DEVELOPMENT OF COLORIMETRIC METHOD FOR THE QUALITATIVE DETECTION OF SORBITOL ADULTERATION IN MILK

THESIS SUBMITED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY
IN DAIRYING
(DAIRY CHEMISTRY)

BY
KARRA. MADHAVI LATHA
B.Tech. (Dairy Technology)

DAIRY CHEMISTRY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL-132001 (HARYANA), INDIA

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DAIRY CHEMISTRY

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This is to certify that the thesis entitled, **"DEVELOPMENT OF COLORIMETRIC METHOD FOR THE QUALITATIVE DETECTION OF SORBITOL ADULTERATION IN MILK"** **submitted by KARRA MADHAVI LATHA** in partial fulfilment of the requirement for the award of the degree of **MASTER OF TECHNOLOGY** in **DAIRY CHEMISTRY** of the **ICAR- NATIONAL DAIRY RESEARCH INSTITUTE**, (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Date: 31/8/ **2021**

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Major Advisor

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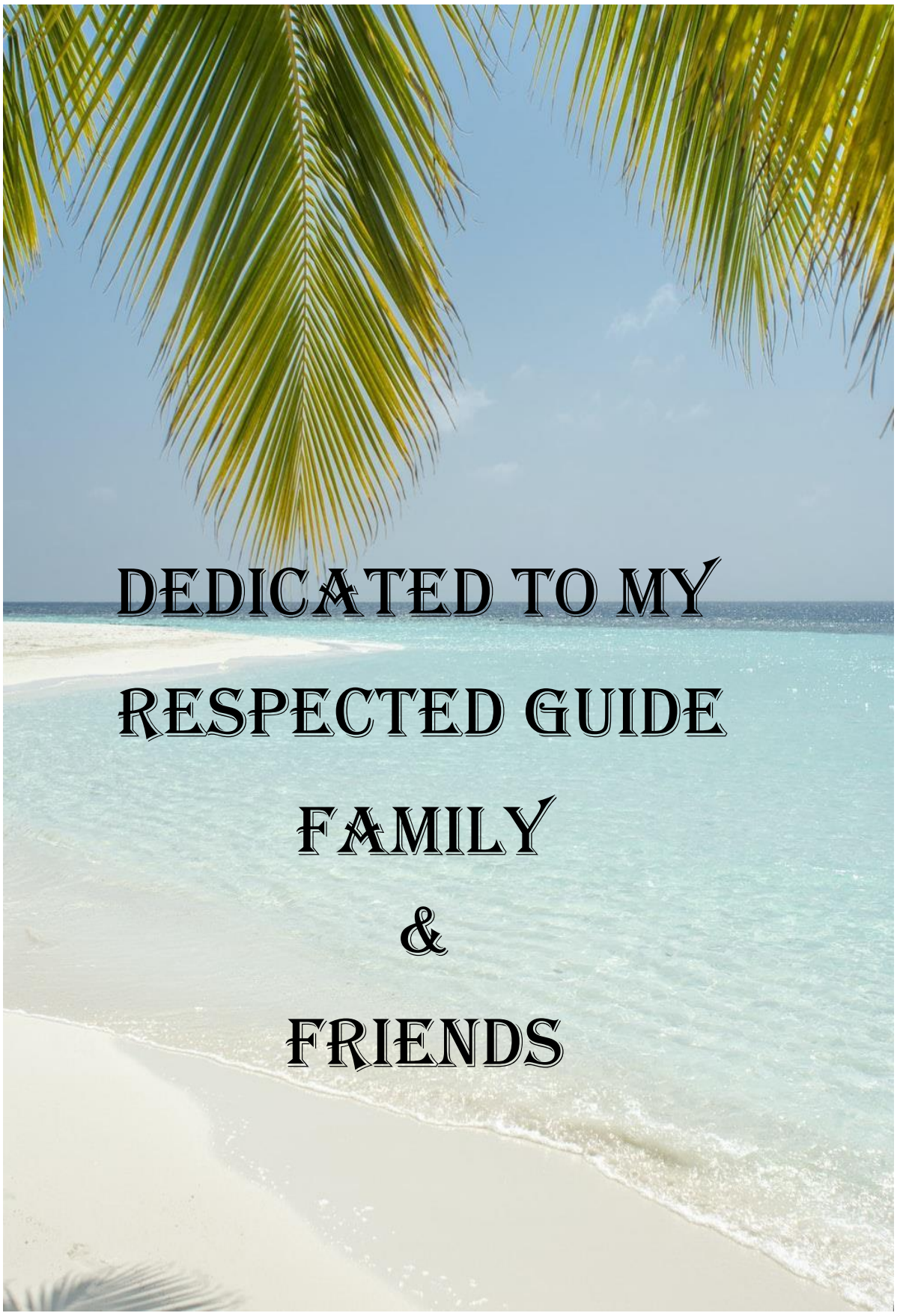
DEDICATED TO MY
RESPECTED GUIDE
FAMILY
&
FRIENDS

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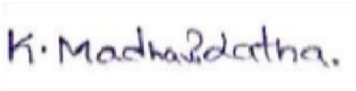

(Karra. Madhavi latha)

ABSTRACT

Milk is considered as complete food having high nutritional value. However, the literature also suggests that it is the most exploited commodity in the dairy industry/ dairy business in India. Sorbitol is one of such recent adulterants which has gained the popularity amongst unscrupulous traders of milk. They add this adulterant to milk to increase the specific gravity of milk so as to mask the lactometer reading. Therefore, in the present study a colorimetric method was developed for qualitative detection of sorbitol in milk using mixed indicator. A suitable coagulant was also selected to improve the sensitivity of the test. The analysis was carried for pure sorbitol solution with different volumes of mixed indicator (30µl, 60µl, 0.1ml and 0.2ml) and milk spiked with sorbitol. Results showed a change of colour from green (control sample i.e., without any sorbitol) to pinkish in sorbitol spiked samples. The colour change was distinct to naked eyes in case of cow, buffalo milk sample spiked with sorbitol @ 5% and 2.5% in case of mixed milk, on adding 60 µl of mixed indicator in the presence of boric acid. To increase the sensitivity of the test, approach of using clear milk filtrate instead of milk was adopted. Acetone was found to be a suitable coagulating agent. Colour change in filtrate was also the same as was in aqueous solutions of sorbitol (green to pink). However, the level of sorbitol detection was improved a lot i.e., 0.5% addition of sorbitol in milk could be detected. The effect of other common adulterants (carbohydrates and sugar alcohols, neutralizers, urea and ammonium sulphate) was also studied. Carbohydrates, urea and ammonium sulphate spiking did not affect the color change in sorbitol spiked samples and thereby the performance of the test. However, the presence of neutralizers showed a considerable effect on the performance of the color based test, both in milk and filtrate obtained after precipitation with acetone.

सारांश

दूध को उच्च पोषण मूल्य वाला संपूर्ण भोजन माना जाता है। हालाँकि, साहित्य यह भी बताता है कि यह भारत में डेयरी उद्योग / डेयरी व्यवसाय में सबसे अधिक शोषित वस्तु है। सोरबिटोल हाल ही में मिलावटी पदार्थों में से एक है जिसने दूध के बेईमान व्यापारियों के बीच लोकप्रियता हासिल की है। वे दूध के विशिष्ट गुरुत्व को बढ़ाने के लिए इस मिलावट को दूध में मिलाते हैं ताकि लैक्टोमीटर रीडिंग को मास्क किया जा सके. इसलिए, वर्तमान अध्ययन में मिश्रित संकेतक का उपयोग करके दूध में सोर्बिटोल के गुणात्मक पता लगाने के लिए एक वर्णमिति विधि विकसित की गई थी। परीक्षण की संवेदनशीलता में सुधार के लिए एक उपयुक्त कौयगुलांट का भी चयन किया गया था। मिश्रित संकेतक (30µl, 60µl, 0.1ml और 0.2ml) के विभिन्न संस्करणों के साथ शुद्ध सोर्बिटोल समाधान के लिए विश्लेषण किया गया था और दूध सोर्बिटोल के साथ मिलाया गया था।

परिणामों ने सोर्बिटोल नुकीले नमूनों में हरे रंग (नियंत्रण नमूना यानी, बिना किसी सोर्बिटोल के) से गुलाबी रंग में परिवर्तन दिखाया। रंग परिवर्तन गाय के मामले में नग्न आंखों के लिए अलग था, बोरिक एसिड की उपस्थिति में मिश्रित संकेतक के 60 µl जोड़ने पर, भैंस के दूध के नमूने में 5% सोर्बिटोल @ 5% और मिश्रित दूध के मामले में 2.5% की वृद्धि हुई। परीक्षण की संवेदनशीलता को बढ़ाने के लिए दूध की जगह साफ दूध छानने का तरीका अपनाया गया। एसीटोन एक उपयुक्त जमावट एजेंट पाया गया। निस्यंद में रंग परिवर्तन भी वैसा ही था जैसा सोर्बिटोल (हरे से गुलाबी) के जलीय घोल में होता था। हालाँकि, सोर्बिटोल डिटेक्शन के स्तर में बहुत सुधार हुआ था यानी दूध में सोर्बिटोल की 0.5% मिलावट का पता लगाया जा सकता था। अन्य सामान्य मिलावटों (कार्बोहाइड्रेट और चीनी अल्कोहल, न्यूट्रलाइज़र, यूरिया और अमोनियम सल्फेट) के प्रभाव का भी अध्ययन किया गया। कार्बोहाइड्रेट, यूरिया और अमोनियम सल्फेट स्पाइकिंग ने सोर्बिटोल नुकीले नमूनों में रंग परिवर्तन को प्रभावित नहीं किया और इस तरह परीक्षण के प्रदर्शन को प्रभावित किया। हालांकि, एसीटोन के साथ वर्षा के बाद प्राप्त दूध और छानना दोनों में, न्यूट्रलाइज़र की उपस्थिति ने रंग आधारित परीक्षण के प्रदर्शन पर काफी प्रभाव दिखाय

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LIST OF ABBREVATIONS AND SYMBOLS

AR	Analytical Reagent
Avg	Average
BIS	Bureau of Indian Standards
cal	Calories
cal/g	Calories /gram
°C	Degree Centigrade
°	Degree
FDA	Food and Drug Administration
Fig	Figure
FSSR	Food Safety and Standards Regulation
GC	Gas Chromatography
GLC	Gas Liquid Chromatography
Gm	Gram
Gm/ml	Gram/millilitre
GRAS	Generally Recognized as Safe
HPLC	High Performance Liquid Chromatography
HILC	Hydrophilic Interaction Liquid Chromatography
ICAR	Indian Council of Agricultural Research
IDF	International Dairy Federation
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
Kg	Kilogram
KJ	Kilo Joules
LOD	Limit of Detection
L	litre
M	Molar
m	Meter
ml	Millilitre
min	Minute
NDDB	National Dairy Development Board
NDRI	National Dairy Research Institute
NADP	Nicotinamide adenine dinucleotide phosphate

N	Normal
Nm	Nanometre
%	Percentage
Rf	Resolution factor
sec	Second
TLC	Thin Layer Chromatography
USDA	United States Department of Agriculture
µm	Micrometre
v	Volume
v/ v	Volume/Volume
w	Weight
w/w	Weight/weight

CHAPTER-1

INTRODUCTION

Milk is the normal mammary secretion derived from complete milking of healthy milch animal without either addition thereto or extraction therefrom unless otherwise provided in these regulations. It shall be free from colostrum (FSSR, 2011). Milk is used for good health and is the form of best staple food for the customers to consume for maintaining health (Hospido *et al.,* 2016).

India is the world's largest milk producer, producing 187.7 million tonnes (NDDB, 2018-19) and accounting for 22% of total milk production. A report from the Department of Animal Husbandry Dairying and Fisheries (2018-19) stated that with the launch of Operation Flood, India became first in global milk production. India's milk production has increased from 22 million metric tonnes in 1970 to 187.7 million metric tonnes in 2018-19. During these five decades, about eight times growth was recorded. According to an economic survey report, India's milk production increased by 35.61 percent in the last six years, reaching 198.4 million tonnes in 2019-2020 (Anon, 2021).

Despite of highest milk producing, about 68.7 percent of the milk in country was found to be adulterated (Animal Welfare Board, 2018). Milk is among the list of most adulterated foods in India (Sahoo *et al.,* 2020). An adulterant means any material which is or could be employed for making the food unsafe or substandard or misbranded or containing extraneous matter (FSSR, 2006).

Perishable nature of milk is the major factor which is responsible for its adulteration (Tipu *et al.,* 2011). In order to keep milk temporarily fresh, middlemen commonly add ice to the milk, which results in dilution of milk solids. Middlemen attempt to counter the dilution by adding vegetable oil, starch, flour, sugarcane, whey powder, skim milk powder, and other ingredients to extend the solid content of the milk (Fakhar *et al.,* 2006). Dilution of milk solids due to the addition of water leads to decreased foaminess, hence detergents are added to milk achieve artificially the lost foamy appearance (walker *et al*., 2014). To extend the shelf life of milk chemicals such as hydrogen peroxide, carbonates, bicarbonates, caustic soda, formalin are also commonly used by unscrupulous traders (Tariq, 2001). Malpractice of adulteration is also done to prevent financial losses incurred due to the spoilage of milk during transportation and sale. Recently sorbitol has emerged as a new tool to manipulate the lactometer reading of the diluted milk so that it goes unchecked on the reception dock or dairy plant laboratory. The lack of suitable detection tests for detection of some emerging adulterants like sorbitol, further encourages the unscrupulous traders. Sugar alcohols,

also known as polyhydric alcohols or polyols, are derived from saccharides through a chemical or biochemical process that converts an aldehyde or ketone group to an alcohol group. These polyhydric alcohols are heat stable and do not take part in Maillard browning process and having general formula of -(CHOH) nH_2 where n=4-6. They are referred to as sugar-free bulk sweeteners because they are low in calories. There are three types of polyols i.e., hydrogenated monosaccharides, hydrogenated disaccharides, and mixtures of hydrogenated saccharides and polysaccharides.

Sorbitol is one of such sugar alcohols which is added to milk to increase the specific gravity of milk and is one of the emerging adulterants affecting the dairy sector (Nuzhat *et al.,* 2018).

Several chromatographic methods like (GC-MS, HPLC, etc.) have been reported previously for the assay of D-sorbitol. However, these methods still have some limitations such as requirement of bulky expensive and elaborate instrumentation, tedious and time-consuming, and financial investment (Fang *et al.,* 2019). The methods reported in the literature are mostly for food products (apart from milk), pharma and wine. The one reported for milk needs validation. Hence there is a need of some simple and rapid method to detect this emerging adulterant in milk. Therefore, the present study has been envisaged with the following objectives to develop a colorimetric method to detect sorbitol in milk.

Objectives:

1. To standardize the method for detection of sorbitol in milk.

2. Effectiveness of qualitative method in presence of other adulterants.

CHAPTER-2

REVIEW OF LITERATUREATE

2.0 REVIEW OF LITERATURE

2.1 Sorbitol

Sorbitol was discovered initially in fresh mountain ash berries by a French chemist named Joseph Boussingault in 1872 (O'Donnell & Kearsley, 2012). It is an isomer of mannitol differ only in the orientation of the hydroxyl group on carbon-2. Sorbitol can exist in different polymeric forms, such as alpha, beta, gamma and delta and E, which is a glass transition form. All these forms have different properties including solubility, melting range and stability. The gamma form of sorbitol is most stable and modern manufacturing technique produces sorbitol powder predominantly in this form.

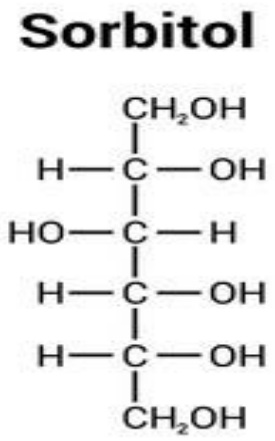


Fig.2.1: Structure of Sorbitol

2.1.1 Production

Sorbitol is produced via two ways i.e., natural (Biochemical) and commercial (Hydrogenation).

Synthesis of sorbitol takes place by catalysis of glucose via NADP – dependent sorbitol 6-phosphate dehydrogenase then the formed sorbitol is further degraded to fructose by $NADP^+$ sorbitol dehydrogenase (Sharma *et al.,* 2014).

Step- 1: Glucose-6-phosphate $\xrightarrow{\text{Sorbitol-6-phosphatse dehydrogenase}}$ sorbitol-6-phosphate

Step-2: sorbitol-6-phosphate $\longrightarrow$ sorbitol

Glucose syrup, invert sugar, and other hydrolysed starches are important raw materials for the manufacturing of sorbitol. Commercial manufacturing of sorbitol uses catalytic hydrogenation of glucose, in this process D-glucose is made to react with hydrogen in water at 120°C under 1500001.15 Torr, for 1 hour. The reaction is driven by a catalyst such as nickel and after completion of reaction the catalyst is filtered out and the solution is purified. The solution is purified in two steps, the first step is to filter the solution through an ion-exchange resin bed to remove gluconate and other ions. In the second step, activated carbon is used to remove any remaining organic impurities from the solution. The commercial 70% sorbitol solution is made by evaporating water under vacuum. Then for further evaporation is done to obtain to at least 99% solids, which is called as crystalline sorbitol (Godswill, 2019).

2.1.2 Storage

Because sorbitol powder compacts easily, careful storage is required to keep the products flowing freely. A shelved storage system should be used instead of stacking pallets of product. The temperature in the room should be kept constant. Bulk storage is normally kept at a temperature of 50–60°C. Syrups with a greater sorbitol content must be handled with extra caution to avoid crystallisation (Deis, 2012).

2.1.3 Physicochemical Properties of Sorbitol

Chemical name of sorbitol is D-glucohexane-1,2,3,4,5,6-hexaol, having a chemical formula of $C_6H_{14}O_6$ with a molecular weight of 182.17 Daltons and melting point of 94-96°C, density is 1.49g/cm^3 and INS and E number of sorbitol is 420. Sweetness is around 60% sweet as sucrose. It provides dietary energy of 2.6 kilocalories (11KJ) per gram versus the average 4 kilocalories (17KJ) for carbohydrates. It is 20-fold more soluble in water than mannitol. It is a colourless and odourless solid obtained in various forms such as flakes, granules, pellets, or powder (Newman *et al.,* 1999).

a) *Heat of a solution*

Sorbitol is having negative heat of solution which causes a colling sensation when placed in mouth as it is an endothermic reaction. Smaller the size of particle faster the solubility so more notable the effect. Mannitol is having less cooling effect than sorbitol and heat of solution of sorbitol is -26.5cal/g at 25°C (Dies, 2012).

b) Hygroscopicity

Sorbitol is very hygroscopic in nature; it starts absorbing moisture when relative humidity reaches out to 65%. So, this can cause a problem when used in foods like candies and especially in tableting where the absorbed moisture can prevent the presses running (Dies, 2012).

c) *Molecular weight*

Sorbitol is having the molecular weight of about 182.17 Daltons. Viscosity, freezing point depression, boiling point elevation and osmatic pressure all are directly related to molecular weight. In ice-cream freezing point depression is an important factor hence sorbitol is use to lower the freezing point of ice-cream (Dies, 2012).

d) *Optical rotation*

The specific rotation of sorbitol in 0.1mg/ml aqueous solution is -1.9°. Optical rotation will be increased to +4.0 to+7.0° by the addition of complexing salts such as borax or ammonium molybdate (Dies, 2012).

e) *Solubility characteristics*

Sorbitol dissolves easily in water, with a solubility of 2.56g/ml H_2O at 25°C. It is almost insoluble in chloroform and ether and only slightly soluble in methanol. It is stable in cold diluted acids and bases, but in acidic or basic media it forms water soluble chelates with many divalent and trivalent metal ions. Sorbitol solutions will discolour when they react with iron oxide (Mullin, 1972).

f) *Crystallographic properties*

Sorbitol exhibits number of crystal forms having number of polymorphs. The gamma form which melts at 101°C, is thermodynamically most stable form at room temperature. Upon standing or stress conditions the sorbitol is converted to gamma form (Dies, 2012).

g) *Glycaemic index (GI)*

The concept of GI was developed over 20 years ago by David Jenkins at university of Toronto as a tool to allow diabetics to manage their diet. It is a method of ranking foods according to the extent to which they raise blood glucose level after consumption. Food contains carbohydrates that breakdown quickly after ingestion giving fast and high

glucose response have the lowest GI values low-55 and bellow, medium-56-69 and high GI-70 and above. GI of sorbitol is very low 10 (Dies, 2012).

h) *Sweetness*

Sorbitol is less sweet than sugar. It is stated that the sweetness is 60% of the sweetness of sucrose. Hydrogenation of a reducing sugar normally results either increase in sweetness or occasionally a slight decrease in sweetness. Hydrogenation of fructose to produce sorbitol produces a very dramatic decreases in sweetness (Newman *et al.,* 1999; Dies, 2012).

