Note: Seliwanoff's test reported by Srivastava (2010) was found to be the best.		

6	**Maltodextrin**	
	(1) Iodine test	Sharma *et al.* (2012)
	(2) Barium chloride test	Draaiyer *et al.*(2009) Anon. (2009)
	Note: Iodine test reported by Sharma *et al.* (2012) was found to be the best.	
7	**Starch**	
	Iodine test	(i) Sommerfeld (1901)
		(ii) BIS (1960), Anon. (2006), Dixit (2012), Sharma *et al.*(2012)
		(iii) Anon.(2009), FSSAI (2016)
		(iv) Sharma *et al.* (2012)
	Note: Iodine test reported by BIS (1961) was found to be the best.	
8	**Common salt (Sodium chloride)**	
	Silver nitrate test	(i) Anon. (2006), Singh *et al.* (2012), Dixit (2012)
		(ii) Sharma *et al.* (2012), FSSAI (2016)
		(iii) Anon. (2009), Srivastava (2010), Kamthania *et al.* (2014)
	Note: Silver Nitrate test reported by FSSAI (2016) was found to be the best.	
9	**Nitrate**	
	(1) Diphenylamine test	(i) FAO (1986)
		(ii) Sharma *et al.*(2012)
		(iii) Srivastava (2010)
	(2) Diphenylamine sulphate test	BIS (1981)
	Note: Diphenylamine test reported by FAO (1986) was found to be the best.	
10	**Sulphate**	
	Barium chloride test	Sharma *et al.* (2012), FSSAI (2016)
	Note: Barium chloride test reported by FSSAI (2016) was found to be the best.	
11	**Hydrogen peroxide**	
	(1) *p*-Phenylendiamine test	(i) BIS (1981), Sharma *et al.* (2012), Kamthania *et al.* (2014), FSSAI (2015)
		(ii) Draaiyer *et al.* (2009)
		(iii) Dixit (2012)
	(2) Iodometry test	(i) Luck (1962)
		(ii) Sharma *et al.* (2012), FSSAI (2016)
	(3) Vanadium pentoxide test	Luck (1962)
	Note: Iodometric test reported by FSSAI (2016) was found to be the best.	

12	Formaldehyde	
	(1) Leach test	(i) Williams & Sherman (1905), BIS (1961), Vishweshwar & Krishnaiah (2005)
		(ii) Farrington and Woll (1918)
		(iii) Sharma *et al.* (2012)
	Note: Leach test reported by BIS (1961) was found to be the best.	
	(2) Hehner Test	(i) BIS (1960), Singh *et al.*(2012), Dixit (2014)
		(ii) Vishweshwar & Krishnaiah (2005)
		(iii) Draaiyer *et al.* (2009)
		(iv) Kamthania *et al.* (2014)
	(3) Chromotropic acid test	BIS (1961)
	(4) Phenylhydrazin hydrochloride test	BIS (1961)
	(5) Methylene blue reduction test	Schardinger (1902) as cited in Fay (1935)
	Note: Hehner test reported by Draaiyer et al. (2009) was found to be the best.	
13	**Neutralizers**	
	(1) Rosolic acid test	(i) Farrington & Woll (1918), BIS (2006), Anon (2006), Draaiyer *et al.*(2009), Singh *et al* (2012), Dixit (2012), Kamthania *et al.* (2014)
		(ii) Paradkar *et al.* (2001)
		(iii) DGHS (2005)
		(iv) Sharma *et al.*(2012), FSSAI (2016)
		(v) Chakraborti & Raghav (2000)
	(2) Methyl alcohol test	Davies (1938)
	(3) pH determination	Davies (1938), BIS (1961)
	(4) Change in pH on boiling	Davies (1938)
	(5) Alkalinity of ash	BIS (1960), Singh *et al.*(2012), Dixit (2012)
	Note: Rosolic acid test reported by DGHS (2005) was found to be the best.	

3. Qualitative Tests for Detection of Adulterants in Milk - Modified by AAU

Upon review and evaluation of qualitative tests available for detection of common adulterants reported in milk, it was observed that there is wide variation related to several test performance parameters like sensitivity, convenience, cost etc. This variation in performance was mainly attributable to variations in the procedures of the test. Further, it also appeared from the survey of literature that scant attention has been paid on systematic work for improving performance of the qualitative tests for detection of adulterants in the milk. Thus, it was envisaged to undertake work for improving some qualitative tests suggested for detection of common adulterants encountered in milk. The qualitative tests were optimized considering three different aspect of the test procedures.

1. To select suitable medium for performing the tests in detection of adulterants.

2. To standardize various chemicals/reagents used in the tests.

3. To optimize different conditions involved in performing the tests.

Considering the requirements for improving the performance of existing qualitative tests reported for common adulterants, including detergent, urea, ammonium salts, glucose, sucrose, maltodextrin, starch, hydrogen peroxide, salt, nitrate, sulphate, formaldehyde and neutralizers were modified.

Sr. No.	Adulterants	Details of tests selected for optimization/ modification	
		Test	**Reference**
1.	Detergent	Methylene blue	Paradkar *et al.* (2000)
2.	Urea	DMAB	FSSAI (2016)
3.	Ammonium salts	Nessler	Sharma *et al.* (2012)
4.	Sucrose	Seliwanoff	Srivastava (2010)
5.	Glucose	Barfoed	Barfoed (1873)
6.	Maltodextrin	Iodine	Sharma *et al.* (2012)
7.	Starch	Iodine	BIS (1960)
8.	Salt	Silver nitrate test	FSSAI (2016)
9.	Nitrate	Diphenylamine	FAO (1986)
10.	Sulphate	Barium chloride	FSSAI (2016)
11.	Hydrogen peroxide	Iodometric test	FSSAI (2016)

12.	Formaldehyde	Leach	BIS (1961)
		Hehner	Draaiyer *et al.* (2009)
13.	Neutralizers	Rosolic acid	DGHS (2005)

Procedures of the qualitative tests optimized by the Anand Agricultural University, Anand are described hereunder. The procedures of the qualitative tests given by FSSAI and other workers are given in the **Chapter 4**.

3.1 DETECTION OF DETERGENTS BY METHYLENE BLUE TEST

Methylene blue is cationic dye which forms complex with anionic detergents. It is normally water soluble compound; however it shows affinity for anionic detergents, if they are present. In this method, detergent is first extracted in chloroform and then methylene blue solution is added. In presence of detergent blue colour is developed in chloroform layer of the sample, whereas blue colour is observed in milk layer in control (pure milk). Chloroform is heavier (density 1.49 g/ml) than milk (density ~1.030 g/ml), hence settles at the bottom. This implies that observation of blue colour in the bottom layer indicates presence of detergents.

Reagents:

1) Methanol (AR)

2) Methylene blue solution: 12.5 mg methylene blue (AR) is dissolved in 100 ml of distilled water. Protect the solution from direct sunlight.