2.1.4 Indian Sorbitol Market

Advanced inorganic produces- sorbitol powder > 99% pure having a cost of 130 Rs/Kg and Amrut international produce 70% pure sorbitol solution, which costs @ 65Rs/L and sorbitol solution >99% purity by A.B. Enterprises costs @ 200Rs/L (Anon, 2021).

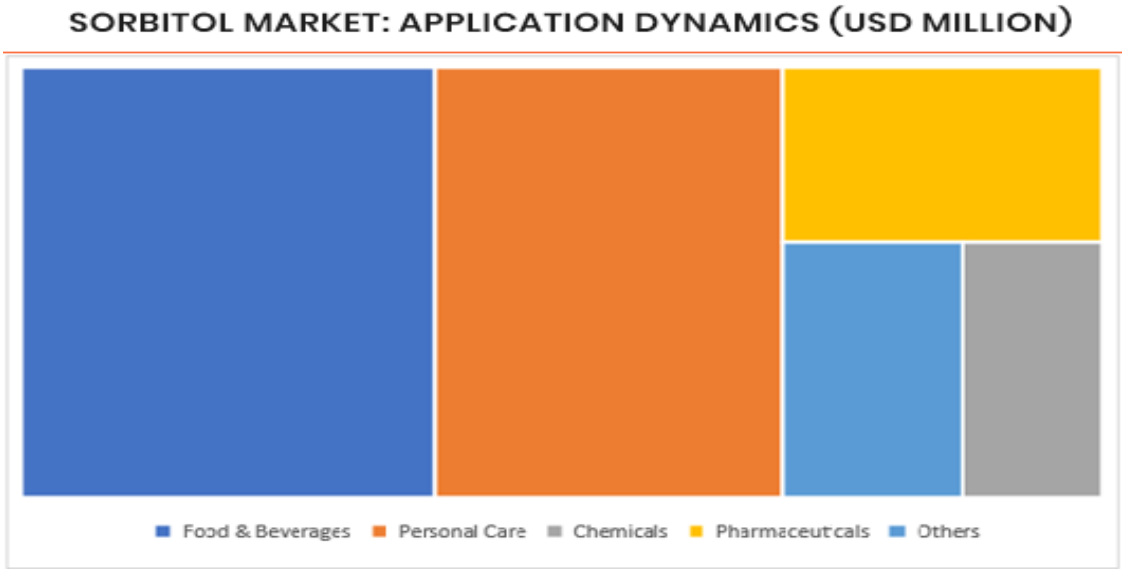


Fig.2.2: Sorbitol market survey

(Anon, 2021)

2.1.5 Applications

Sorbitol is used to extend the shelf life of baked goods. It serves as a humectant, attracting moisture from the environment. It retains the moisture in the baked products and keeps them from drying out. It is added to cooked sausages to increase the flavour

and method of cooking. It is useful in baking since it acts as a plasticizer which helps to slow down the staling process (Dies & Kearsley, 2012).

Because of its viscosity of around 110 centipoise, it is employed as a bodying agent in a wide range of beverages, where it improves the mouth feel. In the production of surimi, a processed fish paste, it is used as a cryoprotective ingredient (Dies & Kearsley, 2012).

It is also utilised as a humectant in some cigarettes and as a raw material for the synthesis of vitamin C.

It is used in the bacterial culture media to distinguish between pathogenic Escherichia coli O157:H7from other strains of E. coli. Because it is unable to ferment sorbitol unlike other E. coli.

Sorbitol and sodium polystyrene sulfonate an ion-exchange resin, are used to treat hyperkalaemia (high bold pressure) In the bowel, the resin exchanges sodium ions for potassium ions, while sorbitol aids in elimination. It is also used to make soft gel capsules for storing single doses of liquid medicines. (Rugolotto et al., 2007).

Used in cosmetics as a humectant and thickener. Sorbitol often used in mouth wash and tooth paste. Some transparent gels can be made only with sorbitol as it has refractive index sufficiently high for transparent formulations.

It exerts laxative effects by drawing water into large intestine, there by stimulating bowel movements (Rapaille *et al.,*2003; Nabors, 2001).

2.1.6 Adverse Health Affects

Effect of sorbitol consumption in 12 diabetes and 23 non-diabetes as a sweetener in dietic foods was studied. It was reported that six diabetics (50%) and thirteen non-diabetics (56%) experienced abdominal symptoms. To examine the link between regular sorbitol consumption and abdominal discomfort, 100 diabetics and 100 non-diabetics were interviewed. They conclude that many diabetics are sorbitol intolerant, and regular sorbitol ingestion may explain certain diabetics' "idiopathic" diarrhoea (Badiga, 1990).

It has been reported that sorbitol added to sodium polystyrene sulfonates might produce gastrointestinal issues such as bleeding, perforated colonic ulcers, and colonic necrosis, especially in uremia patients. Hypovolemia, surgical setting, immunological suppression, peripheral vascular disease, and hypotension after haemodialysis are all risk factors for sorbitol-induced injury (Mohamad *et al.,* 2010).

People with untreated celiac disease often present sorbitol malabsorption as a result of the small bowel damage. Sorbitol malabsorption is an important cause for persisting symptoms in patients already on gluten free diet (Montalto *et al.,*2013).

2.1.6.1 Overdose affects

Injecting large amount of sorbitol can lead to abdominal pain, flatulence and mild to severe diarrhea. Sorbitol ingestion of 20 grams per day lead to severe diarrhea lead to unintended weight loss of 11 kgs in eight months in a woman originally weighing 52 kg. due to the larger molecular weight of sorbitol, after large quantities of sorbitol are consumed only small quantity of sorbitol is absorbed in small intestine then most of sorbitol goes into the colon with resulting gastrointestinal effects (Godswill, 2019).

In a report published in 1985 by the European Union's Scientific Committee on Food, it was noted that consuming more than 50g of sorbitol per day could cause diarrhoea. Foods containing more than 10% sorbitol may be hazardous to one's health and have a laxative impact. Polyols are banned in soft drinks in Europe due to their laxative effects. The European Food Safety Authority issued a health study on polyols like sorbitol in 2011, concluding that they might be used to improve dental health and rebuild tooth enamel (Anon, 2021).

2.1.6.2 Acceptable daily intake

Sorbitol was first used in the USA by the food industry in 1929. The Generally Recognized as Safe (GRAS) list of Food and Drug Administration (FDA) suggests a tolerance of 7% of sorbitol in foods.

The acceptable daily intake (ADI) of sorbitol has been classified as "not specified" by the Joint Food and Agriculture Organization (JECFA), which is the safest category for any food ingredient. There is no established upper limit for sorbitol consumption. However, the FDA warns that consuming more than 30grams of sorbitol per day can cause serious side effects. Sweeteners like sorbitol should be avoided by those who

have nausea, vomiting, or gastrointestinal pain, and pregnant and breastfeeding women should consult their doctor before using them (Embuscado, 2006).

2.2 METHOD OF SORBITOL DETECTION / ESTIMATION

2.2.1 Food and Milk

2.2.1.1 Color based method:

Colorimetric assays have gained great interest because of their inherent advantages, including simple operation, quick response, adaptable sensitivity, and long linear range of the quantitative assay that is based on spectrometry. The added advantage is no need of complicated instrumentation (Zhu and Gao, 2019).

A study conducted in Pakistan was designed to screen the various adulterants in the market milk sold in the vicinity of Hyderabad district of Sindh province in which they found that milk is adulterated with sorbitol @ 3% (Barham *et al*., 2014).

Generally, sorbitol is added to increase the specific gravity of milk and thereby masking the detection of added water through lactometer reading. To detect this adulteration, a color indication method has been listed in the Laboratory manual of quality control of milk (Chaudhary *et al.,* 2015). As per the listed method the sorbitol is made to react with ferric sulphate and sodium hydroxide, the development of brownish color in test tube indicates sorbitol negative and precipitate will settle down. In case of positive samples, green color is formed, precipitate formation is absent. Limit of detection reported was up to 0.25% in raw milk.

Fig.2.3: Test tubes containing negative and positive sorbitol solution

A survey conducted in Pakistan used the above procedure, in which they observed distinct colour (yellow green) in sorbitol containing milk, while the appearance of reddish-orange colour in negative sample. They also observed that pasteurized milk samples showed comparatively better results (Basharat *et al.,* 2019; Shehazadi *et al.,* 2016).

Survey study related to milk adulteration in Pakistan was done using MAT Kit (Milk Adulteration Test) Kit. It was reported that sorbitol was one of the adulterants (Braham *et al*., 2019).

Minsker (1959) developed a method for detecting sorbitol in 4% vinegar. Using a solution of sorbitol in 4 percent acetic acid, corresponding to 10 mg per ml, the process was evaluated with various amounts of sorbitol and reagents under various conditions. The procedure used sorbitol solutions containing 20-300 mg of sorbitol. Evaporate the solution until it is almost dry, then stir in 20 mL strong hydrochloric acid gently until it dissolves. They filtered on asbestos pad in a Gooch crucible and washed it with 50 ml cold water and 50 ml methanol and dried it at 100°C for 1 hour and weighed dissolve the precipitates by washing with chloroform, dry the crucible and weigh compute the weight of precipitate by difference. The huge portion of O-chlorobenzaldehyde (20 drops) required to precipitate 300mg of sorbitol was discovered.

Musto *et al.* (2009) constructed sensor array for detection and identification of wide range of commonly used natural and artificial sweeteners. The array composed of a series of ormosil encapsulated pigments that comprised of indicators immobilized on a hydrophilic porous membrane. This technique allows for rapid interactions between aqueous analytes and hydrophilic indicators. Selectivity relies in part of differences in association constants of boronic acids with diols which results in change in solution pH. Array has excellent reproducibility and long shelf life.

Kumar *et al*. (2015) had conducted a kinetic study that the oxidation of sugar alcohols will lead to the production of either aldehydes or acids by different oxidants. Here they had used Dichloro isocyanuric acid (DCICA) in aqueous acetic acid- per chloric acid media as an oxidant and ruthenium (III) as a catalyst which leads to the production of D-gluconic acid which is the oxidation product of sorbitol, which was identified by spot test. Dinapthol sulphuric acid used for further confirmation of gluconic acid by treating little amount of mixture with a β, β dinaphthol solution in concentrated sulphuric acid followed by heating to 1 h in a water bath at 85°C. Development of green colour indicates the presence D-gluconic acid.

2.2.1.2 Enzymatic method

Umeda *et al.* (2001) developed an improved enzymatic assay of D-sorbitol in human erythrocytes. Samples were prepared by centrifugation, washing with saline, lysed with

distilled water and proteins were precipitated by NaOH and $ZnSO_4$ and again centrifuged which resulted in to a clear supernatant, which was mixed with glycine buffer containing NAD^+ and sorbitol dehydrogenase. Contents were incubated for 30min at 37°C, during the oxidation NAD+ was converted into NADH which was measured fluorometrically. Limit of detection was 0.64µmol/L.

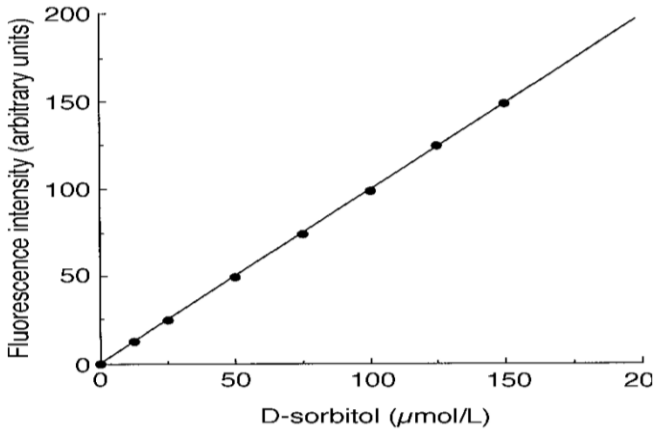


Fig.2.4: Calibration curve of the enzymatic and fluorometric assay of D-sorbitol concentration.

2.2.1.3 Spectroscopy method

Adcock (1957) determined sorbitol in fruit preserve and fruit squash where sorbitol was subjected to oxidation with the periodate in alkaline solution which led to the production of two moles of formaldehyde by oxidation of one mole of sorbitol with periodate. The formed formaldehyde was made to react with chromotropic acid leading to red or wine-coloured complex which was measured at 570 µm.

Graham (1963) used anthrone reagent to detect the amount of sorbitol in food gums. There were some prerequisites for a repeatable quantitative result, such as 0.15 percent anthrone in concentrated sulphuric acid. After adding this acid to the sorbitol solution, it was heated for 60 minutes at 99°C. As a result, colour was produced, which had to be measured after cooling for 30 minutes because colour intensity rises with time. When sorbitol combines with concentrated sulfuric acid, it generates a yellow colour with a 410nm absorption, but when it reacts with sulfuric acid and anthrone, it generates a red yellow colour with a 720nm absorption.

Graham (1964) determined the sorbitol in milk in which proteins were removed by treating with glacial acetic acid and tri carboxylic acid, followed by removal of carbohydrates by treating with acid and alkali and subsequent filtration through ion exchange resins. Filtrate was made to react with the Komarowsky reagent (cyclic aldehyde, thiourea, and concentrated sulfuric acid) leading to the production of red colour having maximum absorption at 540 µm.

2.2.1.4 Polarimetric method

Turner (1964) reported a method of detection of sorbitol in diabetic chocolates. He reported that sorbitol in the presence of molybdate in acid conditions exhibits pronounced dextrorotation. He described that the change in rotation is specific and can be used quantitatively. This change is unaffected by the presence of optically active sugars, so that sorbitol can be determined in their presence. Recoveries were between 98-100%, here chocolate with sorbitol contents ranging from 24-33% were tested.

2.2.1.5 Titrimetric method

2.2.1.5.1 Foods

Todd *et al.* (1938) developed a method for detecting sorbitol in blood and urine samples by collecting samples and treating them with various chemicals to remove all interferences. In a Pyrex tube, they put 5 ml of this sample containing 0.1 to 0.7 mg of sorbitol, 3 ml of (1.8 percent of $C_6N_6FeK_3$ in water), 3 ml of (5 percent of Na_2SO_4 in 3.33N NaOH), and heated for 30 minutes. Then they added 5ml of a 15 percent $ZnC_4H_6O_4$ + 12% percent KI and CH_3COOH solution, which leads to iodine liberation, which was titrated against 0.005N thiosulfate and starch as an indicator, however this method has several drawbacks.

Adcock (1957) determined the sorbitol in food stuffs (fruit squash and preserve) by heating the sorbitol with periodate in presence of sulphuric acid for 20 min. after cooling potassium iodide (KI) and contents were titrated 0.005N sodium thiosulphate using starch as an indicator. The amount of sorbitol equivalent to 1ml of 0.005N sodium thiosulphate, theoretically.

Bark *et al*. (1976) developed a technique to detect sorbitol in foods (blackberry jelly, orange marmalade, chocolate) by causing them to react with sodium periodate,

resulting in the production of heat. This heat was used to determine the end-point of the titration using an enthalpogram, with the break in curvature being considered the endpoint. They also discovered that all of the sugars reacted with periodate more slowly than sorbitol.

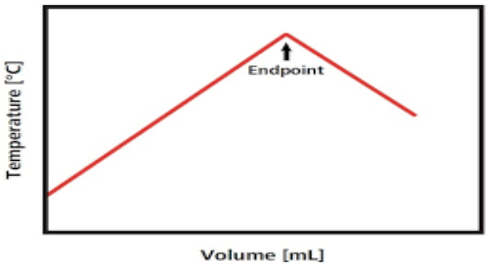


Fig.2.5: Enthalpogram

2.2.1.5.2 Pharma

Pandey *et al.* (2018) used the pyridinium fluorochromate (PFC) reagent to determine the sorbitol. Chromium (VI) is an excellent oxidising agent in PFC. In a conical flask, aliquots containing 1mg of the material were placed, and (PFC) and H_2SO_4 were added. The reaction mixture was allowed to stand for 15 minutes. After the reaction was completed, a solution of potassium iodide (KI) was added, agitated, and allowed to stand for 1 minute. Using starch as an indication, liberated iodine was titrated with 0.01N sodium thiosulphate. The amount of the sample was estimated by the difference in the titre value of sodium thiosulphate solution for the blank and actual experiments.

Calculation

$$\text{mg of sample} = M * N\ (B-S)/n$$

Molecular weight of sample (M), Normality (N), Volume of thiosulphate sol for blank (B), volume of thiosulphate sol for sample (S), Stoichiometry of Reaction (n). One disadvantage is that its reaction time is around 50min.

2.3 Sensors

2.3.1 Medical Applications (Infusion solutions)

Feng *et al.* (2014) prepared polymers by electrochemical polymerization that employs the concept of molecular imprinting for analyte sensing. The frequency shift was directly proportional to the concentration of sorbitol and the detection limit was about 1 mM. These sensors could be stored for more than 4 months at room temperature.

Rattanawaleedirojn *et al.* (2016) developed an electrochemical sensor for sorbitol detection by using Ni-P-TiO_2 as a working electrode. It measured the electrocatalytic activity of oxidation of alcoholic compounds (sorbitol) by linear sweep voltammetry. A low LOD value of 1.0 nm., a wide linear range of 2.0 nm. – 0.2 mM were achieved for sorbitol in pure solution.

Fang *et al.* (2019) reported different types of diboronic acid-based sensors for recognition of D-Sorbitol. They reported that binding of sensor with D-Sorbitol in solution increased the fluorescence by 1.5 times. Sensor had a high binding constant (10922±776 M) and a low limit of detection (6.91×10-7 M) in pH 9 and the response time was 0 to 25 min. However, there are some limitations to the sensors. For example, the pH value required is little higher (pH 9) than PH value of biological fluids like milk which is less than 9. Therefore, when the sensor is applied to detect D-sorbitol in biological fluid, it needs to increase the work of regulating biological fluid pH value.

2.3.2. Foods

Michel *et al.* (1997) prepared a biosensor layer by using multi-enzymatic sequential reaction. They used the compartmentalization of three enzymes viz. sorbitol dehydrogenase (SDH), NAD (P) H: FMN Oxidoreductase, and bacterial luciferase. The range of measurement is from 50 nm. to 2μM with a response time of 4-6 min and optimum pH is around 7.0. Stored either 4 or -20°C for the dehydrogenase and only at -20°C for the bioluminescence system. This activity retained up to 15 days. This technique was employed for food stuffs like sweets and chocolates.

Saidman *et al.* (2000) developed a biosensor by using a carbon paste electrode. This electrode was modified with d-sorbitol dehydrogenase (SDH) and nicotinamide adenine dinucleotide-(NAD^+), immobilised by a layer of a nonconducting poly(o-phenylenediamine) (PPD). Modified carbon paste was packed into the well of the working electrode. The electrodes thus prepared were used for the amperometric measurements of sorbitol in food stuffs (dietetic ice cream, candy) at an applied potential of 0.0 V. The resulting biosensor responded rapidly to sorbitol up to 8×10^{-4} M with a detection limit of 4×10^{-5} M. Electrode did not allow sorbitol detection at very low concentration levels and these electrodes was stable for up to 15 days.

Kant *et al.* (2016) detected D-sorbitol in aqueous infusion samples by employing surface plasmon resonance (SPR) as the sensing principle, wherein, SPR technique

was coupled with fiber optics. The sensitivity was 92.16 nm/g/ml and limit of detection (LOD) was 3.6 ng/ml and fast and sensor display maximum activity at a pH value of 7. So, that it can be used for milk also with little modifications. This technique could be employed for both food and medical applications.

2.4 Sensors detecting sorbitol along with other components in food

Harnsoongnoen *et al*. (2017) developed a magnetic sensing at microwave frequencies for real-time monitoring of sucrose, sorbitol, D-glucose and D-fructose concentrations in food and plants. The magnetic sensor was connected to a Vector Network Analyzer (VNA) and the electromagnetic interaction between the samples and sensor was analysed. The magnitude of the transmission coefficient was used as an indicator to detect the solution sample concentrations ranging from 0.04 to 0.20 g/ml.

Atiqullah *et al.* (2019) had made three different structures of index guided hexagonal shaped Hollow Core Photonic Crystal Fiber (HC-PCF). Specially designed for sensing harmful food additives like saccharin, sorbitol and butyl acetate the sample can be sensed by the interaction of light with the sample. Sensitivity of 88.75%, 87.37% and 86.72% for saccharin, sorbitol, and butyl acetate respectively at the operating wavelength of 1.33 μm.

2.5 Thin Layer Chromatography (TLC)

Coles & Upton (1972) developed a thin layer chromatographic technique for identification of sorbitol and sorbitol solutions. Here the glass plates coated with 250μm silica gel and sprayed with boric acid solutions until saturated and dried. Distilled water was used as a mobile phase. Spots were detected by using alkaline permanganate as a spraying reagent. Rf value of sorbitol was (0.66) and Rf values of contaminants were mannitol (0.77), D-fructose (0.73), maltitol (0.79), D (+)-xylose (0.83), D-glucose (0.86), sucrose (0.91) and maltose (0.99).

For the separation of glucose and sorbitol in diabetic millets (Hadzija *et al.,*1994) utilised cupric ion impregnated silica gel thin layer chromatography plates. They used a variety of solvents as a mobile phase. Spots were detected using a potassium permanganate detecting reagent, which produced yellow spots on a deep-violet backdrop. The Rf values of glucose and sorbitol were 91 and 22, respectively, when the mobile phase was distilled water. When the mobile phase was n-propanol: water

(4:1) had Rf values were 55 and 10, The Rf values in water-ethanol (7:3V/V) were 60 and 5 and in water-ethanol (9:1) were 76 and 9.

Hundley *et al.* (1996) developed a method for detection of sorbitol in bakery products by extracting the sorbitol with water and interferences like reducing sugars were removed by treating with alkali which leads to the production of acids and their salts were removed by strongly basic ion exchange column. Whereas non reducing sugars were inverted by acid. Then the sugar alcohols were treated with periodate to undergo oxidation. The amount of periodate consumed in oxidation were determined by titration with sodium thiosulphate using starch as an indicator. Sorbitol is identified in an additional step by thin layer chromatography where they used isopropyl alcohol: acetone: water (4:2:1: v/v/v) as a mobile solvent and 0.5% KMno4 in 1N NaOH as a spraying agent. The Rf values of sugars and sugar alcohols as follows which sorbitol (Ff 0.38) is separated from dextrose (0.62), sucrose (0.61), fructose (0.47), Lactose (0.47), mannitol (0.52) and glycerol (0.63)

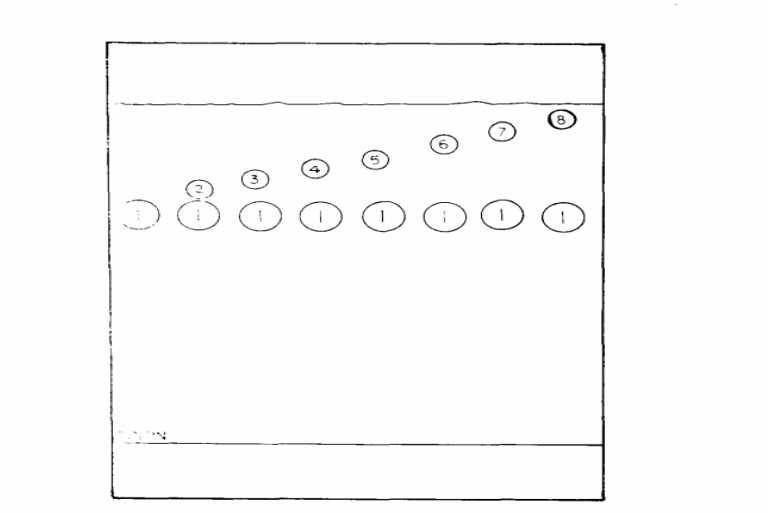


Fig.2.6: Thin layer chromatogram of sorbitol and potential contaminants:1, sorbitol; 2, mannitol; 3, D-fructose; 4, maltitol; 5, D (+)-xylose; 6, D-glucose, 7, sucrose; 8, maltose.

2.6 Paper Chromatography

Hough (1950) used the paper partition chromatography for separation of polyhydric alcohols from leaves. They used different solvent systems as mobile phase and a solution of 5% silver nitrate containing ammonia in excess as detecting reagent to develop the paper chromatogram. The reagent was very sensitive for hexitols and

glycerol, since it detected as little as 1 µg. Rf values of sorbitol with different solvents was as follows: (0.06) in n-butanol saturated with water, (0.21) in n-butanol: ethanol: water (4:1.1:1.9), (0.10) in n-butanol: ethanol: water (4:1:5), (0.21) in benzene: n-butanol: pyridine: water (1:5:3:3) and (0.17) in n-butanol: acetic acid: water (5:1:2).

A new spraying agent for detection of polyols on paper chromatogram was developed by (Hockenhull, 1953). They employed two different solvent systems and a spraying reagent consisting of 1 part 0.05N sodium borate, 2 parts phenol red, and 7 parts methanol was employed to detect spots. Sorbitol Rf values in various solvent systems were (0.92) in n-butanol: pyridine: water (3:2:1.5) and (0.87) in n-butanol: pyridine: water (3:2:1.5). (4:1:1).

A paper chromatography approach for the identification of sugar alcohols was developed by (Cifonelli and Smith, 1954). As irrigating solvents, they employed tert-amyl alcohol, n-propyl alcohol, and water. They sprayed the chromatogram with metaperiodate solution and used the benzidine reagent-B after it had dried (mix 10 vols. of 0.1 M benzidine in 50 percent aq. alcohol with 2 volumes of acetone and 1 volume of 0.2N hydrochloric acid). Spots appeared as a colourless spot-on blue background with Rf value of sorbitol as 0.43.

For the separation of sugar alcohols and glycosides (Cerbulis, 1955) utilised paper chromatography with a solvent mixture of 1-propanol-ethyl acetate-water. The paper chromatogram sheet was placed in a shallow pan containing the p-anisidine reagent. The sheet was heated to 100 degrees Celsius for 10 to 15 minutes until the paper turned light brown and sugar alcohols emerged as a white spot on a light bright backdrop. Sorbitol had an Rf value of 0.31.

2.7 Capillary Zone Electrophoresis (CE)

Capillary electrophoresis (CE) is a family of electrokinetic separation methods performed in submillimeter diameter capillaries and micro and nanofluidic channels. In CE methods, the analyte migrates through electrolyte solutions under the influence of an electric field. Analyte separate according to ionic mobility or partitioning into an alternate phase. To identify biological and pharmaceutical analytes, CE is coupled to a variety of detectors, like fluorescence, mass spectrometry, and electrochemical detection (Huang *et al.,* 2002; Suntornsuk, 2010).

Jian-Guo *et al.* (2007) established a miniaturized CE with electrochemical detection system for the separation and determination of xylitol and sorbitol in three commercial sugar-free gums. These two analytes have been separated within 10 min at a separation voltage of 4 kV in 70 mmol/L NaOH running buffer. Linear response was obtained at the range of 5.0×10^{-5} to 1.0×10^{-2} mol/L and 5.0×10^{-5} to 5.0×10^{-2} mol/L with the detection limits of 5.0×10^{-6} mol/L and 2.5×10^{-6} mol/L for xylitol and sorbitol, respectively. The proposed method was successfully applied to determine the food gum samples with the RSD and average recoveries of 3.7%, 4.5% and 98.1%, 91.1% for xylitol and sorbitol, respectively.

Pospisilova *et al.* (2007) reported, mannitol, sorbitol, and xylitol in the form of anionic borate–polyol complexes were separated and determined using capillary zone electrophoresis with indirect UV detection at 215 nm. The separation was performed in a fused silica capillary at a voltage of 25 kV. The background electrolyte was chosen a 200 mM borate buffer. When utilizing xylitol as the internal standard, the separation of mannitol and sorbitol took 13 minutes. For each analyte, the limit of detection was 30µg/mL. This method was used to determine the concentrations of mannitol and sorbitol in medicinal infusion solutions.

Coelho and De Jesus (2016) developed a method for the determination of erythritol, maltitol, xylitol and sorbitol in sugar free chocolates by combining capillary electrophoresis (CE) with capacitively coupled contactless conductivity detection (C4D). Using bare fused capillary column with length 70 cm and internal diameter 50 µm. At the separation voltage of 25KV. The polyols were extracted from the samples only using ultrapure water and ultrasonic energy. Separation was obtained by dynamic formation of ester borate between the polyols and the borate anions in the alkaline background electrolyte. The limit of quantifications of analytes were erythritol-12.4, maltitol-15.9, xylitol-9.0 and sorbitol-9.0 µg/g.

2.8 Liquid Chromatography

The conventional liquid chromatography is an older version of modern high performance liquid chromatography. It consists of plastic and glass column that can range from few centimetres to several meters around 10-100cm. The movement of flow was by gravity and volumetric flow rates in mL/hr. Separation took hours and sometimes days to complete whereas high-performance liquid chromatography relies

on a pump. It comprises of columns constructed of stainless-steel tubes with an inner diameter of 3-5 mm and a length of 10-30 cm (Karger, 1997).

According to USDA, Meat and Poultry inspection (MPI) regulation limits the quantity of sorbitol in sausages to 2.0%. A liquid chromatographic method for the determination of dextrose, mannitol and sorbitol in meat products was developed (Ali, 1988). Analytes were extracted from comminute meat products with 52% ethanol. After filtration, the extracts were purified by passing through a C_{18} Sep-pack cartridge and two ion exchange resins, after concentration and filtration analytes were analysed by liquid chromatography using a cation exchange analytical column and differential refractometer detector. Samples were fortified with dextrose, mannitol and sorbitol at four different concentrations. Retention time of dextrose-8.50, maltotol-17.00 and sorbitol-22.52 min.

For the identification of sugar alcohols in beverages and foods (Nojiri *et al.,* 1999) used the liquid chromatography (LC) technique. P-nitrobenzoyl chloride (PNBC) was used to make an ultraviolet-absorbing derivative of the sugar alcohols meso-erythritol, xylitol, D-sorbitol, and D-mannitol. 30 percent ethanol was used to extract the sample. An ODS column with acetonitrile water (65 + 35) as the mobile phase was used to identify the derivatives. The method sensitivity is 10 to 1000 times that of gas chromatography with flame ionisation and liquid chromatography with refractive index detection.

2.9 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is an important qualitative and quantitative technique which is most versatile, safest, dependable and fastest chromatographic technique. Added advantage is that it does not require a complicated derivatization step.

Samarco (1982) again used the same technique for the detection of three analytes i.e., mannitol, sorbitol and xylitol in chewing gums and confections where he finds retention times of mannitol, sorbitol and xylitol as 18,26,22, respectively.

Dennis *et al*. (1994) conducted a survey of sorbitol concentration in wines sold in UK. They used high-performance liquid chromatography with refractive index detector as a technique to quantify sorbitol in those wine samples. Most wines were found to be

contain less than 0.2g/L of sorbitol. These concentrations were detected by gas chromatography and confirmed by mass spectrometry. The sorbitol was isolated from three wines by semi-preparative HPLC. The ratios of these isolates were measured by stable isotope ratio mass spectrometry (SIRMS) and were found to be inconsistent with that expected from grape products. The isotope ratios of were further examined by GCSIRMS. This proved to be a rapid and convenient technique and was particularly valuable when limited amounts of analyte were present in the sample.

Grembecka *et al.* (2014) used HPLC-CAD for simultaneous determination of sugars and polyols in food products. They used a column packed with 5 µm shell particles (4.6 × 250 mm) and acetonitrile–water gradient mobile phase at 25 °C. The limit of detection was 0.28 µg/ml and limit of quantification was around 0.93µg /ml. Recovery was around 98.5%, and retention time was 18.33min.

Ma *et al.* (2014) developed HPLC–ELSD method to separate and quantify the sugar compounds of fructose, sorbitol, glucose and sucrose without derivatization in 25 min. Drift tube temperature of the ELSD system was set to 82°C and nitrogen flow rate was 2.0 L/ min. Limit of detection was 0.27mg/ml and limit of quantification 0.91mg/ml. Recovery was 103.23%. retention time was 11.12min.

Spinelli *et al.* (2016) analysed sorbitol as an adulterant in grape juice. They used HPLC-87C column, 300×7.8mm with refractive index (RI) detector. Milli-Q water was used as mobile phase in isocratic flow of 0.60 mL min−1. All juices were diluted twice, filtered through membranes of 13 mm of diameter and 0.8 µm of pore size and injection volume of samples was 20 µL. limit of detection of the method for sorbitol was-6.44mg/L and limit of quantification was 7.09 mg/L and retention time was 22.5 min.

Hadjikinova *et al.* (2017) developed a method enabling the simultaneous determination of sugars and polyols in food products by HPLC-RID. Column operating at 80 °C and RID at 35 °C, mobile phase distilled water with flow rate 0.5 ml/min was used. They observed a limit of detection of 0.17mg/ml, limit of quantification 0.56, repeatability and reproducibility were around 4.2 and 6.5%, recovery rate around 98%, and retention time around 47.14min for sorbitol.

2.10 Gas Liquid Chromatography (GLC)

GLC became the leading technique for the analysis of carbohydrates in foods until the mid-1970s. GC with flame ionization detector (FID) is 10 times more sensitive than HPLC using refractive index detector. As study conducted by (Date *et al.,* 1982), concluded that separation of sorbitol from glucose by HPLC was not possible. Although analysis times were longer for GC than HPLC, GC offered greater sensitivity and better resolution. Generally, Carbohydrates are non-volatile so it is necessary to form chemical derivatives that have enhanced volatility. This is usually done by applying derivatization procedures that convert the hydroxyl groups of the carbohydrate molecules to ether or ester groups.

Hundley (1968) determined the sorbitol in bakery products, wine and vinegar by gas liquid chromatography using flame ionization detector. From bakery products sorbitol is extracted with methanol and wine, vinegar was taken directly. Sugars and sugar alcohols were acetylated by using pyridine then extracted with chloroform. Acetates formed so were determined with gas liquid chromatography by a U-shaped glass column packed with 10% DC-200 and nitrogen as a carrier gas.

A rapid method for the detection of sorbitol in raisins and dietetic foods was developed by Fernandez-Flores and Blomquist (1973). Sorbitol in raisins was extracted with methanol and after adding of lead salt there was a precipitation of interfering organic acids and sorbitol was converted to trimethylsilyl derivatives and they used α-glucoheptose as an internal standard.

Samarco (1977) developed a high-pressure liquid chromatography protocol for the separation and determination of mannitol and sorbitol in sugarless chewing gums. Samples were extracted by using toluene and water and filtered through 0.45µm pore membrane filter. They used Q-15S as a cationic exchange resin as a column, water as the mobile phase and differential refractive index as a detector and average recoveries of mannitol and sorbitol were 98.9 and 99.2% and Standard deviations were 0.35 and 0.98% and time of retentions were 11 and 15 min.

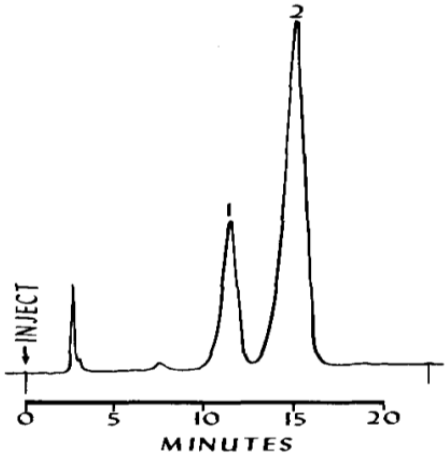


Fig.2.7: Liquid chromatographic separation of 1, mannitol; 2, sorbitol in mixed standard solution

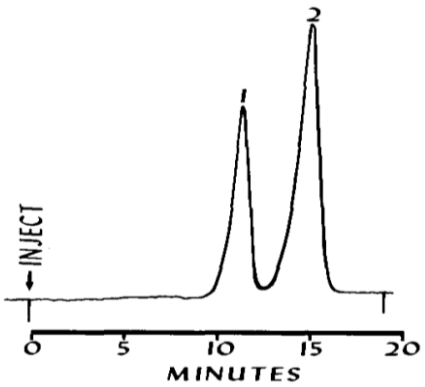


Fig.2.8: Liquid chromatographic separation of 1, mannitol and 2, sorbitol in sugarless chewing gum

For detecting sorbitol in cooked sausages, Moseley *et al.,* (1978) employed a gas chromatographic technique. According to the Department of Agriculture's Meat and Poultry Regulations, this product may contain up to 2% sorbitol. Sorbitol was extracted with water and subsequently freeze dried to remove the water yielding a dry residue. To create the trimethylsilyl (TMS) derivative, the silylation reagent is added to the dry residue and because of that the intermolecular and intramolecular hydrogen bonds are broken and sorbitol becomes volatile after synthesis of the TMS derivative. They used 5% OV-1 column to separate the sorbitol and measure its concentration.

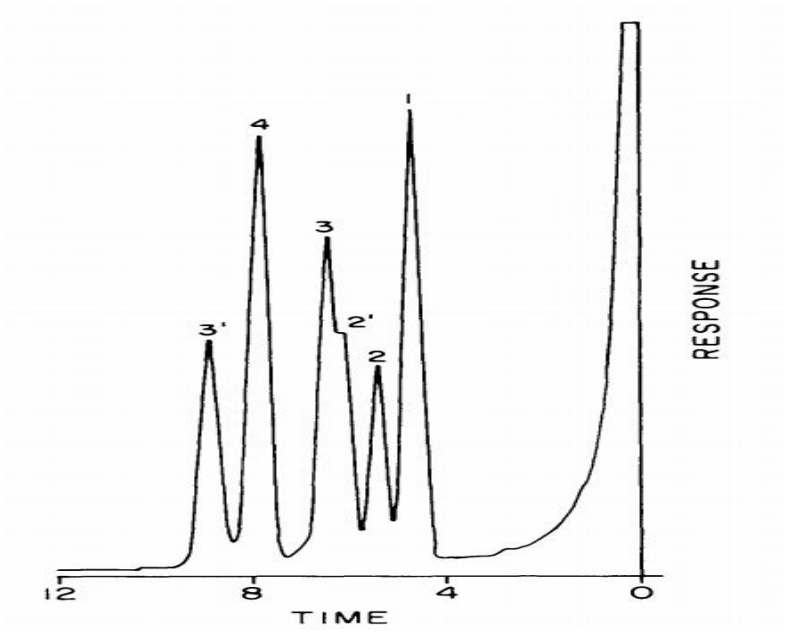


Fig: 2.9 Gas chromatogram of 1,5 µg fructose; 2,5 µg galactose; 3,5 µg dextrose; and 4,5 µg of sorbitol

Daniel *et al.* (1982) developed the method using gas chromatography for the determination of sorbitol, xylitol, and mannitol in chewing gum and sorbitol in mints. To extract the aqueous, chewing gum was separated between methylene chloride and water, and mint is dissolved in water. Then, the extract was dried and the residue is derivatized with the help of pyridine-acetic anhydride to form the corresponding peracetates. These derivatives were quantitated by gas chromatography using 9 ft × 2mm column packed with 10% Silar IOC on Chromosorb. They got an average recovery of these sugar alcohols ranging from 96-102%.

Burda and Collins (1991) detected the adulteration of wine with sorbitol by using gas chromatography. Sorbitol was determined as its trimethylsilyl derivative. Sorbitol was found in 10 wines in the range 3.4-6.7 g/L and the detection limit for sorbitol was found to be 0.1 mg/L.

The GLC method was used to determine the amount of sugar alcohols present in dietetic biscuits (Jones *et al.,* 1996). Individual hexitol concentrations are determined using acetate esters, while overall hexitol concentrations are determined using trimethylsilyl ethers (TMS). The internal standard was 1,4-sorbitan. On a QF-l/BDS column, the acetate esters were identified, whereas the TMS ethers were

chromatographed on a SE-30 GLC column. From laboratory-baked biscuits, total hexitols were recovered at a rate of 97—100%.