3) Chloroform (AR):

   Precaution: Inflammable and toxic on inhalation. Mouth pipetting is not recommended

Procedure:

1) Take 2.5 ml of suspected milk sample in a test tube and add 7.5 ml methanol.

2) Filter the content through Whatman No. 1 filter paper.

3) Take 2 ml filtrate in a test tube.

4) Add 2 ml of methylene blue solution and shake well.

5) Subsequently add 4 ml chloroform and shake well again.

6) Allow the chloroform layer to separate.

7) Compare the colour extracted in the chloroform layer in suspected milk with that for pure milk.

Interpretation : If the methylene blue colour extracted from a suspected sample into the chloroform layer is darker than that extracted from pure milk sample, it indicates the presence of detergent in milk.

Limit of detection : 0.02 g/ 100 ml milk

(**Note**: The method reported by Paradkar *et al.* (2000b) is modified. Methanol was used in place of ethanol. Methylene blue concentration was reduced from 25 mg/100 ml to 12.5 mg/100 ml.)

Benefits of modified method over reported method

-   Better differentiation between adulterated and pure sample

-   Eliminates use of ethanol (a regulated chemical)
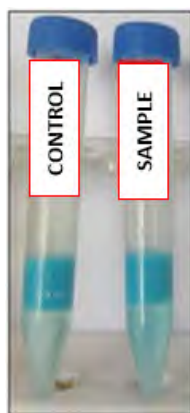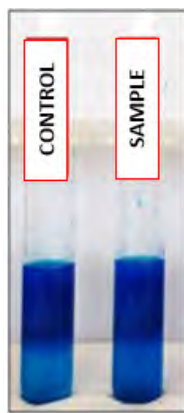
Methylene Blue Test FSSAI (2016)   **Methylene Blue Test Paradkar (2000)**   **Methylene Blue Test Modified-AAU (2018)**

Fig. 1: Test of Detergents

3.2 DETECTION OF UREA BY DMAB TEST

A yellow coloured complex is formed between urea and *p*-dimethyl amino benzaldehyde (DMAB) reagent in low acidic alcoholic solution at room temperature. The intensity of colour can be measured at 440 nm (Lafier, 1996). The colour developed is in proportion to urea content in the sample.

Reagents:

p-Dimethylaminobenzaldehyde reagent (DMAB): The reagent is prepared by dissolving 1.6 g of *p*-dimethylaminobenzaldehyde (AR) in methanol (AR) subsequently adding 10 ml of concentrated HCl (AR) and making volume to 100 ml with methanol.

Procedure:

1) Take 5 ml milk in a test tube.

2) Add 5 ml DMAB reagent.

3) Mix the content and observe the colour.

Interpretation : Formation of distinct yellow colour indicates the presence of added urea in milk sample. Pure milk shows light yellow colour due to natural urea.

Limit of detection : 0.2 g/ 100 ml milk
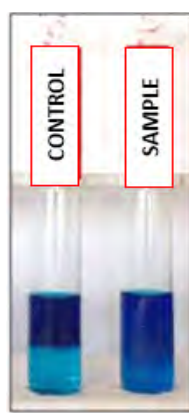
Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample.

- Eliminates use of ethanol (a regulated chemical).

(**Note**: The method reported by FSSAI (2016) is modified. Ethanol was replaced with methanol. Instead of 1 ml each of milk sample and DMAB reagent, 5 ml of each is taken.)

DMAB Test
FSSAI (2016)

DMAB Test (Milk)
Modified- AAU (2018)

Fig. 2: Test of Urea

3.3 DETECTION OF AMMONIUM SALTS BY NESSLER'S TEST

Nessler's test is one of the classical methods for qualitative and quantitative analysis of ammonia and ammonium ions. Nessler's reagent is an alkaline solution of potassium mercuric iodide (K_2HgI_4). On reaction with ammonium ion Nessler's reagent produces a yellowish brown colour (Sarkar and Ghosh, 1956). The intensity of the colour is directly proportional to the amount of ammonia/ammonium ion present.

Reagents:

1) Nessler's reagent: Dissolve the following chemicals separately.
   a) 8.0 g of mercuric chloride (AR) in 150 ml distilled water.
   b) 60.0 g of sodium hydroxide (AR) in 150 ml distilled water.
   c) 16.0 g of potassium iodide (AR) in 150 ml distilled water.

   Add reagent 'a' to reagent 'b' and mix well. To this mixture, add reagent 'c', mix and dilute the contents to 500 ml with distilled water. Leave this solution undisturbed and decant the clear upper layer of the solution. Store in a stoppered amber glass bottle.

   Note: Alternatively commercially available (readymade) Nessler's reagent can also be used.

2) Citric acid solution (5%): Dissolve 5 g citric acid monohydrate (AR) in distilled water and make up the volume to 100 ml with distilled water.

Procedure:

1) Take 20 ml milk in a conical flask.
2) Warm the milk to 70-80 °C either on direct flame or water bath.
3) Add 5% citric acid solution drop wise in the milk with gentle stirring till visible coagulation occurs. Flask if stirred vigorously will result in fine curd particles and which may impact colour observation.
4) Filter the content using Whatman No. 1 filter paper.
5) Take 5 ml of filtrate into a test tube.
6) Add 0.4 ml of Nessler's reagent.
7) Observe the colour without shaking the test tube.

Interpretation : Carefully observe instant development of orange colour in milk adulterated with ammonium salts. Whereas pale yellow colour indicates unadulterated milk.

Limit of detection : 0.02 g/ 100 ml milk

Benefits of modified method over reported method.

- Improved sensitivity over the reported method.
- Better differentiation between adulterated and pure sample.

(**Note**: The method reported by Sharma *et al.* (2012) is modified. Milk is coagulated using citric acid and filtrate is used for testing. The amount of reagent added is reduced to 0.4 ml from 1 ml.)

Phenol Test
FSSAI (2016)

Nessler's Test
Sharma *et al.* (2012)

Nessler's Test
Modified- AAU (2018)

Fig. 3: Test for Ammonium Salts

3.4 DETECTION OF SUCROSE BY SELIWANOFF'S TEST

Sucrose is a disaccharide containing glucose and fructose (a ketose sugar). Seliwanoff test is used for detection of ketoses. The dilute hydrochloric acid used in Seliwanoff reagent along with heat leads to hydrolysis of sucrose and subsequent dehydration of fructose. Further keto group more actively attacks resorcinol in comparison to aldehyde group. The dehydration product 5-hydroxymethylfurfural condenses with resorcinol forming cherry red colour. Ketoses react rapidly in comparison to aldoses because dehydration of aldoses to 5-hydroxymethylfurfural proceeds in a much slower way than the reaction of ketoses.

Reagents:

Resorcinol solution (0.05%): The reagent is prepared by dissolving 0.05 g of resorcinol (AR) in 100 ml hydrochloric acid (The acid is prepared by taking 30 ml conc. HCl and diluting to 100 ml with distilled water.).