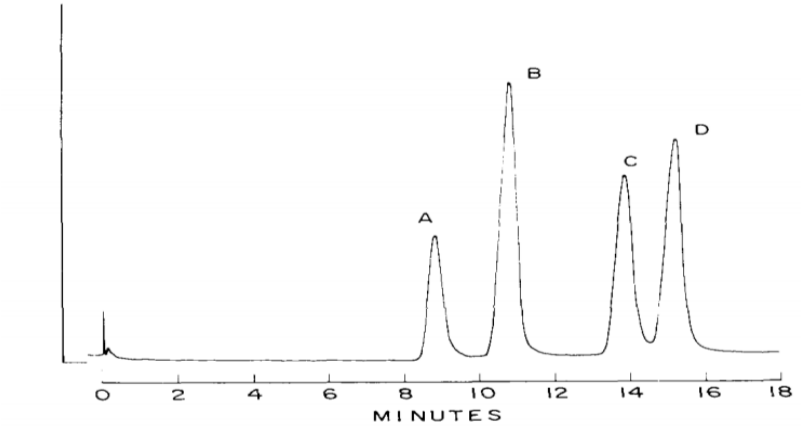


Fig.2.10 GLC separation of the acetate esters of 1,4-sorbitan (A), xylitol(B), D-mannitol (C), D-sorbitol (D)

Monosaccharides such as dextrose, galactose and fructose normally added to the product do not interfere with the sorbitol analysis. Many of monosaccharides gave 2 peaks, which was attributed to formation of alpha and beta forms in water. Analytes were measured by a flame ionized detector.

2.11 Hydrophilic Interaction Liquid Chromatography (HILC)

Stander *et al.* (2013) conducted a survey of fruit juices from South Africa that had been adulterated by the addition of sugar or less expansive fruit juices. Hydrophilic interaction liquid chromatography-mass spectrometry with electrospray ionisation in the negative mode and ultraviolet light detection was used to analyse artificial sweeteners and colours. They were able to distinguish the fruit juices based on the presence of marker chemicals. The samples were prepared by diluting them 10 times in 50% acetonitrile, vertexing, and centrifuging them. As mobile phases, 10mM ammonium acetate in water and 10mM ammonium acetate in 95 percent acetonitrile were used. Limit of detection of sorbitol was 2.0 mg/L, limit of quantification 5.0 mg/L and time of retention of sorbitol was 6.49 min.

CHAPTER-3

MATERIALS AND METHODS

The present study was carried out for development of colorimetric method for the qualitative detection of sorbitol in milk. Effectiveness of the developed colorimetric method was checked in the presence of other adulterants. This chapter includes materials and methods used during course of this study.

3.1 Materials

3.1.1 Collection of milk samples

Fresh pooled milk sample of cow milk, buffalo milk was collected from Livestock Research Centre, National Dairy Research Institute, Karnal. To prepare mixed milk sample, cow and buffalo milk collected in the study were mixed in 1:1 proportion.

3.1.2 Chemicals

Sodium hydroxide (NaOH) pellets (EMPLURA, Merck specialties Pvt. Ltd., Mumbai, India), Ethyl alcohol absolute (Jiangsu Huxari International Trade Co. Ltd, China), Sodium bicarbonate ($NaHCO_3$), Sodium carbonate (Na_2CO_3) (Hi Media, Mumbai-India)

Sucrose (Merck Life Science, Pvt. Ltd., Mumbai-India), Maltodextrin, Starch and maltitol (Hi Media, Mumbai-India), Glucose (Qualigens Fine Chemicals, Mumbai, India) and sorbitol (Sigma Aldrich, USA).
Ammonium sulphate (AR grade, Sigma Aldrich, USA).
Urea (NH_2-CO-NH_2) (Thermo Fisher Scientific Pvt. Ltd., Mumbai, India).
Acetic acid (CH_3COOH), Zinc Acetate monohydrate and Phosphotungstic acid monohydrate (Thermo Fisher Scientific Pvt. Ltd., Mumbai, India).
Acetone (Loba Chemie, Pvt. Ltd., Mumbai, India).
Boric acid (Hi Media, Mumbai-India), Ferrous sulphate (Thermo Fisher Scientific Pvt. Ltd., Mumbai, India).

3.1.3 Reagents

Boric acid (1%): Exactly one gram of boric acid was dissolved in distilled water and volume was made to 100ml with distilled water.

Acetic acid (10%): Ten ml of glacial acetic acid was diluted and volume was made to 100 ml with distilled water.

Trichloro acetic acid (15%): Fifteen grams of trichloro acetic acid was diluted and made to 100 ml with distilled water.

Biggs Szijarto reagent: Twenty-fivegrams of Zinc acetate dihydrate ($Zn(CH_3COOH)_2.2H_2O$ and 12.5 g of Phosphotungstic acid monohydrate ($W_{12}O_{36}.H_3PO_4.H_2O$) was dissolved in about 100ml of distilled water in a 200ml one-mark volumetric flask.20 ml of glacial acetic acid (CH_3COOH) was added to it. Diluted to the 200 ml mark with the distilled water and mixed. At 4°C solution can be stored no longer than one week.

Ethanol (95%): Ninty-five ml of absolute ethanol was diluted and volume made to 100ml with distilled water.

Mixed indicator (0.1%): Exactly 80mg of methyl red and 20 mg methylene blue mixed indicator was weighed and dissolved in small quantity of 95% ethanol, finally the volume was made to 100ml with 95% ethanol. Sodium hydroxide (NaOH 10%): 10 grams of sodium hydroxide pellets were weighed and diluted and volume made up to 100ml with distilled water.

Ferric sulphate (1%): One gram of ferric sulphate was weighed and dissolved in small quantity of distilled water, finally the volume was made to 100ml with distilled water.

3.2 Apparatus and glassware

Funnels (small and large), measuring cylinders (50, 100, 250, 500ml), volumetric flasks (10, 25, 50, 100, 250ml), glass test tubes were purchased from Borosil Laboratory Glass Co. India.

Thermometer (Jain Scientific and Glassware Works Pvt. Ltd., Mumbai, India).

3.2.1 Equipment

Hot Air Oven with Thermostat: Metrex Scientific Instruments Ltd., New Delhi.

Electric Heater: Vikrant, Jain Enterprises, India.

Electronic Balance: Precisa XB 220A, Switzerland. Filter Paper: Whatman filter paper (NO.40 and 42) (GE healthcare, UK Ltd).

Water Bath with Thermostat: The laboratory Glassware Co., Ambala Cantt, India.

pH meter (M-420, Cyberscan pH Tutor, EUTECH Instruments, Thermo Fisher Scientific, Mumbai, India).

vertex shaker (Remi laboratory instruments, Mumbai, India).

Auto pipettes (100-1000µl (BRAND Transferpette, Germany)

(20-200µl (LabQuest Borosil, Pune, India)

(10-100 µl (LabQuest Borosil, Pune, India)

3.3 Methodology

3.3.1 Standardization of the quantity of mixed indicator solution to develop colour in sorbitol solution of varied concentrations prepared in distilled water.

3.3.1.1 Protocol

Sorbitol solution of different concentration viz. 0.1%, 0.25%, 0.5%, 1% was prepared in water. One millilitre of each solution was pipetted out in different glass test tubes followed by the addition of 1.0ml of (1%) Boric acid solution in water. (Pure Distilled water) was taken as a control.

Then different quantities of indicator i.e., 30µL,60µL,0.1ml,0.2ml,0.3ml were added to the test tubes containing sorbitol solution. Observed the development of colour in each tube and compared with the control.

Table:3.1 Procedure used for different concentrations of sorbitol in distilled water

Test tube no.	1	2	3	4	5
Control	1ml	-	-	-	-
% Sorbitol in distilled water (D.W)	-	0.1	0.25	0.5	1.0
	-	1ml	1ml	1ml	1ml

Boric acid (1%)	1ml	1ml	1ml	1ml	1ml
Mixed indicator	30 µl	30 µl	30 µl	30 µl	30 µl

Similarly performed the whole experiment with 60µl, 0.1ml, 0.2ml and 0.3ml quantities of indicator.

3.3.2 Standardization of the quantity of mixed indicator in cow milk samples containing varied quantities of sorbitol

3.3.2.1 Milk Sample Preparation:

Cow milk sample was mixed thoroughly by pouring it into clean dry beaker and back till the homogeneous mixture was formed and brought to a temperature of 25°C. Then milk was checked for its pH, which usually ranges from 6.6 to 6.8. Then the milk was spiked at different levels of sorbitol i.e., 0.1%, 0.25%, 0.5%, 1.0%, 1.5%, 2.0%,2.5%,5.0%,7.5%,10%.

3.3.2.2 Protocol

One ml of the milk sample containing different concentrations of sorbitol was pipetted out into a clean and dry test tube. Pure milk i.e., without any sorbitol spiking was taken as a control sample.

One ml of the 1% boric acid solution in water was added to each test tube. Followed by the addition of 30µL,60µL, o.1ml,0.2ml. of the mixed indicator to each test tube. Observed the development of colour in each tube and compared with that of the control pure milk sample.

Table:3.2 Procedure used for different concentrations of sorbitol in cow milk

Test tune no.	1	2	3	4	5	6	7	8	9	10	11

Control (D.W)	1ml	-	-	-	-	-	-	-	-	-	-
% Sorbitol in cow milk	-	0.1	0.25	0.5	1.0	1.5	2.0	2.5	5.0	7.5	10
	-	1ml	1m	1ml	1ml	1ml	1ml	1ml	1ml	1ml	1ml
Boric acid (1%)	1ml	1ml	1ml	1ml	1ml	1ml	1ml	1ml	1ml	1ml	1ml
Mixed indicator	30µl	30µl	30µl	30µl	30µl	30µl	30µl	30µl	30µl	30µl	30µl

Similarly performed the whole experiment with 60µl, 0.1ml and 0.2ml quantities of indicator in buffalo and mixed milks also.

3.4 To check the effect of reagents to be used for milk coagulation on the development of colour in aqueous sorbitol solution

The reagents which were planned to be used for the precipitation of proteins from the milk samples were as follows:

1. Distilled water
2. Sorbitol
3. Boric acid (1%)
4. Acetic acid (10%)
5. Trichloro acetic acid (TCA-15%)
6. Biggsszijarto
7. Acetone
8. Mixed indicator

3.4.1 Glacial acetic acid (10%)

Two ml of 10% glacial acetic acid solution was added to ten ml of 1% sorbitol solution and contents were mixed and one ml of this solution was pipetted out in the test tube

followed by the addition of one ml of 1% boric acid solution and then 60µL of indicator. Compared the developed colour with the control which was without acetic acid.

3.4.1.1 Trichloro acetic acid (TCA-15%)

One ml of 1% sorbitol solution was added to five ml of volumetric flask and then made up the volume with 15% of trichloro acetic acid. One ml of this solution was pipetted out in the test tube followed by the addition of one ml of 1% boric acid solution then 60µl of indicator. Compared the developed colour with the control which was without trichloro acetic acid.

3.4.1.2 Biggsszijarto reagent

Three ml of 1% sorbitol solution was added to ten ml volumetric flask and made up the volume with Biggs Szijarto reagent. One ml of this solution was pipetted out in the test tube followed by the addition of one ml of 1% boric acid solution and then 60µl indicator. Compared the developed colour with the control which was without biggsszijarto.

3.4.1.3 Acetone

Ten ml of acetone was added to five ml of 1% sorbitol solution and contents were mixed. One ml of this solution was pipetted out in the test tube followed by the addition of one of 1% boric acid solution and then 60µl indicator. Compared the developed colour with the control which was without acetone.

3.4.2 To check the effect of reagents to be used for milk coagulation on the development of colour in milk containing different concentrations of sorbitol

3.4.2.1. Glacial acetic acid (10%)

Ten ml sample of cow milk having varied concentrations of sorbitol was taken into a 100ml beaker. Fifty ml of hot water (40°C) was added and mixed the contents then two ml of 10% acetic acid solution was added, mixed the contents gently and kept it undisturbed for 10min. Filtered the contents using Whatman No 40-filter paper and collected the filtrate. Pure cow milk i.e., without sorbitol spiking was used as control. Each one ml of the clear filtrate was taken into a test tube followed by the addition of one ml of 1% aqueous boric acid solution. Then, 60µl of the mixed indicator was added

and contents were mixed gently. Observed the change in colour in control and test samples.

3.4.2.2 Trichloro acetic acid (TCA-15%)

Two ml of cow milk sample having varied concentrations of sorbitol was treated with ten ml of 15% of trichloro acetic acid and mixed the contents gently and kept it undisturbed for 10 min. Filtered the contents using Whatman No 40-filter paper and collected the filtrate. Pure cow milk i.e., without sorbitol spiking was used as control. Each one ml of the clear filtrate was taken into a test tube followed by the addition of one ml of 1% aqueous boric acid solution. Then 60µl of the mixed indicator was added to develop the colour and contents were mixed gently. Observed the change in colour in control and test samples.

3.4.2.3. Biggsszijarto reagent

Three ml of cow milk sample having varied concentrations of sorbitol was taken into 10 ml volumetric flask followed by the addition of Biggsszijarto solution slowly and made up the volume. Contents were mixed gently and kept it undisturbed for 10 min. Filtered the contents using Whatman No 42-filter paper and collected the filtrate. Pure cow milk i.e., without sorbitol spiking was used as control. One ml of the clear filtrate was taken into a test tube followed by the addition of one ml of 1% aqueous boric acid solution. Then 60µl of the mixed indicator was added and contents were mixed gently. Observed the change in colour in control and test samples.

3.4.2.4 Acetone reagent

Five ml of cow milk sample having varied concentrations of sorbitol was treated with ten ml of acetone and mixed the contents gently and kept it undisturbed for 10 min. Then Filtered the contents using Whatman No 42-filter paper and collected the filtrate. Pure cow milk i.e., without sorbitol spiking was used as control. Each one ml of the clear filtrate was taken into a test tube followed by the addition of one ml of 1% aqueous boric acid solution. Then 60µl of the mixed indicator was added to develop the colour and contents were mixed gently. Observed the change in colour in control and test samples.

3.5 Sorbitol adulterated milk samples spiked with other carbohydrates (sucrose, starch, glucose, maltodextrin and maltitol), **neutralizer, ammonium sulphate and urea**

Pure cow milk and samples spiked with sorbitol i.e., 0.25% as a lower concentration and 2.5% as an upper concentration. These spiked samples were then further spiked with same concentrations of different adulterants like carbohydrates (sucrose, starch, glucose, maltodextrin and maltitol), neutralizers (sodium carbonate, sodium bi carbonate and sodium hydroxide), urea and ammonium sulphate.

Each concentration of sorbitol along with other adulterated milk was taken into a clean and dry test tubes along with pure cow milk as a control.

Boric acid (1%) concentration one ml was added to each test tubes. Then selected indicator quantity i.e., 60µL, was added. Observed the development of colour in each tube with that of the control of pure milk sample.

Table:3.3 Procedure used for different concentrations of sorbitol, carbohydrates, neutralizers urea and ammonium sulphate and their conjunction with sorbitol in cow milk

Component	S		CH		N		U		A. S		S+O	
%	0.25	2.5	0.25	2.5	0.25	2.5	0.25	2.5	0.25	2.5	0.25	2.5
Test tube no	1	2	3	4	5	6	7	8	9	10	11	12
Quantity ml	1	1	1	1	1	1	1	1	1	1	1	1
Boric acid (1%) ml	1	1	1	1	1	1	1	1	1	1	1	1
Indicator µl	60	60	60	60	60	60	60	60	60	60	60	60

S=Sorbitol, CH= Carbohydrates, N= Neutralizers, U= urea, A. S= Ammonium sulphate, O= Other adulterants

3.5.1.2 Qualitative detection of added adulterants in milk filtrates obtained from acetone by using developed reagents

To five ml of milk sample 10ml of acetone was added and contents were mixed by inverting the contents/ vertexing. The contents were then filtered through Whatman NO.42 filter paper. Filtrate was collected in a clean and dry test tube. One ml of this filtrate was taken and one ml of aqueous boric acid solution (1%) was added. Contents were mixed followed by the addition of 60µL of mixed indicator. Observed the development of colour in each tube with that of the control of pure milk sample filtrate.

CHAPTER-4

RESULTS AND DISCUISION

This chapter covers the findings of present study conducted to (i) standardize the method for detection of sorbitol in milk and (ii) Effectiveness of qualitative method in presence of other adulterants. The results presented in this chapter are discussed in two phases as per the objectives given.

4.1 Proposed hypothesis of the color based method for sorbitol detection in milk

It is stated that sorbitol in the presence of boric acid gives rise to an acidic complex (Nose and Zenki, 1991). Keeping this in mind it was contemplated that in case, sorbitol is present in milk then it will also form an acidic complex on addition of boric acid and use of a dye sensitive to a particular pH will show a color change.

Sorbitol + Boric acid = acidic complexes

↓ Indicator dye

Colour development

Hence, suitable combination of pH sensitive dyes which was standardized in the previous work carried out in the lab used in the present study.

4.2 Standardization of the quantity of mixed indicator solution to develop colour in sorbitol solution of varied concentrations prepared in distilled water

a) 30µl indicator

C **0.1%** **0.25%** **0.5%** **1.0%**

b) 60µl indicator

C **0.1%** **0.25%** **0.5%** **1.0%**

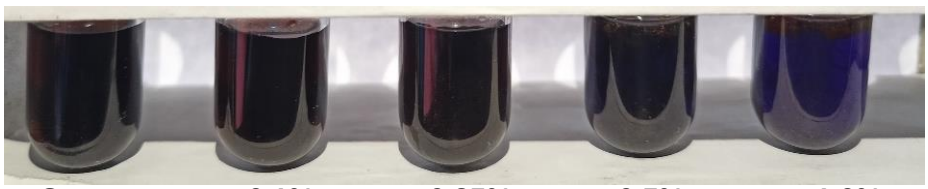
c) 0.1ml indicator

C 0.1% 0.25% 0.5% 1.0%

d) 0.2ml indicator

C 0.1% 0.25% 0.5% 1.0%

d) 0.3ml indicator



C 0.1% 0.25% 0.5% 1.0%

Fig:4.1 Effect of concentration of sorbitol in distilled water with varied volumes (a) 30µl (b) 60µl (c)0.1ml (d) 0.2ml of indicator on the intensity and hue of color

It is evident from the results (Fig 4.1) that addition of mixed indicator up to the level of 0.1 ml showed a green color in case of control i.e., distilled water without any sorbitol. However, as the volume of mixed indicator addition was increased the color started getting dark and at 0.3 ml level it was almost dark purple. In case of sorbitol solution having concentration of 0.1 to 1.0 % in distilled water, the change in color and its intensity was very evident as the volume of mixed indicator was increased. From the results it is clear that addition of 60 µl of the mixed indicator in sorbitol solution showed a clear distinction in color from the control. It is also evident from the results that as the concentration of sorbitol increased the intensity of the color was also increased and in 1.0 % sorbitol solution a dark pink color was observed. This confirmed that 60 µl of the mixed indicator was optimum to detect sorbitol if added at as low as 0.1% in water. Using the same reagents, Nose and Zenki (1991) detected boron in eye lotions based on the complexation reaction between D-sorbitol and boric acid followed by the acid-base reaction of Methyl Orange.

4.2.1 Standardization of quantity of mixed indicator to develop colour in cow milk samples containing varied concentrations of sorbitol

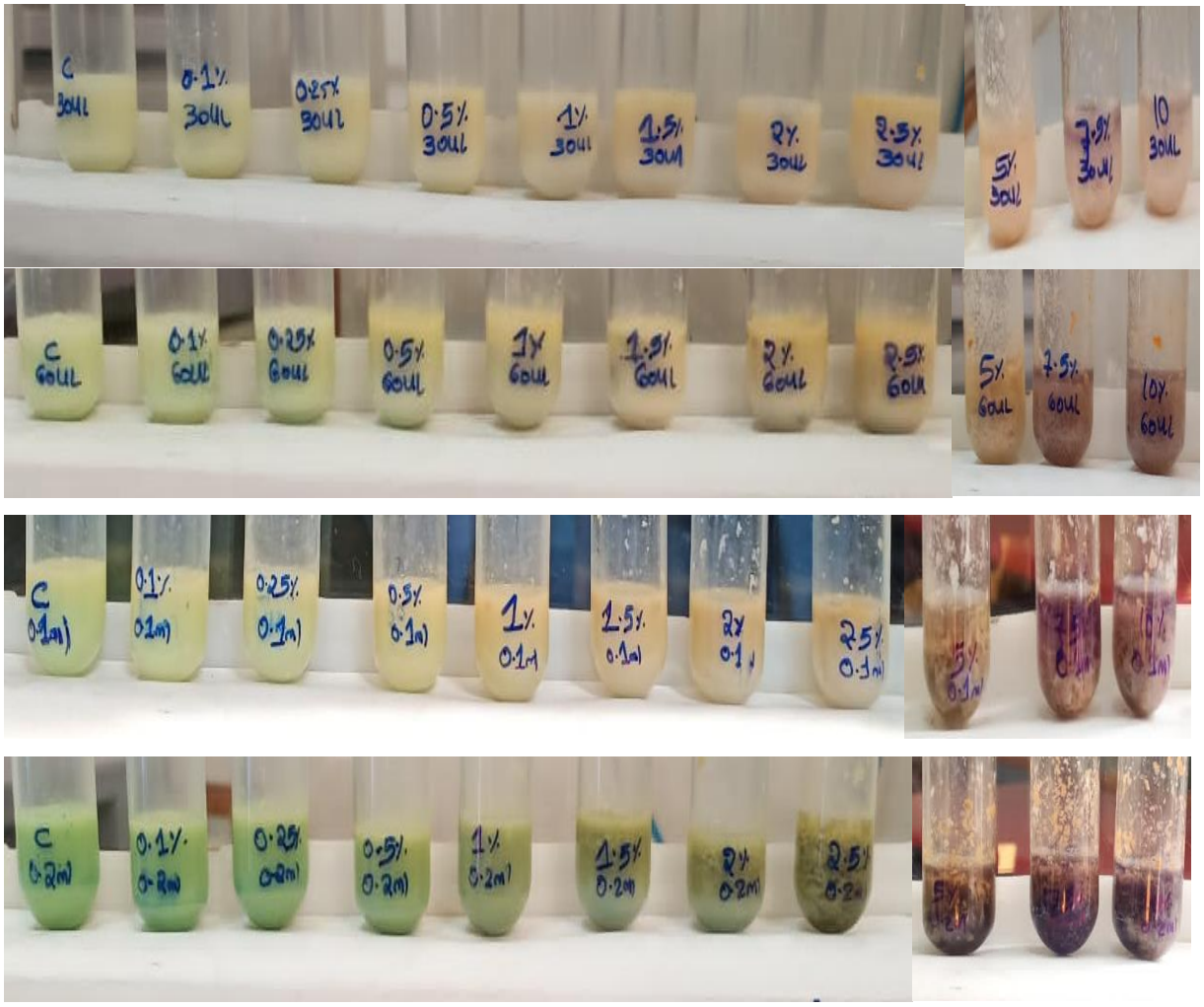


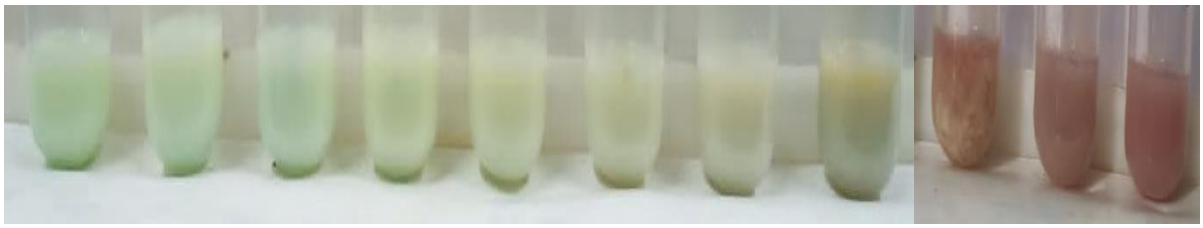
Fig:4.2 Effect of concentration of sorbitol in cow milk with varied volumes of mixed indicator on the intensity and hue of color

Fig 4.2 depicts the development of color on addition of different volume (30µl, 60µl, 0.1ml and 0.2ml) of mixed indicator in cow milk samples spiked with varied amount of sorbitol. It is evident from the results that in all the cases there was a change in color from green (control sample i.e., without any sorbitol) to pinkish. The color change was distinct to naked eyes in case of milk sample spiked with sorbitol @ 5%. In this case also it was observed that 60µl of the mixed indicator was optimum for the development of distinct color. The level of detection of sorbitol with naked eyes was found to be 5%. (Basharat *et al.,* 2019; Shehazadi *et al.,* 2016) reported detection of sorbitol in milk using two different unknown reagents in which they observed distinct colour (yellow green) in sorbitol containing milk, while the appearance of reddish-orange colour in

negative sample. (Chaudhary *et al.,* 2015) reported sorbitol detection in milk by the method in which sorbitol is made to react with ferric sulphate and sodium hydroxide, the development of brownish color in test tube indicates sorbitol negative and precipitate will settle down. In case of positive samples, green color is formed, precipitate formation is absent. Limit of detection reported was up to 0.25% in raw milk. Compared to these methods, developed method showed lesser sensitivity in case of milk samples where as in case of filtrate that is obtained from acetone showed the LOD is same as above method.

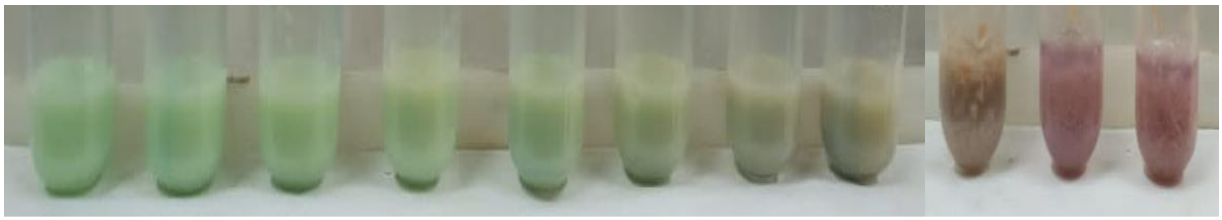
4.2.2 Standardization of quantity of mixed indicator to develop colour in buffalo milk samples containing varied concentrations of sorbitol

a)



C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

b)



C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

c)

C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

d)

C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

Fig:4.3 Effect of concentration of sorbitol in buffalo milk with varied volumes (a) 30µl (b) 60µl (c)0.1ml (d) 0.2ml of indicator on the intensity and hue of color

In this experiment buffalo milk spiked with sorbitol was evaluated for the development of color in spiked samples by using varied levels of mixed indicator. It can be seen from the results (Fig 4.3) that control milk sample were of light green to dark green color with increasing the quantity of indicator. On the contrary in case of spiked samples, green color showed a change from green to pinkish. Results depicted in fig 4.3, revealed that color of the dye changed towards pink and as the volume of the dye was increased the intensity of the color increased. However, in this case also it was found that 60µl of the mixed dye were optimum to bring a change in the color of spiked samples. Visual limit of detection in this case was also found to be 5% as was the case with cow milk.

4.2.3 Standardization of quantity of mixed indicator to develop colour in mixed milk samples containing varied concentrations of sorbitol

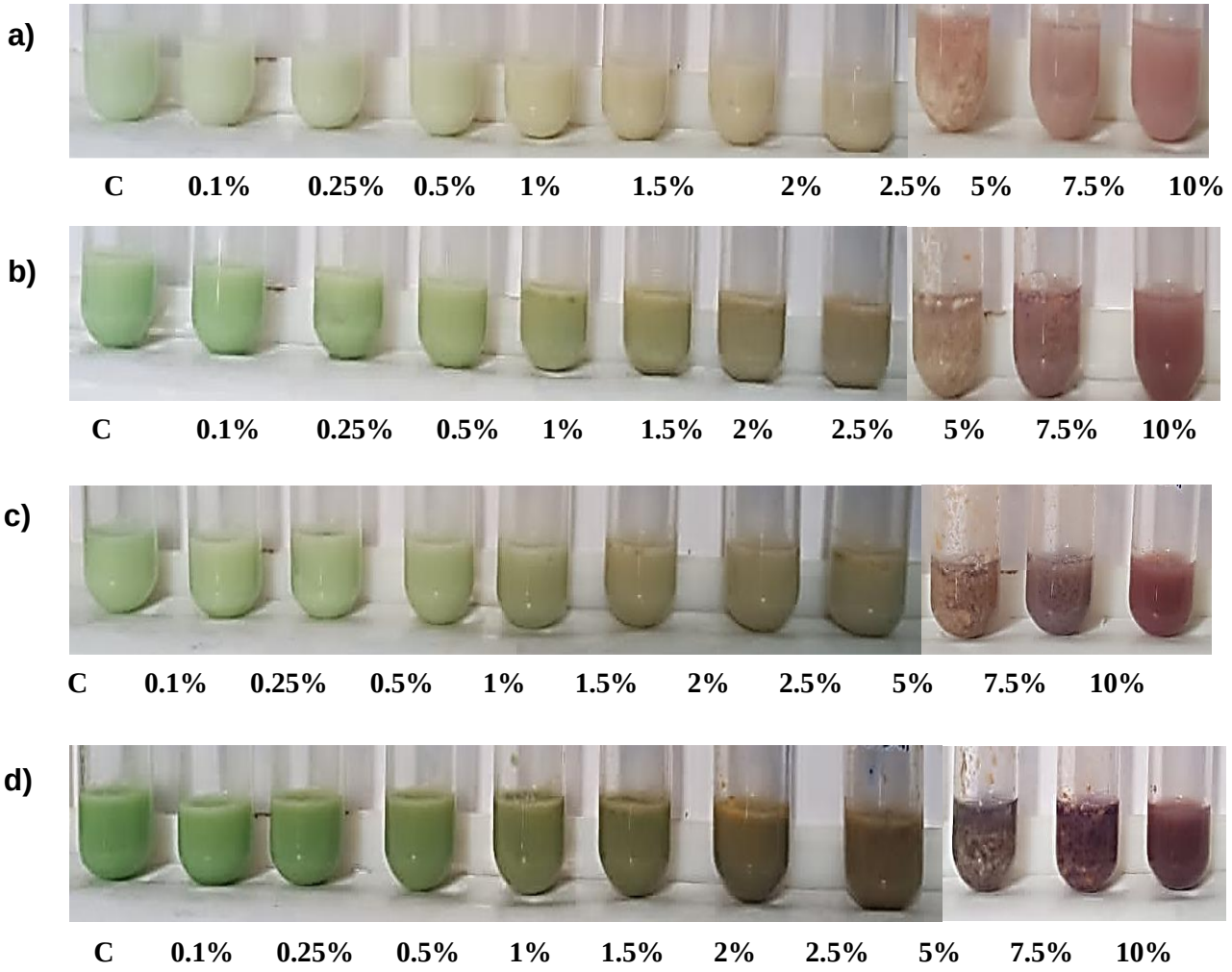


Fig:4.4 Effect of concentration of sorbitol in mixed milk with varied volumes (a) 30µl (b) 60µl (c)0.1ml (d) 0.2ml of indicator on the intensity and hue of color

In this experiment mixed milk (cow: buffalo in 1: 1ratio) was used to see the color change in spiked samples. It is evident from the results (Fig 4.4) that at all the levels of mixed dye addition, the trend in the change of color was similar to the change in the sorbitol spiked cow and buffalo milk. However, the level of detection was slightly less i.e., at 2.5% level of sorbitol addition could be detected easily with naked eyes. In this case also the level of dye addition found to be optimum was 60µl.

4.3 Effect of reagents to be used for milk coagulation on the development of colour without sorbitol

C **Acetic acid** **Biggss** **Acetone** **TCA**

Fig:4.5 Effect of in different acids without sorbitol with 60µl indicator

It is clear from the results obtained and discussed in the previous section that the level of detection of sorbitol in milk ranged from 2.5 to 5%. This could be due to the interference of milk proteins in the development of sharp change in color, which is difficult to observe with naked eyes. Therefore, in this section of the effect of coagulants (TCA, acetone and acetic acid-Dr.M.K. Srivastava, 2010 and biggsszijarto-ISO 22662:2007) contemplated to be used for the precipitation of milk proteins was evaluated. It is evident from the results (Fig 4.5) that there was an appearance of green colour in (Distilled water + Boric acid) control test tube. Presence of acetic acid (Distilled water + Acetic acid), violet colour was observed. Similarly, dark violet color was appeared in the presence of TCA (Distilled water + TCA) and pink colour in the presence biggsszijarto (Distilled water + Biggs) and green colour in the presence of acetone (Distilled water + acetone) on adding 60µl of the mixed indicator standardized in the present study. As it is seen that the color that was developed in acetone was almost similar to control. So, in order to perform further studies, acetone was selected as a coagulant agent for experiments in milk.

4.3.1 Effect of reagents to be used for milk coagulation on the development of colour in aqueous 1% sorbitol solution

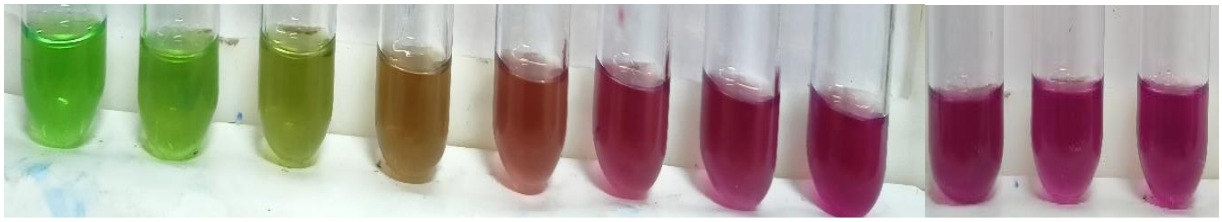
-c **+c** **Acetic acid** **TCA** **Biggss** **Acetone**

Fig:4.6 Effect of concentration of sorbitol (1%) in different acids with 60µl indicator

It is evident from the results depicted in the above section that the most suitable coagulant was acetone. However, to evaluate the effect of these coagulants in the presence of sorbitol was also evaluated and the results have been presented in this section. In these experiments 1% sorbitol solution was used in place of distilled water and the study was carried out. It is clear from the results (Fig 4.6) that there was an appearance green colour in negative test tube (distilled water + boric acid) and violet pink colour in positive (sorbitol+ boric acid) test tube, violet colour in presence of acetic acid (sorbitol + acetic acid), pinkish violet in presence of TCA (sorbitol + TCA), pink colour in presence of biggsszijarto (sorbitol + pink) and pink with tinge of brown in presence of acetone (sorbitol + acetone) on adding the 60µl of mixed indicator. These findings clearly demonstrated that the acetone would be the most suitable coagulant for the precipitation of caseins and use the acetone filtrate or acetone layer to develop the colour which is clearly visible to naked eyes.

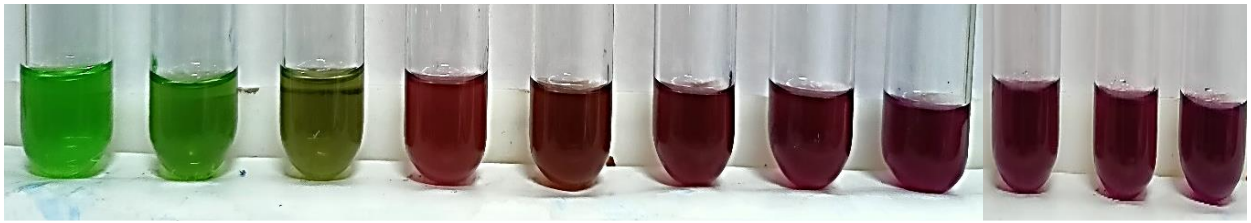
4.4 Effect on the visual limit of detection of sorbitol in milk (cow and buffalo) after coagulation with acetone

a)



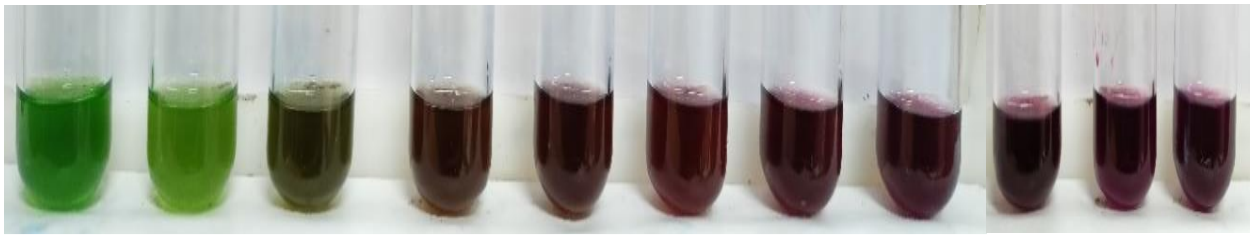
C **0.1%** **0.25%** **0.5%** **1%** **1.5%** **2%** **2.5%** **5%** **7.5%** **10%**

b)



C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

c)



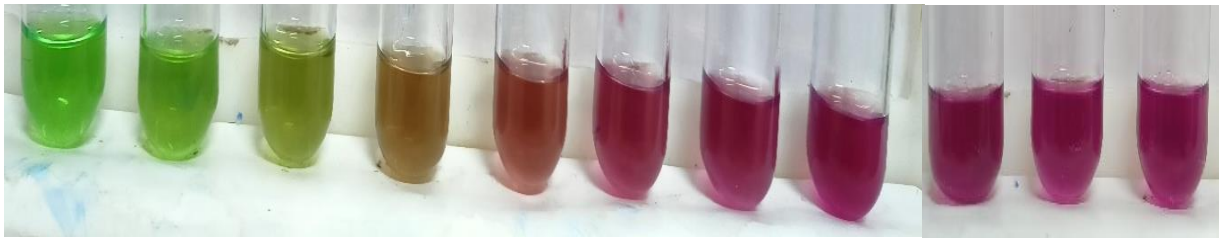
C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

d)

C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

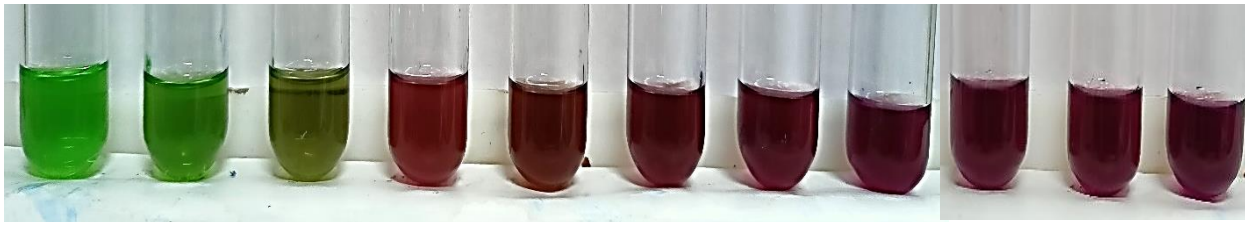
Fig:4.7 Effect on the limit of detection of sorbitol in cow milk after coagulation with acetone with varied volumes (a) 30µl (b) 60µl (c)0.1ml (d) 0.2ml of indicator on the intensity and hue of color.

a)



C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

b)



C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

c)

C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

d)

C 0.1% 0.25% 0.5% 1% 1.5% 2% 2.5% 5% 7.5% 10%

Fig:4.8 Effect on the limit of detection of sorbitol in buffalo milk after coagulation with acetone with varied volumes (a) 30µl (b) 60µl (c)0.1ml (d) 0.2ml of indicator on the intensity and hue of color

It is evident from the results (Fig 4.7) that the change in color was more vivid in the acetone layer collected after the coagulation of milk. It is clear from the results that addition of 60 µl of the mixed dye was optimum to bring a perceptible change in the color in sorbitol spiked samples. In case of control sample, the color was bright green in acetone layer collected after the precipitation of milk proteins with acetone. It is also evident from the results that the level of sorbitol detection was improved a lot and now the addition of 0.5% of sorbitol was possible to detect with naked eyes. Similar trend was observed in case of acetone layer collected after the precipitation of sorbitol spiked samples of buffalo milk. (Fig 4.8).