Procedure (using milk as medium):

1) Take 3 ml milk and 5 ml resorcinol solution in a test tube.

2) Keep the content in boiling water bath for 6 min.

3) Cool the tubes immediately after heating under tap water to retard the rate of reaction, which if not done would narrow the colour difference between negative and positive samples.

4) Observe for colour development.

Procedure (using whey as medium):

1) Take 3 ml milk and 5 ml resorcinol solution in a test tube. (quantity of both milk and reagent can be doubled proportionately to get sufficient filtrate.)

2) Mix and filter the content using Whatman No. 1 filter paper.

3) Keep the filtrate in boiling water bath for 4 min.

4) Cool the tubes immediately after heating under tap water to retard the progress of reaction, which if not done would narrow the colour difference between negative and positive samples.

5) Observe for colour development.

Interpretation : Development of red colour indicates adulteration of sucrose in milk. The intensity of red colour increases with increase in the sucrose content in the milk. Pure milk remains light in colour.

Limit of detection :

0.1g/ 100 ml milk (When test is performed in milk)

0.06 g/100 ml milk (When test is performed in whey)

Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample.

(**Note**: The method reported by Srivastava (2010) is modified. Different concentration of hydrochloric acid (30 ml conc. hydrochloric acid diluted to 100 ml) was used instead of reported concentration (1 part HCl: 2 parts distilled water). The test was also modified using whey as medium.)
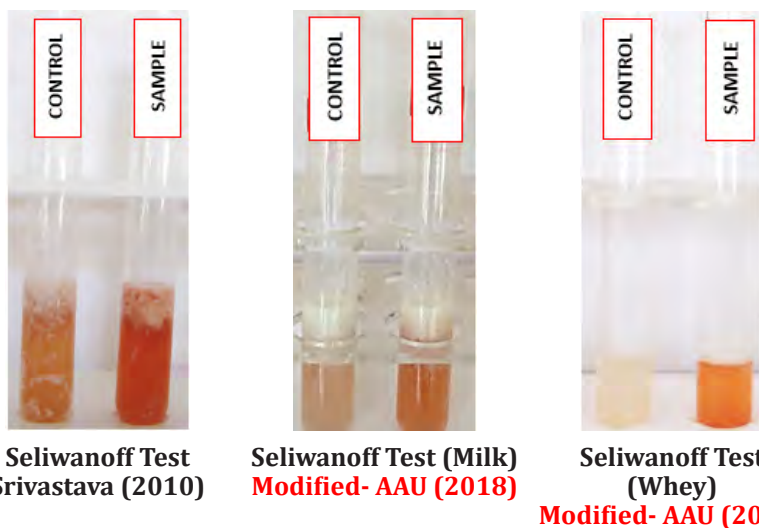
Seliwanoff Test Srivastava (2010)

Seliwanoff Test (Milk) Modified- AAU (2018)

Seliwanoff Test (Whey) Modified- AAU (2018)

Fig. 4: Test for Sucrose

3.5 DETECTION OF GLUCOSE BY BARFOED TEST

Barfoed's test is routinely used for detection of extraneous glucose in milk. By means of the Barfoed's reaction it is possible to differentiate reducing monosaccharaides from reducing disaccharides as monosaccharaides can reduce copper fast enough in comparison to disaccharides. The formation of green, red, or yellow precipitate is a positive test for reducing monosaccharides.

Reagents:

Barfoed reagent: Dissolve 13.3 g of copper acetate (AR) in distilled water, subsequently add 2.0 ml of lactic acid (AR) and make up the total volume to 200 ml.

Procedure (using milk as medium):

1) Take 1 ml milk in a test tube.

2) Add 2 ml Barfoed reagent.

3) Keep the test tube in boiling water bath for 6 min.

4) Cool the test tube to room temperature under tap water.

5) Observe for colour development.

Procedure (using whey as medium):

1) Take 1 ml milk and 2 ml Barfoed reagent in a test tube (quantity of both milk and reagent can be doubled proportionately to get sufficient filtrate).

2) Mix the content and filter using Whatman No. 1 filter paper.

3) Keep test tube contianing filtrate in water bath for 4 min.

4) Cool the test tube to room temperature using water.

5) Observe for colour development.

Interpretation : Development of green colour indicates presence of glucose in milk.

Limit of detection :

0.1 g/100 ml of milk (milk as medium)

0.15 g /100 ml of milk (whey as medium)

Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample.
- Better clarity in whey as compared to milk.
- Eliminates large number of costly chemicals.
- One step procedure, improved convenience.

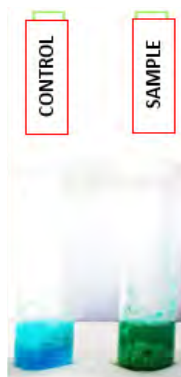(**Note**: The method of Barfoed (1873) was modified. Acetic acid in Barfoed reagent was replaced with lactic acid. Phosphomolybdic acid reagent was eliminated. The test was also modified using whey as medium.)

| Barfoed Test Barfoed (1873) | Modified Barfoed Test FSSAI (2016) | Barfoed Test (Milk) Modified- AAU (2018) | Barfoed Test (Whey) Modified- AAU (2018) |

Fig. 5: Test for Glucose

3.6 DETECTION OF MALTODEXTRIN BY IODINE TEST

Similar to starch maltodextrin also forms complex with iodine. However, instead of blue colour, red to brown colour is observed. This is because the complex formed is dependent on chain length of dextrins. The chain length more than 45 DP (degree of polymerization) gives blue colour, whereas lesser DP gives red to brown colour complex, as in the case of maltodextrin.

Reagents:

1) Iodine solution (1%): Dissolve 2.5 g potassium iodide (AR) in 100 ml distilled water. Then, add 1 g pure iodine crystals (AR). Prepare iodine solution at least a day before as iodine dissolves slowly.

2) Citric acid (5%): Dissolve 5 g citric acid monohydrate (AR) in distilled water and make up the volume to 100 ml.

Procedure:

1) Take 20 ml milk in a conical flask.

2) Warm the milk to 70-80 °C either on direct flame or boiling water bath.

3) Add 5% citric acid solution drop wise in the milk with gentle stirring until clear coagulation occurs (Approximate consumption of citric acid required would be 1.5-2 ml.). Flask, if stirred vigorously will result in fine curd particles and which may impact colour observation.

4) Filter the content using Whatman No. 1 filter paper.

5) Take 5 ml filtrate in a test tube.

6) Add 0.25 ml of 1% iodine solution.

7) Mix the content and observe the colour.

Interpretation : Development of red-brown colour indicates adulteration of milk with maltodextrin. Pure milk remains yellow in colour.

**Limit of detection :** 0.1 g /100 ml milk

Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample.

- Significant improvement in sensitivity.

(**Note**: The test reported by Sharma *et al.* (2012) was modified wherein 0.05 N iodine solution was replaced with 1% iodine solution. The test was performed in whey instead of milk.)