4.5 Effect of common adulterants on the effectiveness of the standardized color based qualitative method of sorbitol detection in milk

To achieve this objective, the color based sorbitol detection method standardised in the present investigation was evaluated in the presence of the following adulterants:

Carbhohydrates (Glucose, Sucrose, Starch, maltodextrin, and maltitol)

Neutralizers (Sodium hydroxide, Sodium carbonate, Sodium bicarbonate) Ammonium sulphate and urea

4.5.1 Effect of carbohydrates

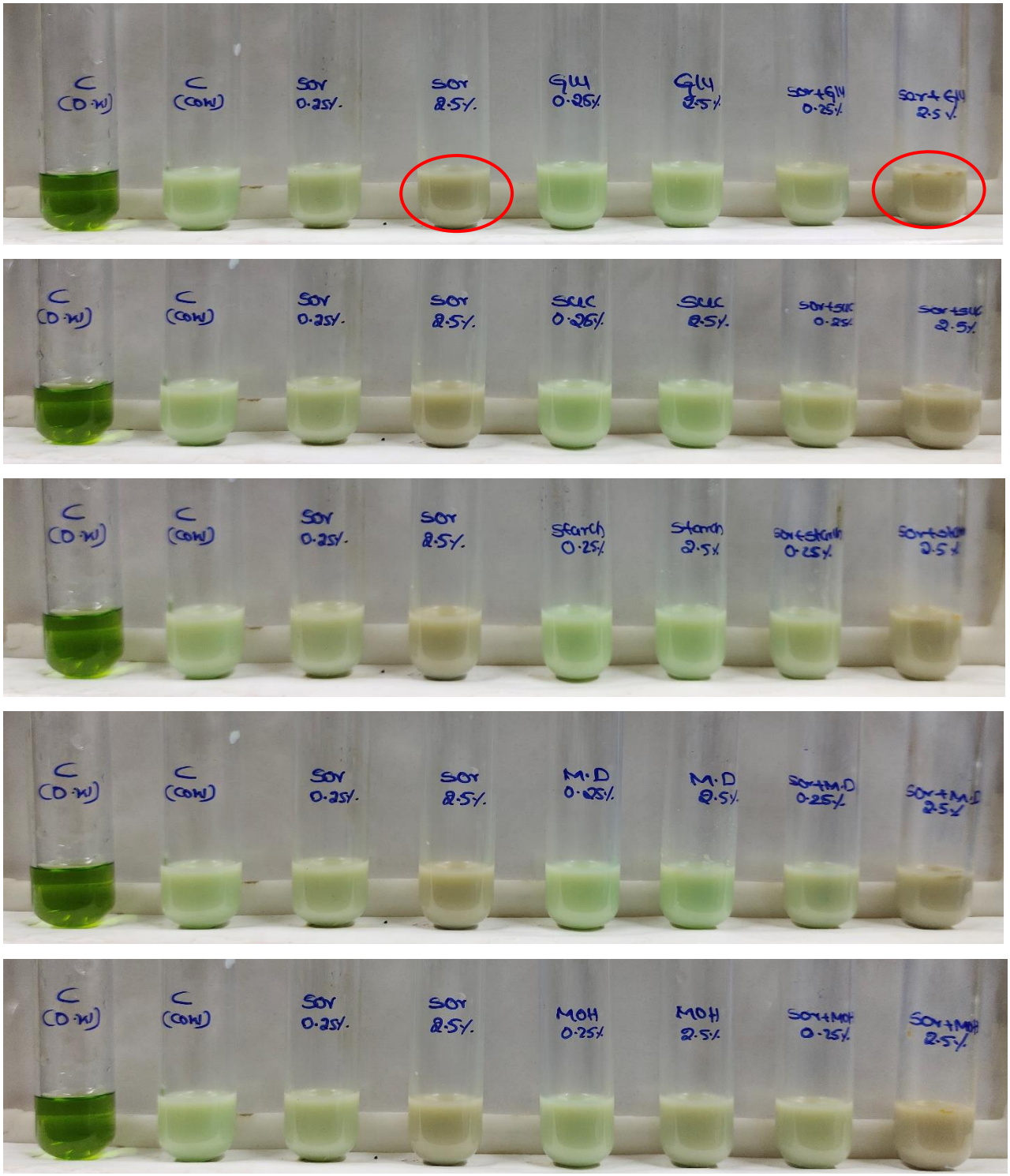


Fig:4.9 Effect of concentration of sorbitol along with other carbohydrates in cow milk with 60µl of indicator on the intensity and hue of color

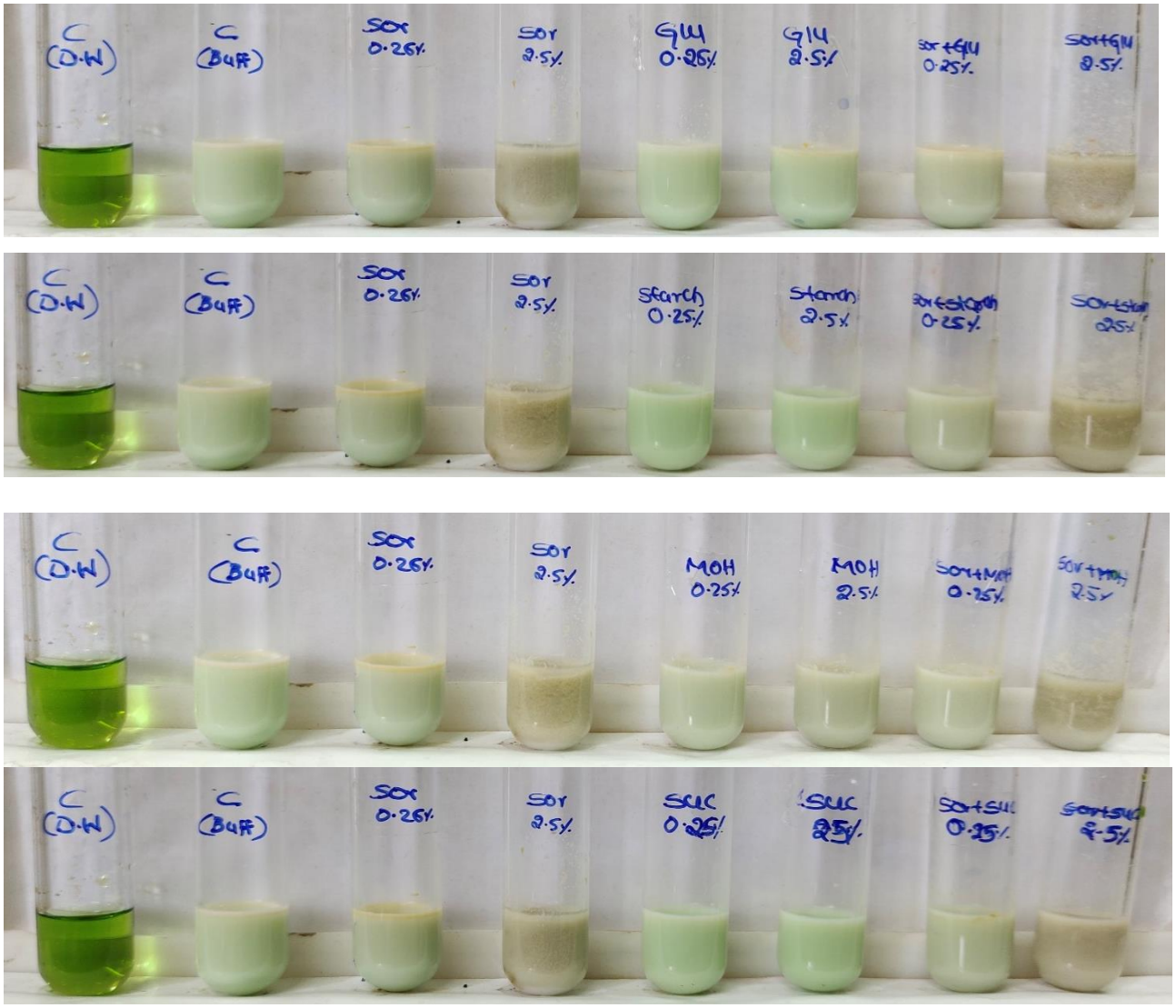
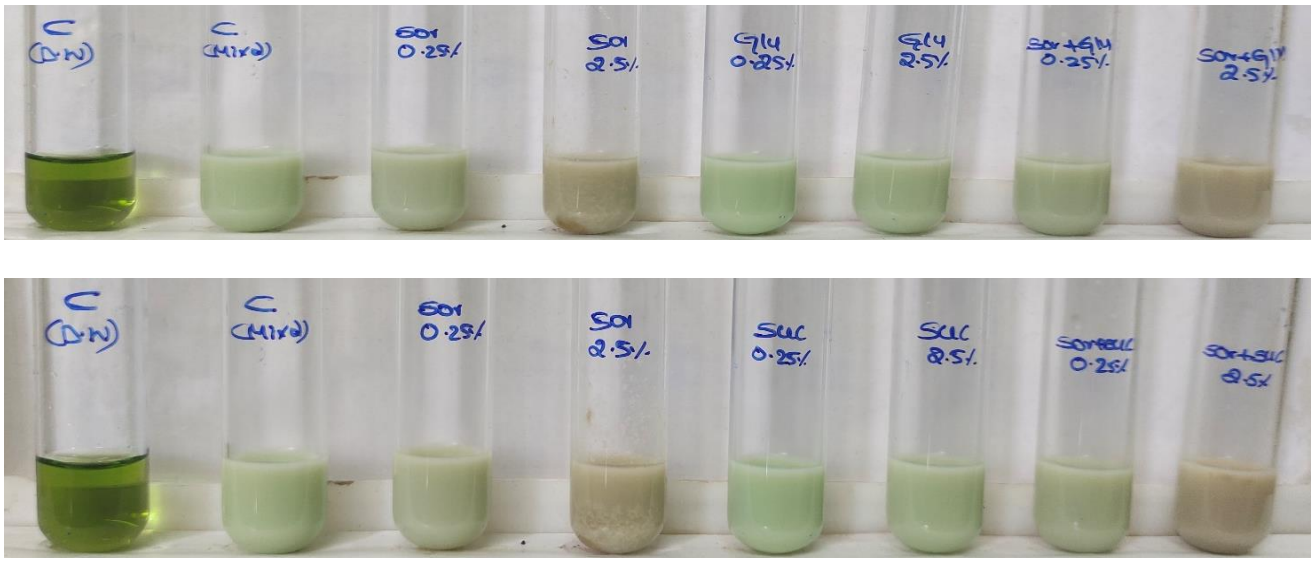


Fig:4.10 Effect of concentration of sorbitol along with other carbohydrates in buffalo milk with 60µl of indicator on the intensity and hue of color



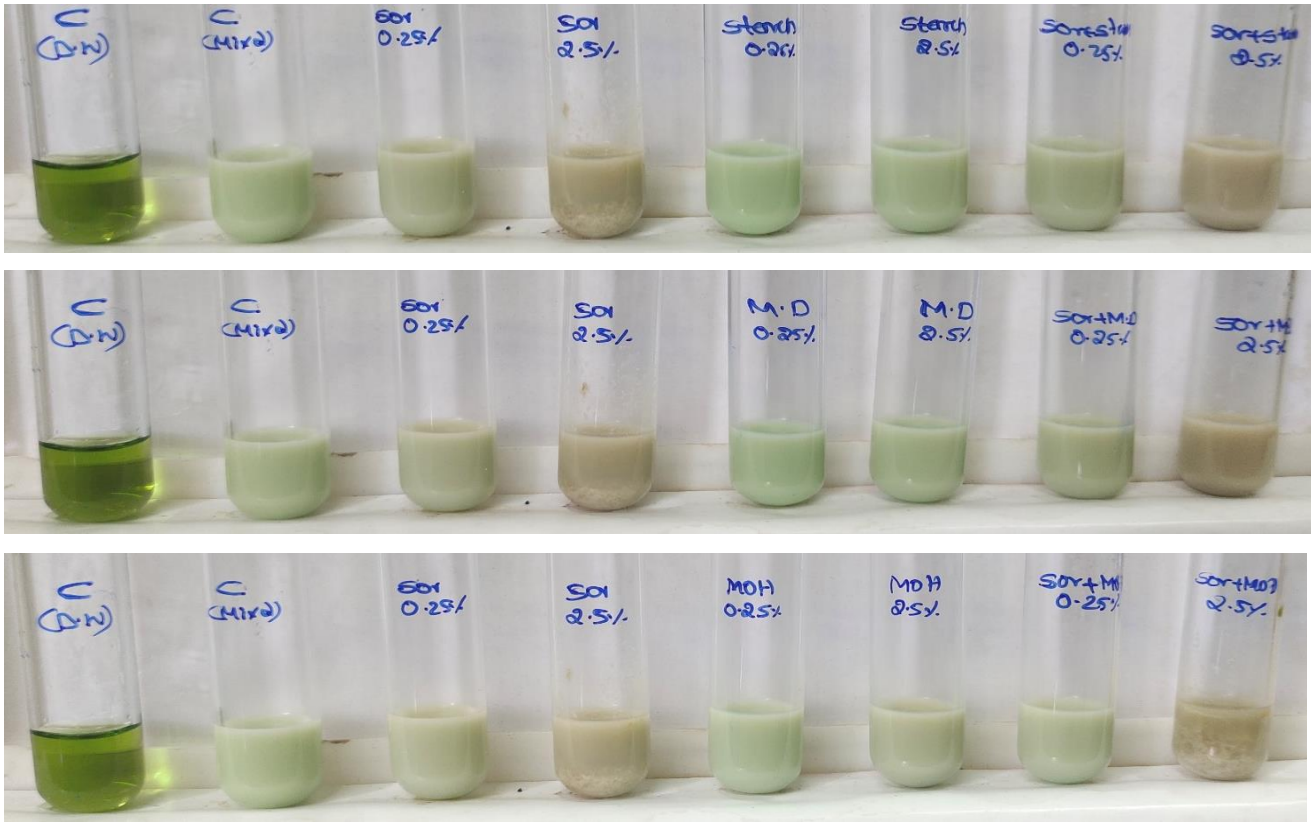


Fig:4.11 Effect of concentration of sorbitol along with carbohydrates in mixed milk with 60µl of indicator on the intensity and hue of color

It is evident from the pictures depicting the color change (Fig 4.9) that glucose spiking did not result any change in color on the addition of 60µl of the mixed dye and color was almost similar to the control milk without any sorbitol. However, the sorbitol in conjunction with glucose showed the disappearance of green color and a color turned out to be on brownish side. The level of sorbitol which could be comfortably detected in the presence of glucose was 2.5%. Similar observations were recorded in the case of milk samples tested wherein sorbitol was present in conjunction with other carbohydrates (Sucrose, Starch, maltodextrin, maltitol). In these cases, also the level of sorbitol which could be detected was 2.5% in milk. Similar trend was observed in case of buffalo and mixed milks. (Fig 4.10 and Fig 4.11).

4.5.1.1 Effect of neutralizers

Neutralization of milk especially in summer months is the regular problem in the milk supply chain. Milk vendors invariably add neutralizers to check the acidity of the milk because of the lack of chilling facility at their end. Therefore, the neutralizers effect was evaluated on the performance of the standardized color based test for the detection of

sorbitol in milk. The above-mentioned (Section 4.5) neutralizers were added at different rate to control milk as well as sorbitol spiked milk samples.

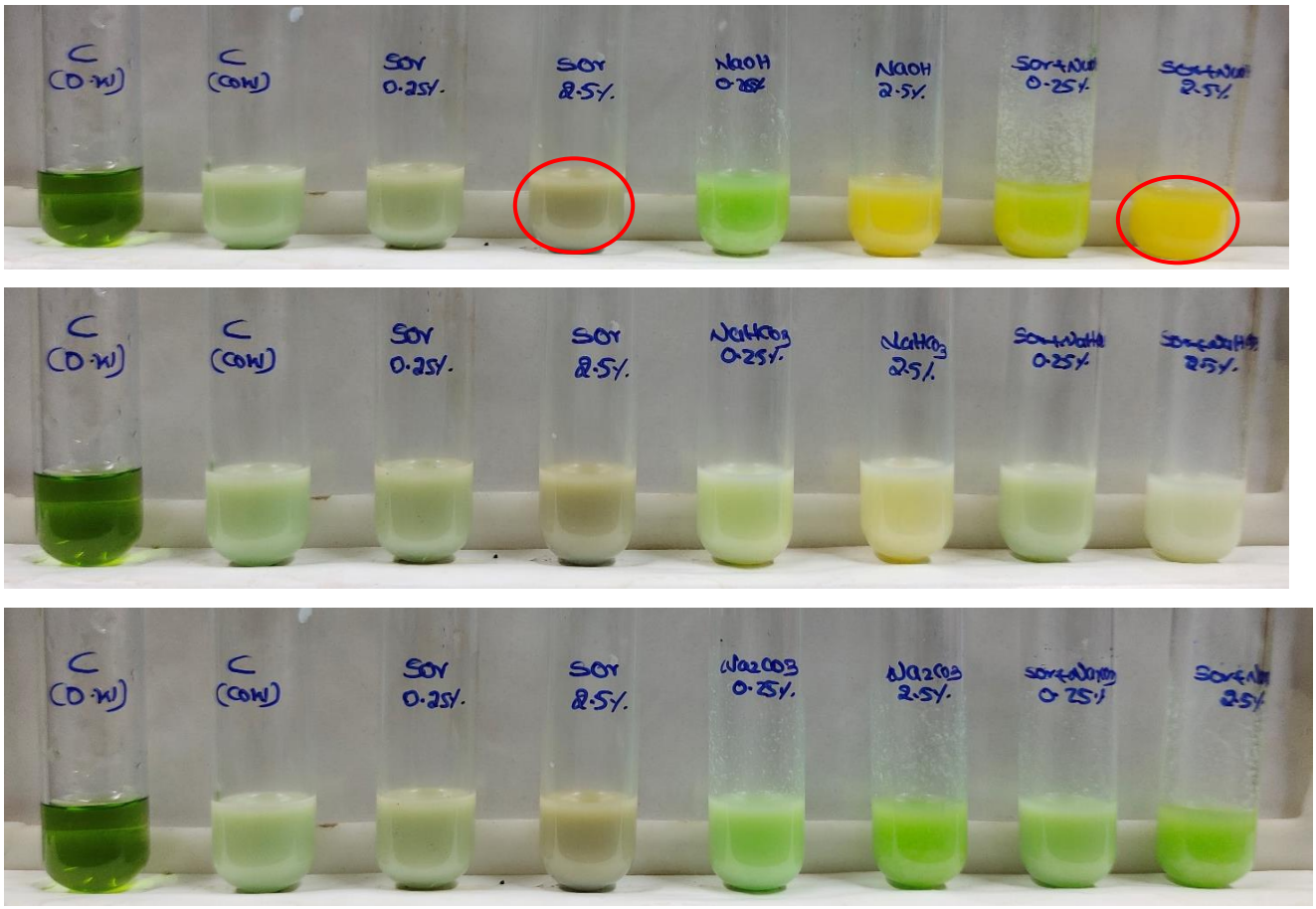
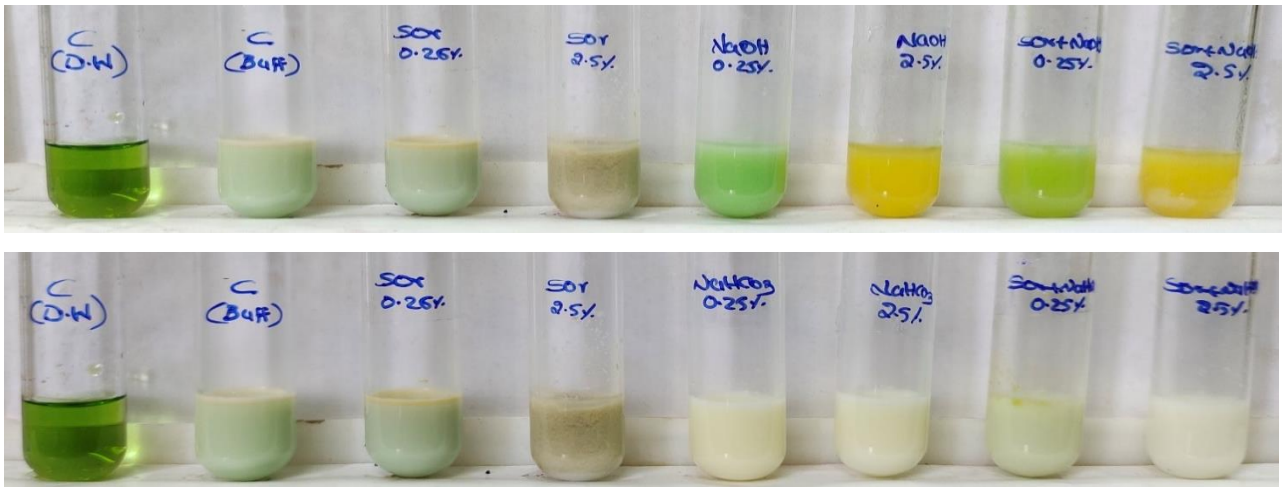


Fig:4.12 Effect of concentration of sorbitol along with neutralizers in cow milk with 60µl of indicator on the intensity and hue of color