Iodine Test (Milk)
Sharma *et al.* (2012)
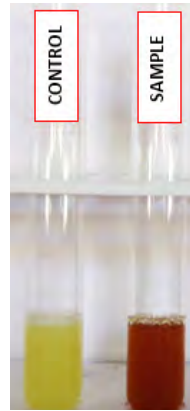
Iodine Test (Whey)
Modified- AAU (2018)

Fig. 6: Test for Maltodextrin

3.7 DETECTION OF STARCH BY IODINE TEST

The development of blue colour on addition of iodine solution in starch containing milk is due to complex formation between iodine and amylose component of starch. The other component, amylopectin, gives a red-purple colour which is much less intense than the amylose. The acidic condition in the reagent mixture accentuates the blue colour, whereas alkali reduces its intensity, the blue colour disappears above a pH of about 9.5. Heating the solution containing starch-iodine complex also destroys the colour although reversibly.

Reagents :

1) Iodine solution: Dissolve 2.5 g potassium iodide (AR) and 1 g of pure iodine crystals (AR) in 100 ml distilled water. Prepare iodine solution at least a day before as iodine dissolves slowly.

2) Acetic acid (10%): Dissolve 10 ml glacial acetic acid (AR) in distilled water and make up the volume to 100 ml.

3) Citric acid solution (5%): Dissolve 5 g citric acid monohydrate (AR) in distilled water and make up the volume to 100 ml with distilled water.

Procedure (using milk as medium):

1) Take 3 ml milk in test tube.
2) Bring the milk to boil on a direct flame or on a boiling water bath.
3) Cool the test tube to room temperature under tap water.
4) Add a drop of 10% acetic acid in the test tube.
5) Add 0.2 ml of iodine solution.
6) Mix the content and observe colour.

Procedure (using whey as medium):

1) Take 20 ml milk in a conical flask.
2) Bring the milk to boil on a direct flame or on a boiling water bath.
3) Add 5% citric drop wise till visible coagulation. (Approximate consumption of citric acid would be 1.5-2 ml.)
4) Filter the content using Whatman No. 1 filter paper. Let the filtrate cool to room temperature.
5) Take 3 ml filtrate in another test tube and add 0.1 ml of iodine solution.
6) Mix the content and observe colour.

Interpretation : Blue/Dark blue colour formation indicates adulteration of milk with starch. Whereas pure milk remains yellow due to colour of iodine.

Limit of detection :

0.02 g/ 100 ml milk (When test is performed in milk)

0.01 g /100 ml milk (When test is performed in whey)

Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample.
- Better sensitivity
- Reduces the chances of interference of neutralizers on the detection of starch.

(**Note**: The method reported by BIS (1961) is modified. Addition of 10% acetic acid is recommended to reduce possible interference of neutralizers and improve differentiation between adulterated and

pure sample. The test was also modified using whey as medium.)
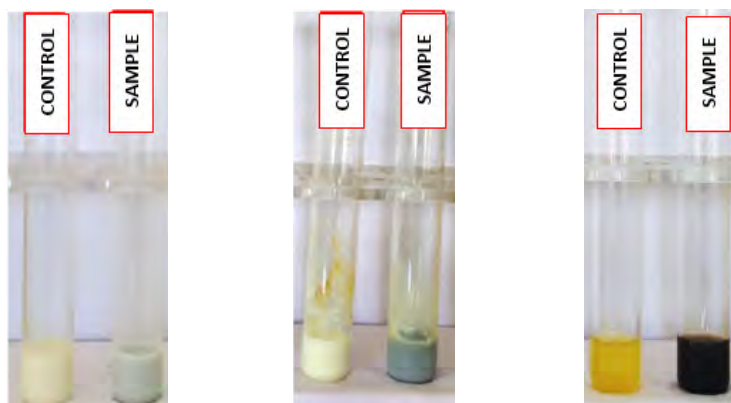
**Iodine Test
BIS (1960)**　　**Iodine Test (Milk)
Modified- AAU (2018)**　　**Iodine Test (Whey)
Modified- AAU (2018)**

Fig. 7: Test for Starch

3.8 DETECTION OF SODIUM CHLORIDE BY SILVER NITRATE TEST

The chloride ion (Cl^-) from sodium chloride reacts with silver ion (Ag^+) of silver nitrate forming white precipitates of silver chloride. Simultaneously water soluble sodium nitrate is also formed. After the Ag^+ from silver nitrate has complexed with all the available chloride in the sample, the Ag^+ reacts with chromate from silver chromate added in the reaction mixture; forming an orange coloured precipitates of silver chromate.

Reagents :

1. Silver nitrate solution (0.1N): The reagent is prepared by dissolving 16.987 g silver nitrate (AR) in 1000 ml distilled water.

2. Potassium chromate solution (5%): The reagent is prepared by dissolving 5 g potassium chromate (AR) in 100 ml distilled water.

Procedure :

1. Take 5 ml milk in test tube.

2. Add 0.5 ml of 5% potassium chromate solution.

3. Add 2 ml 0.1 N silver nitrate and mix the contents.

4. Observe for colour change.

Interpretation : Yellow colour indicates adulteration of milk with common salt (sodium chloride). Unadulterated milk gives chocolate or reddish brown colour.

Limit of detection : 0.04g /100 ml of milk

Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample.

(**Note:** The method reported by FSSAI (2016) is modified. 5% potassium chromate is used instead of 10 %.)
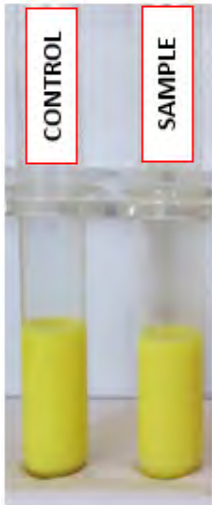
**Chromate Test
FSSAI (2016)**
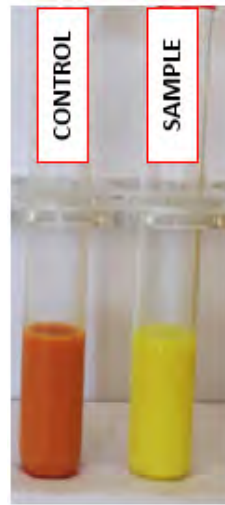
**Chromate Test (Milk)
Modified- AAU (2018)**

Fig. 8: Test for Salt (Sodium Chloride)

3.9 DETECTION OF NITRATE BY DIPHENYLAMINE TEST

The test consists of adding a solution of diphenylamine in sulphuric acid to milk. Nitrates are considered as oxidising agent. Under the conditions of test, diphenylamine is oxidized by nitrate to the intensely blue quinone-immonium salt *via* diphenyl benzidine.

Reagents:

1) Diphenylamine solution: The reagent is prepared by dissolving 0.085 g diphenylamine in 50 ml distilled water and gradually 450 ml of concentrated sulphuric acid is added with constant stirring. During preparation of reagent the content is kept cool by dipping in cold water. Diphenylamine solution should be prepared freshly and shall be colourless.