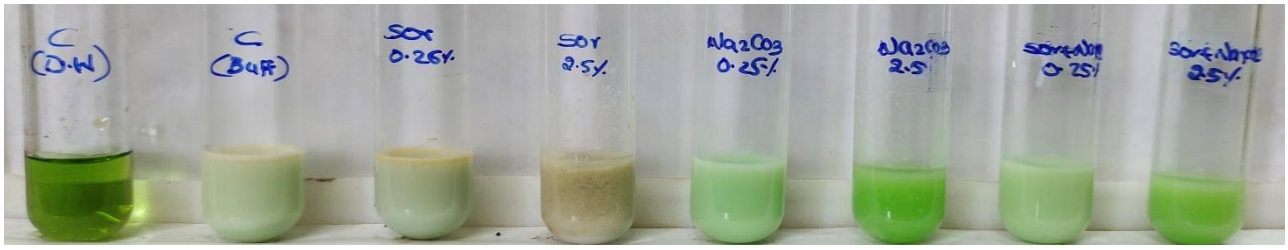


Fig:4.13 Effect of concentration of sorbitol along with neutralizers in buffalo milk with 60µl of indicator on the intensity and hue of color

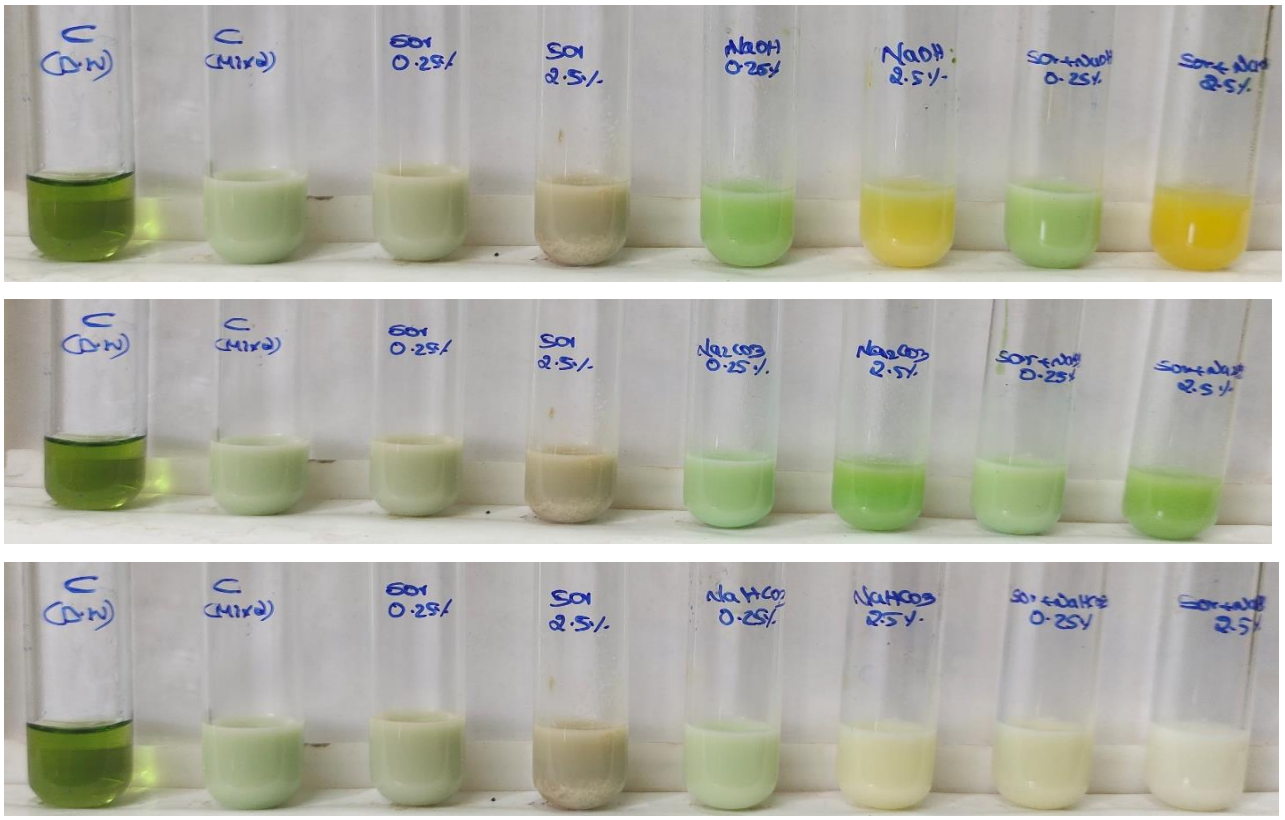


Fig:4.14 Effect of concentration of sorbitol along with neutralizers in mixed milk with 60µl of indicator on the intensity and hue of color

It is evident from the results (Fig 4.12) that sodium hydroxide containing milk samples turned yellow in color when mixed dye was added. Similarly in sorbitol spiked samples also wherein neutralization was done with sodium hydroxide the color developed was yellow. In case of milk samples neutralized with Sodium bicarbonate, the color change was not as it was observed in only sorbitol spiked samples. Similarly, in case of the samples neutralized with sodium carbonate, the color developed was green as was the case in control samples without sorbitol. These results, clearly demonstrated that the neutralization showed a considerable effect on the performance of the color based test standardized in the present study. Similar trend was observed in case of buffalo milk

and mixed milks samples neutralized with studied neutralizing agents (Fig 4.13 and Fig 4.14).

4.5.1.2 Effect of ammonium sulphate and urea

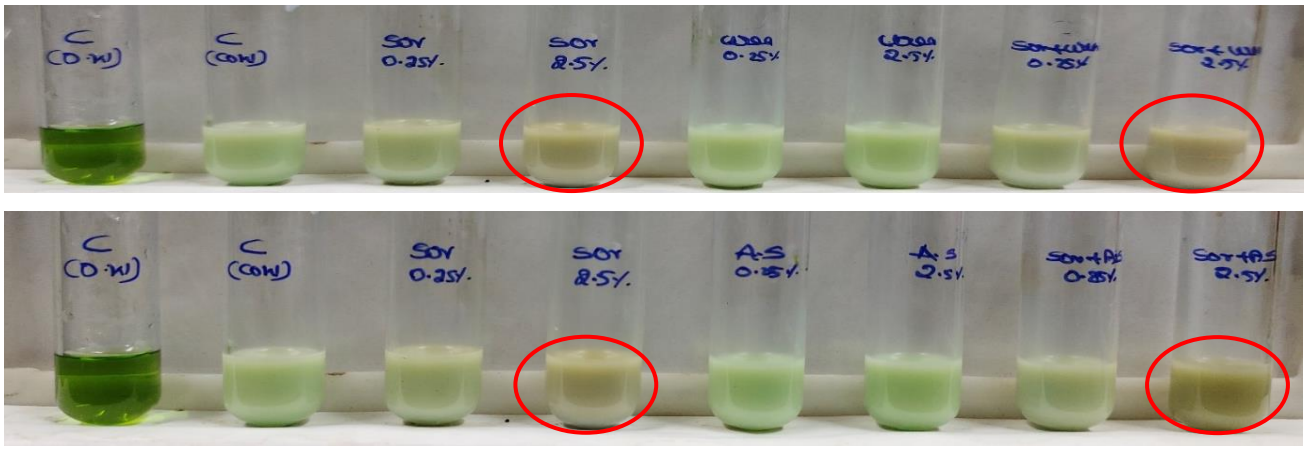


Fig:4.15 Effect of concentration of sorbitol along with urea and ammonium sulphate in cow milk with 60µl of indicator on the intensity and hue of color

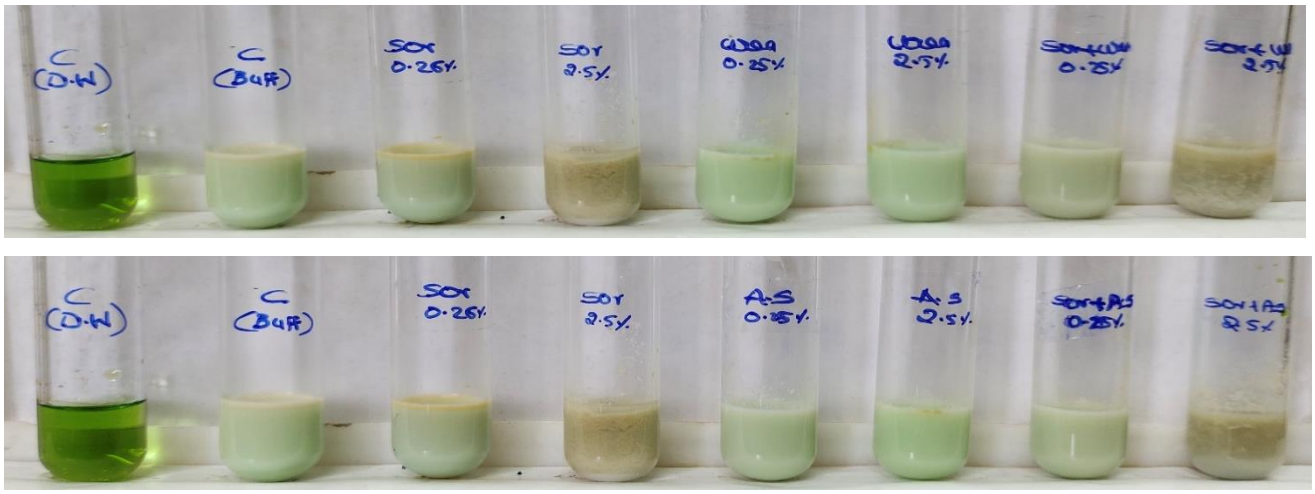
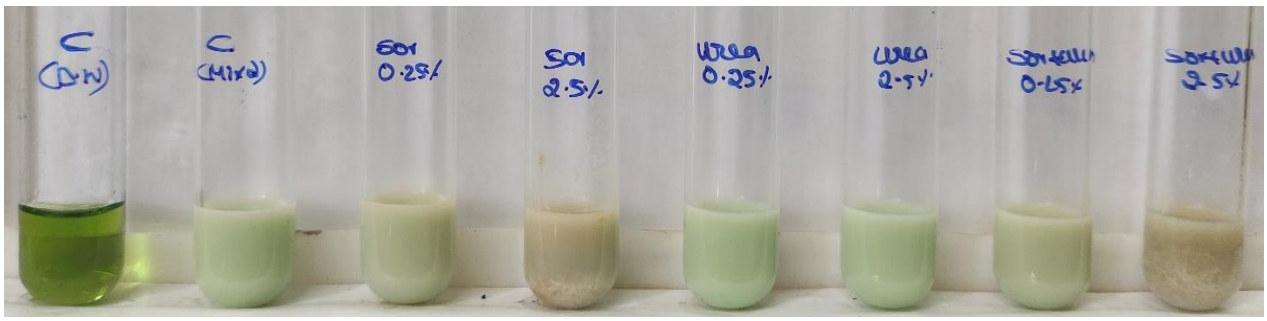


Fig:4.16 Effect of concentration of sorbitol along with urea and ammonium sulphate in buffalo milk with 60µl of indicator on the intensity and hue of color



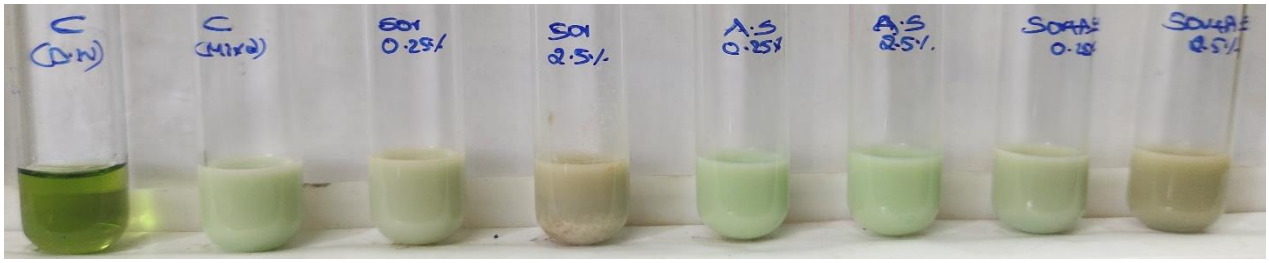
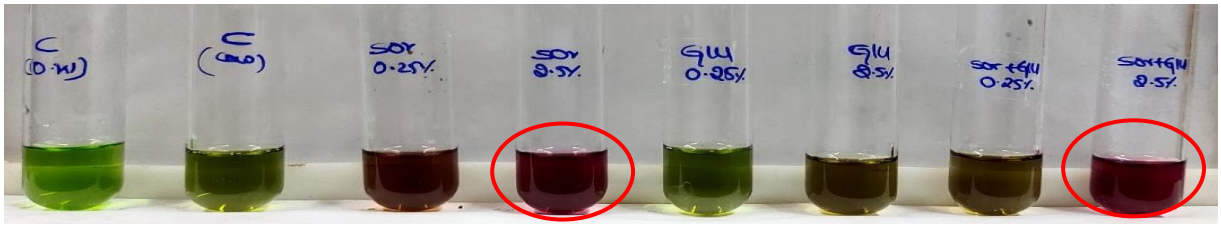


Fig:4.17 Effect of concentration of sorbitol along with urea and ammonium sulphates in mixed milk with 60µl of indicator on the intensity and hue of color

These two adulterants also fall under the category of common milk adulterants; hence their effect was also studies. It is evident from the results depicted in fig 4.15 that there was a development of light brownish color in the samples spiked with sorbitol as well as samples containing sorbitol and urea or ammonium sulphate. Test standardized in the present investigation was capable of detecting sorbitol presence in the milk even in the presence of urea or ammonium sulphate and the visual limit of detection was found to be the 2.5%. Similar trend was observed in case of buffalo milk and mixed milks (Fig 4.16 and Fig 4.17). Therefore, it can be concluded that presence of ammonium sulphate and urea did not show any adverse effect on the performance of the standardized test. Developed method showed lesser sensitivity in case of milk samples where as in case of filtrate that is obtained from acetone showed equivalent sensitivity to (FSSAI, 2015) Using DMAB reagent this method is based on the principle that urea forms a distinct yellow complex with DMAB in a low acidic solution at room temperature where LOD IS 0.25%.

4.5.2 Effect of carbohydrates, neutralizers, ammonium sulphate and urea on the detection limit in the acetone layer collected after precipitation of casein from neutralized milk (cow, buffalo and mixed) spiked with sorbitol:

4.5.2.1 Effects of carbohydrates



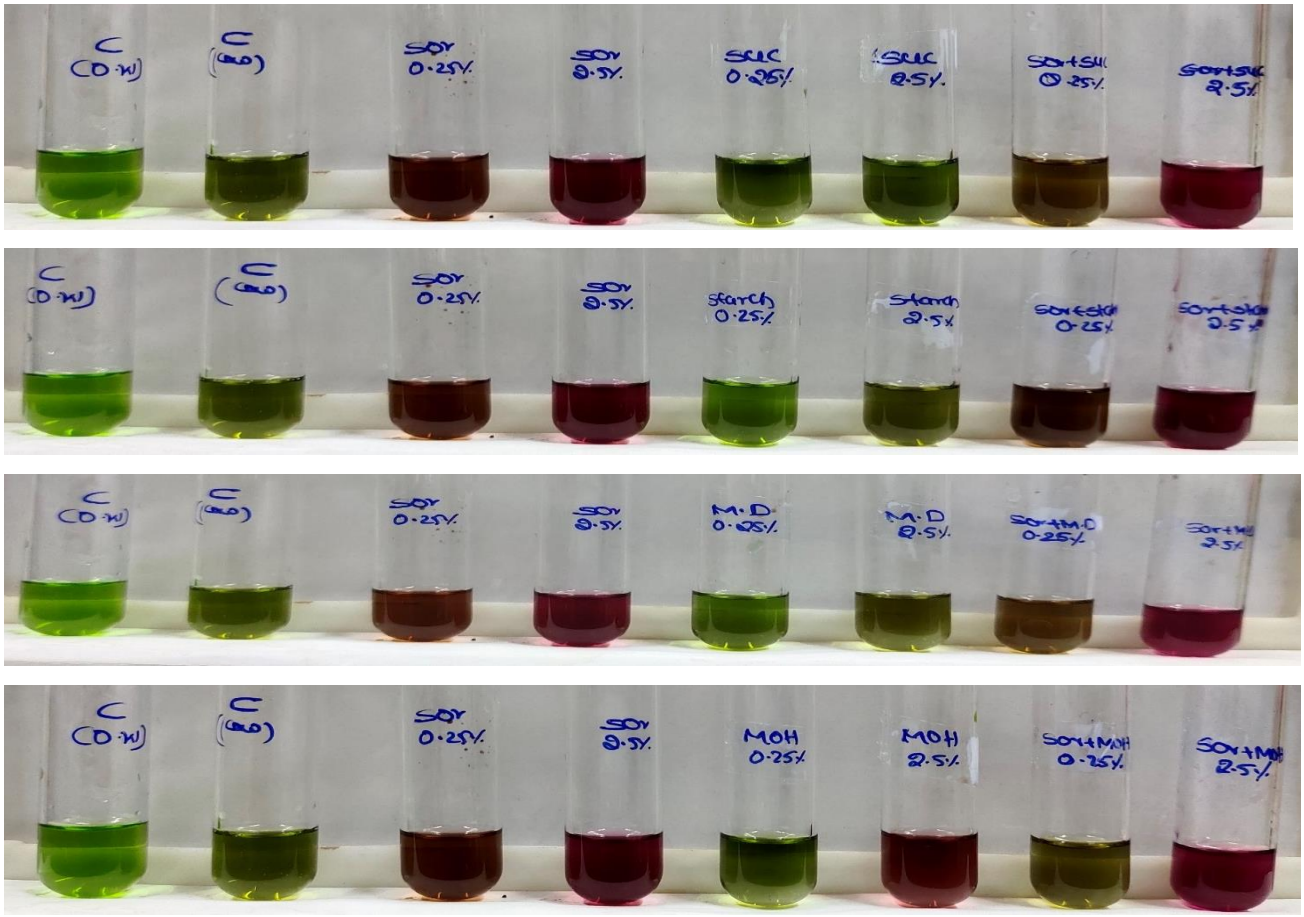
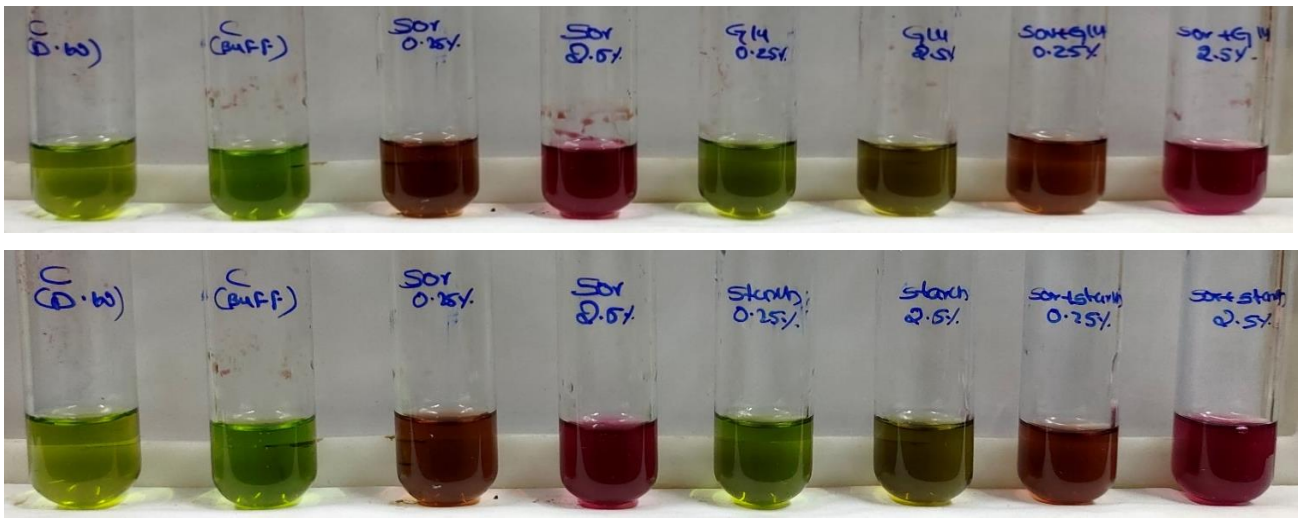