2) Acetic acid (10%): Take 10 ml glacial acetic acid (AR) in 100 ml volumetric flask and make up the volume with distilled water.

Procedure :

1) Take 20 ml milk in a conical flask.

2) Warm the milk to 70-80 °C either on direct flame or boiling water bath.

3) Add 10% acetic acid drop wise in the milk with gentle stirring till visible coagulation.

4) Filter the content using Whatman No. 1 filter paper.

5) Take 2 ml diphenylamine solution in a test tube.

6) Add 1 ml filtrate in test tube containing diphenylamine solution.

7) Observe for ring formation at the junction of two solutions.

Interpretation : Formation of blue ring at the junction of two solutions indicates adulteration of milk with nitrate or surface water.

Limit of detection : 0.002g /100 ml milk

Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample

- Elimination of toxic chemicals from precipitating reagent

(**Note**: The test reported by FAO (1986) was modified. The milk is precipitated using 10% acetic acid instead of precipitating reagent specified in the original method. The procedure of the test is also modified.)
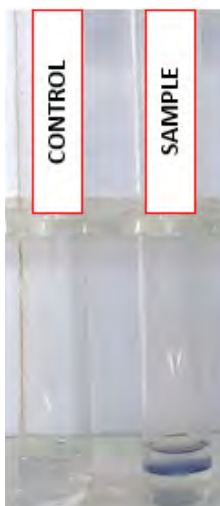
Diphenylamine Test FSSAI (2016)   **Diphenylamine Test FAO (1986)**   **Diphenylamine Test Modified- AAU (2018)**

Fig. 9: Test for Nitrate

3.10 DETECTION OF SULPHATE BY BARIUM CHLORIDE TEST

Barium chloride test is one of the commonly used methods for detection of sulphate adulteration in milk. The barium ion (Ba^{2+}) reacts with sulphate ion (SO_4^{2-}) to give white precipitates of barium sulphate ($BaSO_4$). Using this test, presence of added sulphate like ammonium sulphate, sodium sulphate, zinc sulphate, magnesium sulphate, etc. to milk can be confirmed by observing milky-white precipitates.

Reagents :

1) Barium chloride solution (5%): Dissolve 5.0 g barium chloride (AR) in distilled water and make up the final volume to 100 ml.

2) Lactic acid solution (5%): Take 5 ml lactic acid (AR) in 100 ml volumetric flask and make up the volume with distilled water.

Procedure :

1) Take 20 ml milk in a conical flask/test tube bring it to boil on direct flame.

2) Add 2 ml of 5% lactic acid in hot milk and filter the content using Whatman No. 42 filter paper.

3) Take 2 ml filtrate in a separate test tube.

4) Add 0.2 ml of 5% barium chloride and observe for turbidity development.

Interpretation : Formation of turbidity after barium chloride solution addition indicates adulteration of milk with sulphate.

Limit of detection : 0.015 g /100 ml milk

Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample.

- Improved sensitivity.

- Elimination of TCA (hazardous chemical).

(**Note**: The method reported by FSSAI (2016) was modified. The coagulating agent (24% TCA) is replaced with lactic acid.)

Barium Chloride Test
FSSAI (2016)

Barium Chloride Test
Modified- AAU (2018)

Fig. 10: Test for Sulphate

3.11 DETECTION OF HYDROGEN PEROXIDE BY IODINE TEST

Hydrogen peroxide presence can be detected iodometric test. Hydrogen peroxide oxidises iodide to iodine. Starch forms a deep, dark blue complex with minute amounts of triiodide ions that are formed only in the presence of both iodine and iodide in solution. Thus, formation of blue colour indicates presence of hydrogen peroxide.

Reagents:

1. Potassium iodide solution (20%): Weigh 20 g of potassium iodide (AR) and dissolve it in distilled water to obtain 100 ml

solution. The solution should be prepared fresh before every use.

2. Starch solution (1%): Take 1 g of soluble starch and make paste using cold water. Transfer the paste to 100 ml volumetric flask and make the volume to 100 ml using boiling distilled water. Cool and decant the clear solution. The solution should be prepared fresh before every use.

3. Starch-potassium iodide reagent: The reagent is prepared by mixing equal volumes of 20 per cent potassium iodide solution and 1 per cent starch solution. The solution should be prepared fresh before every use.

4. Acetic acid (10%): Dissolve 10 ml glacial acetic acid in water and make up the volume to 100 ml.

Procedure :

1. Take 20 ml milk in a conical flask.
2. Add 2 ml 10% acetic acid.
3. Mix the content and filter using Whatman No. 1 filter paper.
4. Take 1 ml filtrate and add 1 ml starch-potassium iodine reagent.
5. Observe for colour development.

Interpretation : Appearance of bluish black colour indicates the presence of hydrogen peroxide in the milk sample whereas control milk sample remains colourless.

Limit of detection :

Modified test: 0.015 g /100 ml of milk

Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample.
- Improved sensitivity.
- Method uses relatively benign chemicals in place of potentially harmful chemical paraphenylenediamine.

(**Note:** The method reported by FSSAI (2016) was modified. The test was performed in whey instead of milk.)
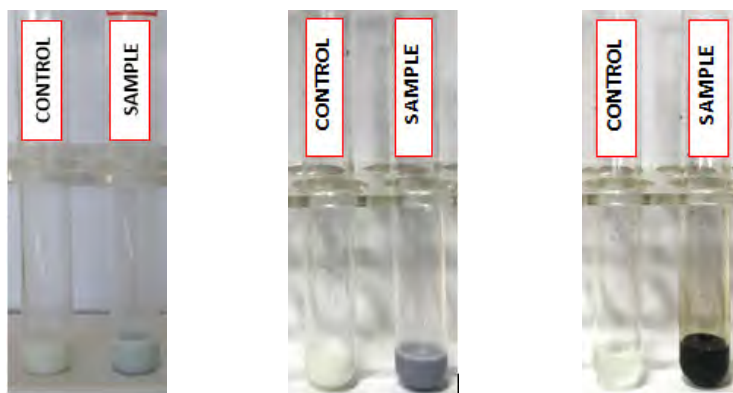
p-Phenylenediamine Test Draaiyer (2009) **Iodometric Test** Sharma *et al.* (2012) **Iodometric Test** Modified- AAU (2018)

Fig. 11: Test for Hydrogen Peroxide

3.12 DETECTION OF FORMALDEHYDE BY HEHNER TEST

In the Hehner test for detection of formaldehyde in milk, concentrated sulphuric acid and ferric chloride is used. The test is an aldehyde-oxidation reaction of an aromatic amine. Ferric chloride acts as oxidising agent for formaldehyde. The formaldehyde reaction depends on the presence of the tryptophan in the protein molecule. The violet colour develops as a result of the reaction of oxidised formaldehyde with tryptophan. The intensity of the reaction with different proteins varies in direct proportion to the amount of tryptophan present in the protein molecule.

Reagents :

1) Ferric chloride (10%): Take 10 g of ferric chloride (AR) in 100 ml volumetric flask and make up the volume with distilled water.

2) Sulphuric acid (80%): Add 80 ml concentrated sulphuric acid (AR) into 20 ml distilled water.

Procedure :

1) Take 5 milk sample in a test tube.

2) Add 5 ml distilled water and 0.1 ml of 10% $FeCl_3$ solution.

3) Mix the content and add 10 ml H_2SO_4 (80%) from the side of the test tube.

Interpretation : Violet ring at the junction of two layers indicates presence of formaldehyde.

Limit of detection

0.0005 ml formalin/ 100 ml milk

Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample.
- Improved sensitivity.

(**Note:** The test reported by Draaiyer *et al.* (2009) was modified. Instead of Gerber sulphuric acid, 80% (v/v) sulphuric acid is used.)

Hehner Test
Draaiyer (2009)

Hehner Test
Modified- AAU (2018)

Fig. 12: Test for Formaldehyde

3.13 DETECTION OF FORMALDEHYDE BY LEACH TEST

In Leach test for detection of formaldehyde, concentrated hydrochloric acid is used in place of sulphuric acid (which is used in Hehner test). The contents are heated to enhance oxidation and resultant colour development.

Reagent :

1) Ferric chloride (10% w/v): The reagent is prepared by dissolving 10 g of anhydrous ferric chloride (AR) in distilled water and volume is made up to 100 ml.

2) Hydrochloric acid containing ferric chloride: The reagent is prepared by adding 1 ml of 10% ferric chloride solution in a 500 ml volumetric flask and making up the volume with concentrated HCl (AR).

Procedure (using milk as medium) :

1) Take 5 ml milk in a test tube.

2) Add 3 ml hydrochloric acid containing ferric chloride.

3) Heat on direct flame for 1 min and observe the colour.

Procedure (using whey as medium) :

1) Take 5 ml milk in a test tube.

2) Add 3 ml hydrochloric acid containing ferric chloride. (Amount of milk and reagent can be increased proportionately to get sufficient filtrate)

3) Filter the content using Whatman No. 1 filter paper.

4) Take filtrate in a test tube and heat on a direct flame for 1 min.

Interpretation : Appearance of violet colour indicates presence of formaldehyde in milk.

Limit of detection :

0.002 ml formalin / 100 ml milk (When test is performed in milk)

0.0005 ml formalin / 100 ml milk (When test is performed in whey)

Benefits of modified method over reported method

- Better differentiation between adulterated and pure sample.

- Improved sensitivity.

(**Note:** The method reported by BIS (1961) was modified. Instead of 10 ml milk and 5 ml reagent; 5 ml milk and 3 ml reagent is recommended respectively. The test is also modified using whey as medium.)

Leach Test
BIS (1961)

Leach Test (Milk)
Modified- AAU (2018)

Leach Test (Whey)
Modified- AAU (2018)

Fig. 13: Test for Formaldehyde

3.14 DETECTION OF NEUTRALIZERS BY PHENOL RED TEST

Phenol red is used as a pH indicator and shows colour transition from yellow to red over the pH range 6.8 to 8.2. However, at pH value greater than 8.2 phenol red turns bright pink. As neutralization increases the pH of milk (beyond 6.8), it can be detected using phenol red indicator.

Reagents :

Phenol red solution: 0.05 g of phenol red is dissolved in 20 ml ethanol and volume is made up to 100 ml using distilled water.

Procedure :

1) Take 4 ml milk in a test tube.
2) Add 1 ml phenol red solution.
3) Mix the content and observe the colour.

Interpretation : Development of pink colour indicates presence of sodium hydroxide (NaOH), whereas orange colour indicates sodium carbonate (Na_2CO_3) or sodium bicarbonate ($NaHCO_3$) in milk. Milk without added neutralizers shows yellow colour.

Limit of Detection :

NaOH: 0.04 g/ 100 ml milk

Na_2CO_3: 0.08 g/ 100 ml milk

$NaHCO_3$: 0.2 g/ 100 ml milk

Benefits of developed method over reported method

- Better differentiation between adulterated and pure sample.
- Improved sensitivity.
- Elimination of ethanol (a regulated chemical)

Rosolic acid Test
DGHS (2005)

Phenol Red Test
Developed by AAU (2018)

Fig. 14: Test for Neutralizers

4. Qualitative Tests for Detection of Adulterants in Milk – Existing Methods

The procedures of qualitative tests given by FSSAI, other organizations and researchers are given hereunder.

4.1 TESTS FOR DETECTION OF DETERGENTS

4.1.1 Methylene blue test (FSSAI, 2016)

Alkyl benzene sulphonic acid (ABS) or anionic detergent may be present in milk due to intentional addition of detergent in milk or due to insufficient rinsing of dairy equipments. The following 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 intense blue colour in lower layer indicates the presence of detergent in milk. The developed test is sensitive to detect anionic detergent up to 0.0125% (12.5 mg/100 ml).

Reagents :

1) Methylene blue solution (12.5 mg/100 ml): 12.5 mg is dissolved in 100 ml of distilled water. Protect the solution against direct sunlight.

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

Procedure :

Pipette 1 ml of suspected milk sample into a 15 ml test tube. Add 1 ml of methylene blue solution followed by addition of 2 ml chloroform. Vortex the contents for about 15 sec and centrifuge at about 1100 rpm for 3 min. Note the intensity of blue colour in lower and upper layer. Relatively, more intense blue colour in lower layer indicates

presence of detergent in milk, whereas, intense blue colour in upper layer indicates absence of detergent in milk. The method can detect presence of 0.15% level of laboratory grade detergent (e.g. labolene) in milk.

4.1.2 Methylene blue test (Paradkar *et al.,* 2000)

Procedure :

To 2.5 ml of a suspected sample and 2.5 ml of pure milk in separate test tubes, 7.5 ml of ethanol is added to precipitate the protein, which is then filtered off. To 2 ml of the filtrate, 2 ml of methylene blue solution (25 mg/100 ml of water) is added and the mixture is shaken well. Then 4 ml of chloroform is added and the mixture is shaken again. The chloroform layer is allowed to separate. If the methylene blue colour extracted from a suspected sample into the chloroform layer is greater than that extracted from an authentic milk sample, it indicates the presence of detergent in milk.

4.2 TEST FOR DETECTION OF UREA

4.2.1 DMAB test (FSSAI, 2016)

This method is based on the principle that urea forms a yellow complex with DMAB in a low acidic solution at room temperature.

Reagents :

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

Procedure :

Mix 1 ml of milk with 1 ml of 1.6% DMAB reagent. Distinct yellow colour is observed in milk containing added urea. The control (pure milk) shows a slight yellow colour due to presence of natural urea. The limit of detection of method is 0.2%.

4.3 TESTS FOR DETECTION OF AMMONIUM SALTS

4.3.1 Phenol test (FSSAI, 2016)

Reagents :

1)   2% Sodium hydroxide

2)   2% Sodium hypochlorite

3)   5% Phenol solution

Procedure :

Take 1.0 ml of milk add 0.5 ml of 2% sodium hydroxide, 0.5 ml of 2% sodium hypochlorite and 0.5 ml of 5% phenol solution. Heat for 20 seconds in boiling water bath, bluish colour turns deep blue in presence of ammonium sulphate. The development of pink colour shows that the sample is free from ammonium sulphate.

4.3.2 Nessler's test (FSSAI, 2016)

Reagents :

1) Nessler's reagent: Dissolve the following chemicals separately.

   a) 8.0 g of mercuric chloride (AR) in 150 ml distilled water.

   b) 60.0 g of sodium hydroxide (AR) in 150 ml distilled water.

   c) 16.0 g of potassium iodide (AR) in 150 ml distilled water.

   Add reagent 'a' to reagent 'b' and mix well. To this mixture, add reagent 'c', mix and dilute the contents to 500 ml with distilled water. Leave this solution undisturbed and decant the clear upper layer of the solution. Store in a stoppered amber glass bottle.

Procedure :

Take 5 ml of milk sample in a test tube. Add 1 ml of Nessler's reagent. Mix the contents of the tube thoroughly and observe the colour change. The control milk sample gives slight greyish colour. At low concentration of ammonium compounds, brownish shade appears which is distinguishable at 0.15% followed by yellowish colour and then orange colour development at higher concentration. The limit of detection of method is 0.15%.

4.4 TESTS FOR DETECTION OF SUCROSE

4.4.1 Modified Seliwanoff's test (FSSAI, 2016)

Fructose in cane sugar (sucrose) reacts with resorcinol in hydrochloric acid to give red colour.

Reagents :

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.

(**Note :** The resorcinol flakes should be white in colour.)

Procedure :

Take 1 ml of milk in a test tube. Add 1 ml of resorcinol solution and mix. Place the tube in boiling water bath for 5 min. Withdraw the tube and observe the colour. Appearance of deep red colour indicates presence of sucrose. In pure milk samples no such red color is developed and sample remains white in nature. The limit of detection of method is 0.1%.

4.4.2 Seliwanoff's test (Srivastava, 2010)

Reagents :

Seliwanoff Reagent: Resorcinol solution (0.05%): Dissolve 0.05g of resorcinol in 100 ml HCl (1:2).

Procedure :

Take 3 ml of milk in a test tube and add 5 ml of Seliwanoff reagent and place the test tube in boiling water bath for 5 minutes. Appearance of deep red colour indicates presence of sucrose.

4.5 TESTS FOR DETECTION OF GLUCOSE

4.5.1 Barfoed test (FSSAI, 2016)

Reagents :

1) Modified Barford's reagent: Dissolve 24 g of copper acetate in 450 ml of boiling distilled water. Add 25 ml of 8.5% acetic acid, shake, cool to room temperature and make up to 500 ml. After sedimentation, filter the reagent and store in dark coloured bottle.

2) Phosphomolybdic acid: 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 all the 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.

Procedure :

Take 1 ml of milk sample in a test tube. Add 1 ml of modified Barford's reagent. Heat the mixture for exact 3 min in a boiling water bath. Rapidly cool under tap water. Add one ml of phosphomolybdic acid reagent to the turbid solution. Observe the colour. Immediate formation of deep

blue color after adding phosphomolybdic acid reagent indicates the presence of added glucose in the milk sample. In case of pure milk, only faint bluish color can be observed due to the dilution of Barford's reagent. The limit of detection of method is 0.1%.

4.5.2 Barfoed test (Barfoed, 1873)

Reagent :

Barfoed's reagent: Dissolve 13.3 g copper acetate in 200 ml of water; add 1.8 ml of glacial acetic acid.

Procedure:

Add 1 ml of the test solution to 2 ml of Barfoed's reagent, boil for one minute and allow to stand. Formation of red precipitates indicating presence of glucose as a result of its reduction. No reduction indicates lactose, or maltose or both.

Note: Barfoed test was developed for analysis solutions containing sugar and in its origincal form it can not be applied for detection of glucose in milk.

4.6 TEST FOR DETECTION OF MALTODEXTRIN

4.6.1 Iodine test (Sharma *et al.,* 2012)

Reagents :

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.

Procedure :

1) Take about 5 ml milk sample in a test tube.

2) Add 2 ml of detecting reagent to the tube.

3) Mix well.

4) Observe the change in colour.

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.

Limit of detection : 0.3 g /100 ml milk

4.7 TESTS FOR DETECTION OF STARCH

4.7.1 Iodine test (FSSAI, 2016)

Reagents :

Iodine solution: Iodine solution: Dissolve 2.6 g of iodine and 3 g of potassium iodide in a sufficient quantity of water and make up to 200 ml.

Procedure :

Take about 5 ml of milk in a test tube. Bring to boiling condition and allow the test tube to cool to room temperature. Add 1-2 drops of iodine solution to the test tube. Development of blue colour indicates presence of starch which disappears when sample is boiled and reappears on cooling. The limit of detection of method is 0.02%.

4.7.2 Iodine test (BIS, 1960)

Reagents :

Iodine solution (1%): The reagent is prepared by dissolving 2.5 g potassium iodide in 100 ml distilled water. Then, 1 g of pure iodine crystal is added and content is mixed well to prepare clear solution.

Procedure :

Place in a test-tube about 3 ml of well-mixed sample. Bring it to boil by holding the tube over a flame. Allow to cool to room temperature. Add a drop of one percent iodine solution. Presence of starch is indicated by the appearance of a blue colour which disappears when the sample is boiled and re-appears on cooling.

4.8 TEST FOR DETECTION OF SODIUM CHLORIDE

4.8.1 Silver nitrate test (FSSAI, 2018)

Reagents :

1) Silver nitrate solution (0.1N): The reagent is prepared by dissolving 16.987 g silver nitrate (AR) in 1000 ml distilled water.

2) Potassium chromate solution (10%): The reagent is prepared by dissolving 10 g potassium chromate (AR) in 100 ml distilled water.

Procedure :

1) Take 5 ml milk in test tube.

2) Add 2 ml 0.1 N silver nitrate and mix the contents.

3) Mix the content thoroughly.

4) Add 0.5 ml of 10% potassium chromate solution.

5) Observe for colour change.

Interpretation : Appearance of chocolate brown precipitate indicates the absence of dissolved chloride in milk and appearance of yellow colour indicates presence of dissolved chloride.

Limit of detection : 0.02g /100 ml of milk

4.9 TESTS FOR DETECTION OF NITRATE

4.9.1 Diphenylamine test (FSSAI, 2016)

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

Reagents :

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

Procedure :

Take 2 ml of milk in a test tube. Rinse the tube with the milk and drain the milk from the test tube. Add two-three drops of the reagent along the side of the test tube. Note the developed colour. Deep blue colour will be formed in presence of nitrate in the milk sample. Pure milk sample will not develop any colour.

4.9.2 Diphenylamine test (FAO, 1986)

Under the conditions of test, diphenyl amine is oxidized by nitrate to the intensely blue quinone-immonium salt *via* diphenyl benzidine.

Note: Care must be taken to rinse all glassware to ensure the absence of even traces of nitrate. The test must not be conducted near sources of nitrous fumes such as reagent bottles of concentrated nitric acid. The filter papers used must also be checked for nitrates and washed prior to use if necessary.

Reagents :

1) Diphenylamine solution: Weighs 0.085 g diphenylamine and dissolve in 50 ml water. Slowly add 450 ml of concentrated sulphuric acid with shaking, keeping the solution cool.

2) Precipitating reagent: Dissolve 20 g of mercuric chloride and 5 g of ammonium chloride in water, add 20 ml of concentrated hydrochloric acid and dilute to 100 ml with water.

Procedure :

To 5 ml of milk in a test-tube add 6 or 7 drops of the precipitating reagent and shake occasionally for about 2 minutes. Pipette 2 ml of the diphenyl amine solution into the bottom of another test-tube without allowing any of the solution to touch the walls of the tube. Place a filter paper in this tube and incline it so that the filtrate from the precipitated milk runs gently down the side of the tube and forms a layer on top. When about 1 ml of filtrate is collected, remove the filter paper and examine the filtrate/diphenyl amine interface over a white surface. In the absence of nitrates, there is no colour, and some yellow or brown colour may appear when the tube is rotated. In the presence of nitrates a blue colour develops either immediately or on rotation of the tube. Carry out a blank with genuine milk. The test detects down to about 0.1 micrograms/ml in the filtrate.

Interpretation :

It is generally accepted that nitrate does not occur in normal milk, but is often present in drinking water, so the test, when positive, serves as confirmation of the addition of water to milk.

4.10 TEST FOR DETECTION OF SULPHATE

4.10.1 Barium chloride test (FSSAI, 2016)

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

Reagents :

1) 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.

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

Procedure :

Take 10 ml of milk in a 50 ml stoppered test tube. Add 10 ml of TCA solution. Filter the coagulated milk through Whatman No. 42 filter paper. Take 5 ml of clear filtrate. Add few drops of barium chloride solution. Observe for any visible precipitates in the tube. Formation of milky-white precipitates indicates the presence of added sulfates like ammonium sulfate, sodium sulfate, zinc sulfate, magnesium sulfate, etc. to milk. The limit of detection of this method is 0.05%.

4.11 TESTS FOR DETECTION OF FORMALDEHYDE

4.11.1 Hehner test (FSSAI, 2016)

Reagents :

Concentrated sulphuric acid.

Procedure :

Take milk sample (2 ml) in a test tube and add 2 ml of sulphuric acid (90%) containing traces of ferric chloride from the side of the test tube slowly. Formation of purple ring at the junction indicates formaldehyde is present in milk. If sucrose is present, distil the milk sample (25 ml) and then carry out the test on the distillate by taking 2-3 ml of distillate and adding 2 ml of formaldehyde free milk. The violet coloration does not appear usually when relatively large quantities of formaldehyde are present.

Precaution: If H_2SO_4 is added from the top and not from the side of the test tube, it may burn the milk solids and affect the end result.

4.11.2 Hehner test (Draaiyer *et al.,* 2009)

Reagents :

1) Sulphuric acid: Density 1.807 - 1.812 g/ml at 27 °C. It should be colourless.

2) Ferric chloride (10% w/v): The reagent is prepared by dissolving 10 g of anhydrous ferric chloride (AR) in distilled water and volume is made up to 100 ml.

Procedure :

Mix 5 ml milk with 5 ml water in a graduated test tube. Add one drop 10% ferric chloride solution to 10 ml sulphuric acid (90%) in another test tube. Gently pour the acid carefully down the side of the test tube

with the milk-water mixture so that it forms a layer at the bottom without mixing with the milk. A violet, or blue colour, at the junction of the two liquids indicates the presence of formaldehyde. The test will detect about 1 ml of 40% formaldehyde solution in 100 litres of milk, *i.e.* about 10 ppm. A green or brown colour indicates absence of formaldehyde.

4.11.3 Leach test (BIS, 1961)

Reagents :

1) Hydrochloric acid : Specific gravity 1.16

2) Ferric chloride (10% w/v): The reagent is prepared by dissolving 10 g of anhydrous ferric chloride (AR) in distilled water and volume is made up to 100 ml.

Procedure :

Mix in a casserole about 10 ml of milk with an equal volume of concentrated hydrochloric acid containing one millilitre of ferric chloride solution to each 500 ml of acid. Heat slowly but directly over a gas flame for about 5 minutes to 80 to 90°C. Rotate the casserole to break up the curd. A violet colour indicates the presence of formaldehyde.

4.12 TEST FOR DETECTION OF NEUTRALIZERS

4.12.1 Rosalic acid test (DGHS, 2005)

Reagents :

1) Rosalic acid solution (1.0 %, w/v): Take 1g of rosalic acid powder; dissolve it in ethyl alcohol and make up the volume to 100 ml in volumetric flask.

2) 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.

Procedure :

To 10 ml of milk add equal volume of 95% alcohol in a test tube. Add a few drops of 1% alcoholic solution (w/v) rosalic acid. If alkali is present, a rose red colour appears whereas pure milk shows only a brownish colour.

4.12.2 Rosalic acid (FSSAI, 2016)

Neutralizers (NaOH, 0.1% for Na_2CO_3 and 0.2% for $NaHCO_3$) are added to milk to neutralize the developed acidity in milk. Rosalic acid method can be used for the detection of presence of these neutralizers in milk. The other method available for detection of neutralizers in milk is through determination of alkalinity of ash.

There are two versions of this method. Both the variants are capable of detecting neutralizers in milk.

Version 1.

Reagents:

1) 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 100 ml.

2) 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.

Procedure :

To 10 ml of milk add equal volume of 95% alcohol in a test tube. Add a few drops of 0.1% alcoholic solution (w/v) rosalic acid. If alkali is present a rose red colour appears whereas pure milk shows only a brownish colour. The limit of detection of method is 0.1% for NaOH, 0.1% for Na_2CO_3 and 0.2% for $NaHCO_3$.

Version 2.

Reagents :

Rosalic acid solution (0.05%, w/v): First prepare 60% (v/v) ethyl alcohol solution by mixing 60 ml ethyl alcohol (95%) and 40 ml distilled water. Weigh 50 mg of rosalic acid powder and dissolve it in small quantity of 60% ethyl alcohol and make up the volume to 100 ml with 60% ethyl alcohol.

Procedure :

Take 2 ml milk sample in a test tube and add 2 ml rosalic acid solution. Mix the contents. If alkali is present in milk, a rose red colour appears whereas pure milk shows only a brownish colour.

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