Fig:4.18 Effect of concentration of sorbitol along with carbohydrates in cow milk filtrates that was obtained from acetone with 60µl of indicator on the intensity and hue of color



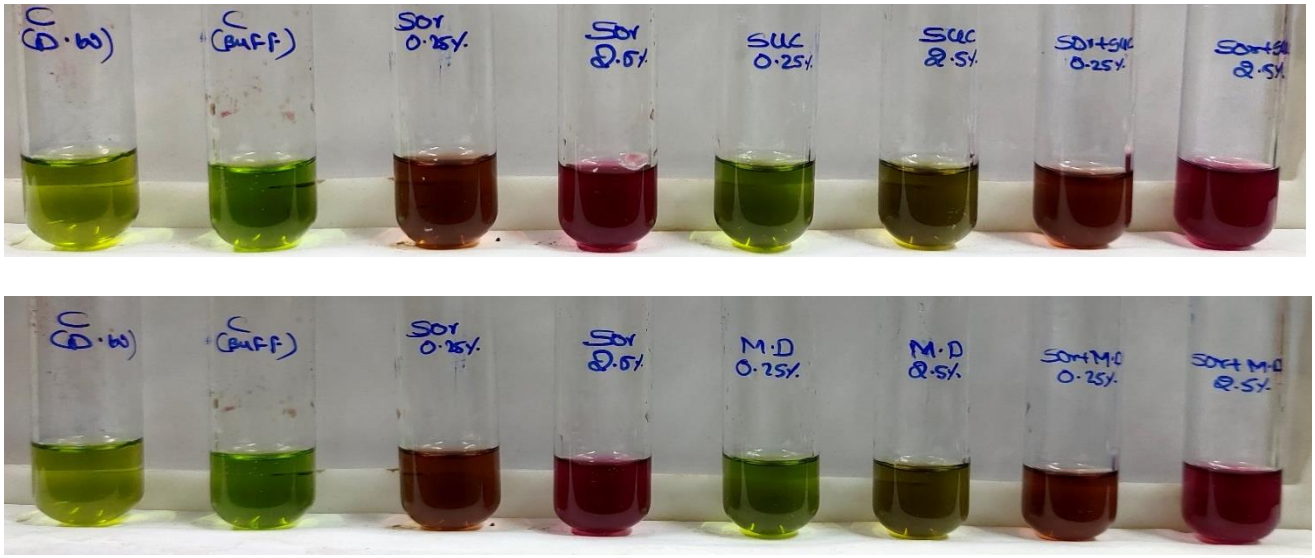
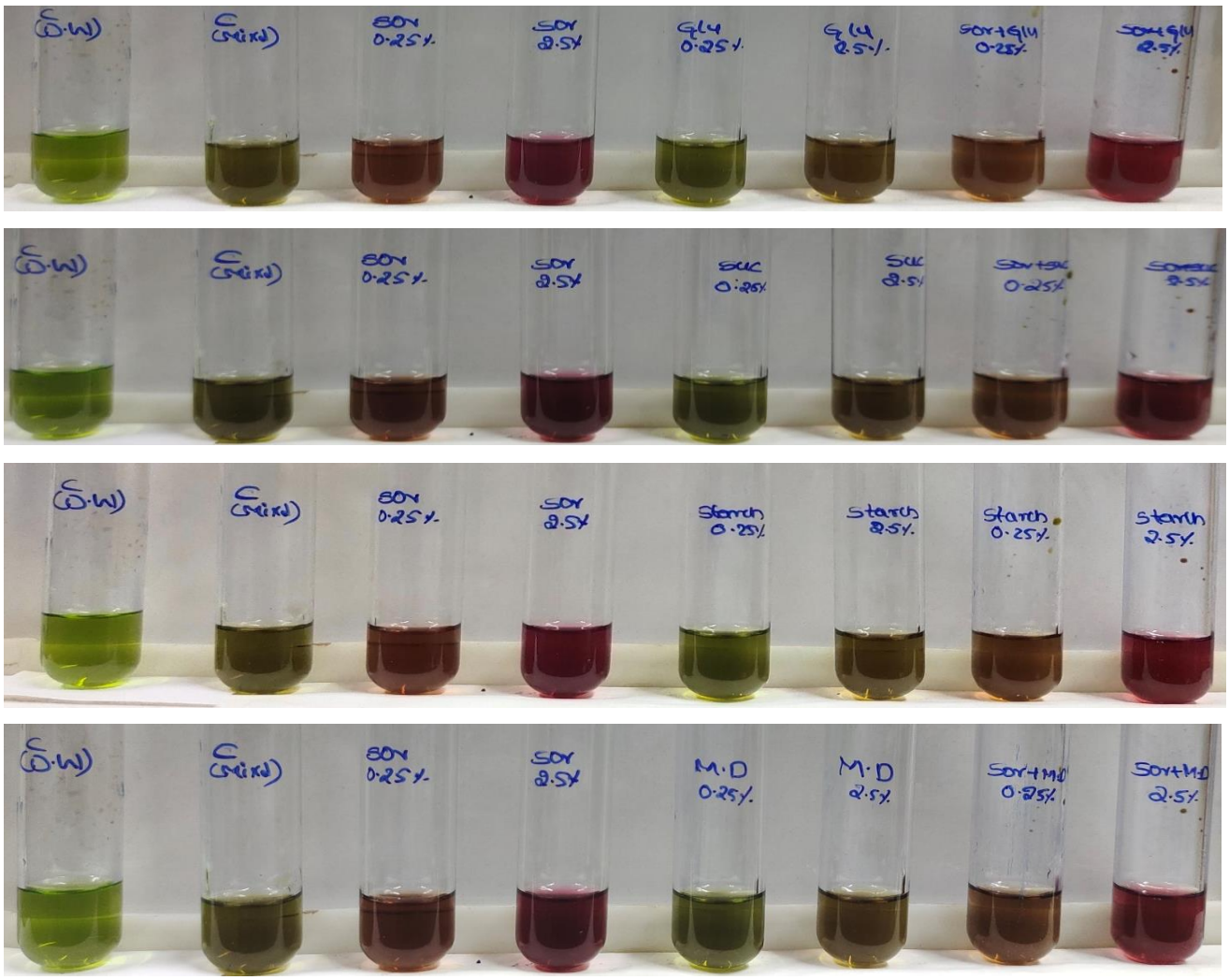


Fig:4.19 Effect of concentration of sorbitol along with carbohydrates in buffalo milk filtrates that was obtained from acetone with 60µl of indicator on the intensity and hue of color



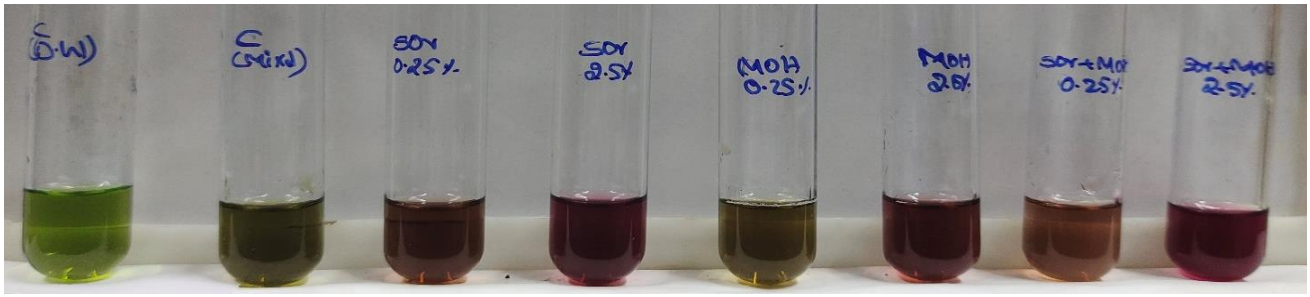
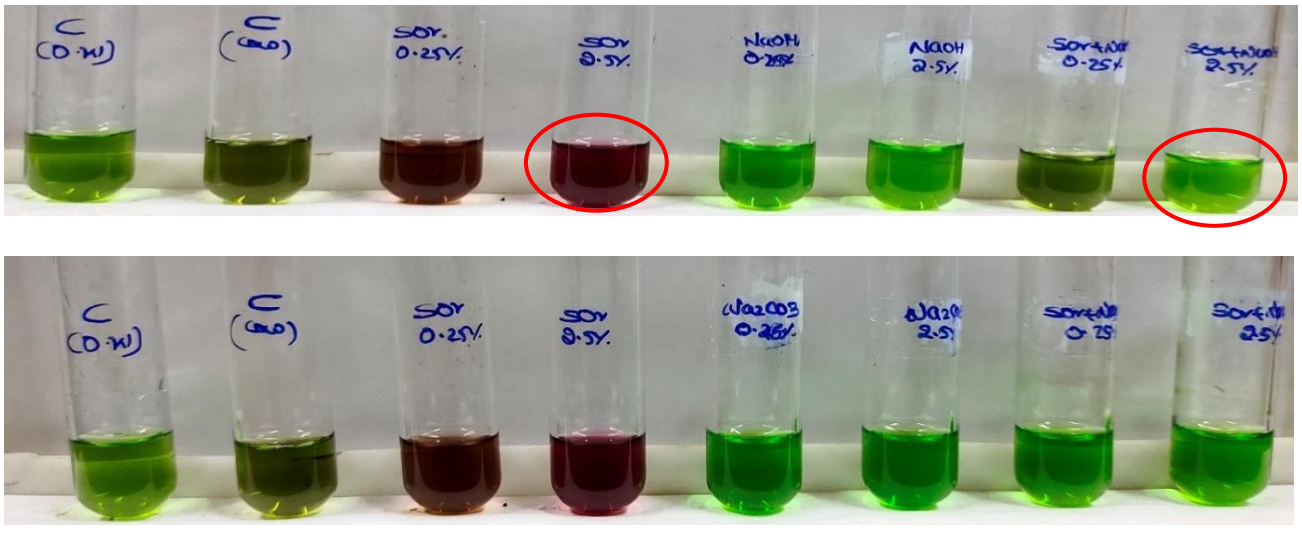


Fig:4.20 Effect of concentration of sorbitol along with carbohydrates in mixed milk filtrates that was obtained from acetone with 60µl of indicator on the intensity and hue of color

In the previous section it was observed that the visual limit of detection was improved many folds, when acetone layer collected after coagulating the caseins from milk was used to develop the color. It is evident from the results (Fig 4.18) that there was a sharp color change from green to maroonish in the milk filtrate of milk samples spiked with sorbitol in conjunction with other carbohydrates. The visual level of detection of sorbitol in conjunction with other carbohydrates studied in the present investigation was found to be 0.25%. It was already known from the previous experiments carried out in the study that carbohydrates did not interfere with sorbitol and thereby performance of the standardized color based test was not adversely affected. Similar results were obtained in case of buffalo milk and mixed milk samples spiked with sorbitol in conjunction with other carbohydrates (Fig 4.19 and Fig 4.20).

4.5.2.2 Effects of neutralizers



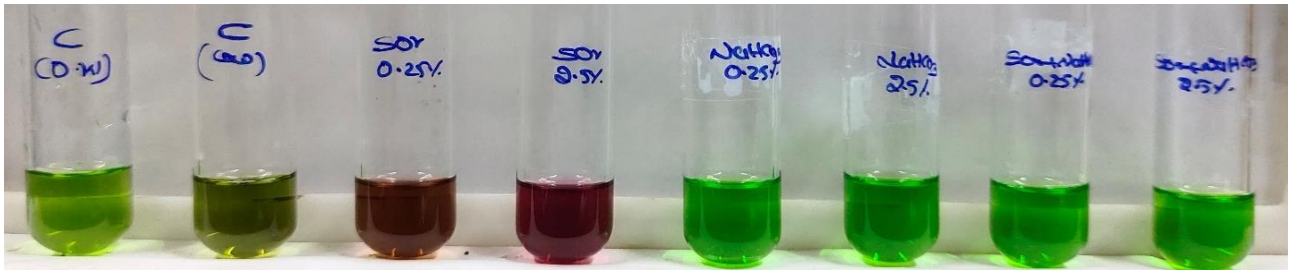


Fig:4.21 Effect of concentration of sorbitol along with neutralizers in cow milk filtrates that was obtained from acetone with 60µl of indicator on the intensity and hue of color

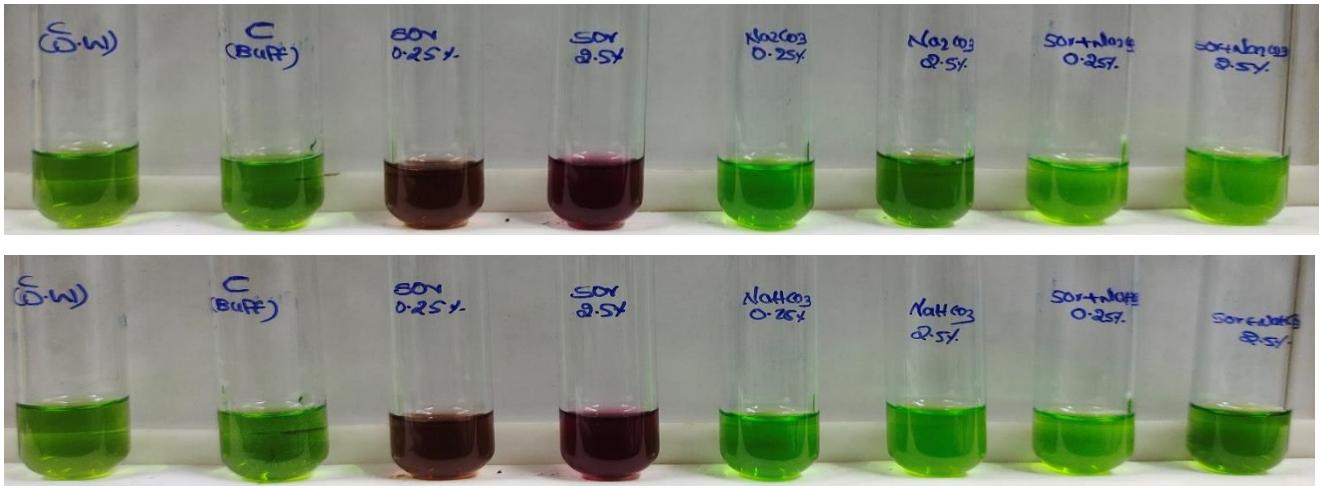


Fig:4.22 Effect of concentration of sorbitol along with neutralizers in buffalo milk filtrates that was obtained from acetone with 60µl of indicator on the intensity and hue of color

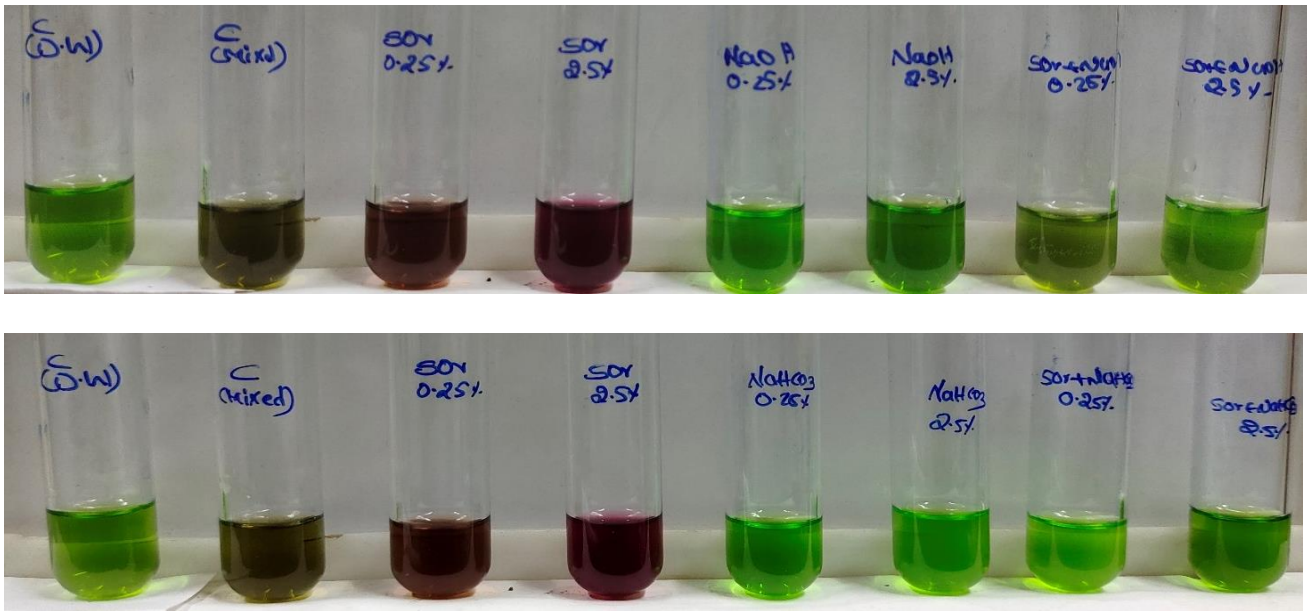


Fig:4.23 Effect of concentration of sorbitol along with neutralizers in mixed milk filtrates that was obtained from acetone with 60µl of indicator on the intensity and hue of color

It was already evident from the experiments carried out in the present investigation that neutralizers adversely affected the performance of the color based test developed to detect sorbitol in milk. However, the test was performed in the acetone collected after the coagulation of the caseins from the control and sorbitol spiked cum- neutralized samples. It is evident from the results (Fig 4.21) that color in the collected acetone layer of sorbitol spiked neutralized milk samples remained green like control milk samples without any sorbitol and neutralizer. This set of experiment further confirmed that the test will not lead to the detection of sorbitol in neutralized milk samples. Similar observations were recorded in the neutralized buffalo and mixed milk samples spiked with sorbitol (Fig 4.22 and Fig 4.23).

4.5.2.3 Effects of ammonium sulphate/ or urea

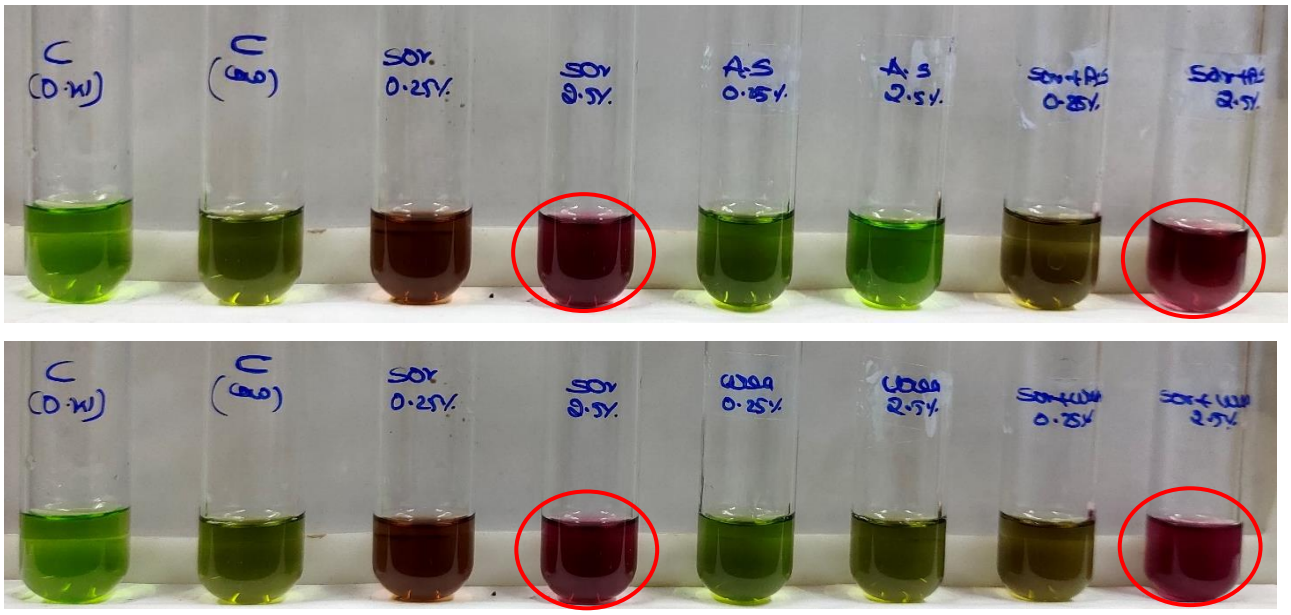


Fig:4.24 Effect of concentration of sorbitol along with urea and ammonium sulphate in cow milk filtrates that was obtained from acetone with 60µl of indicator on the intensity and hue of color

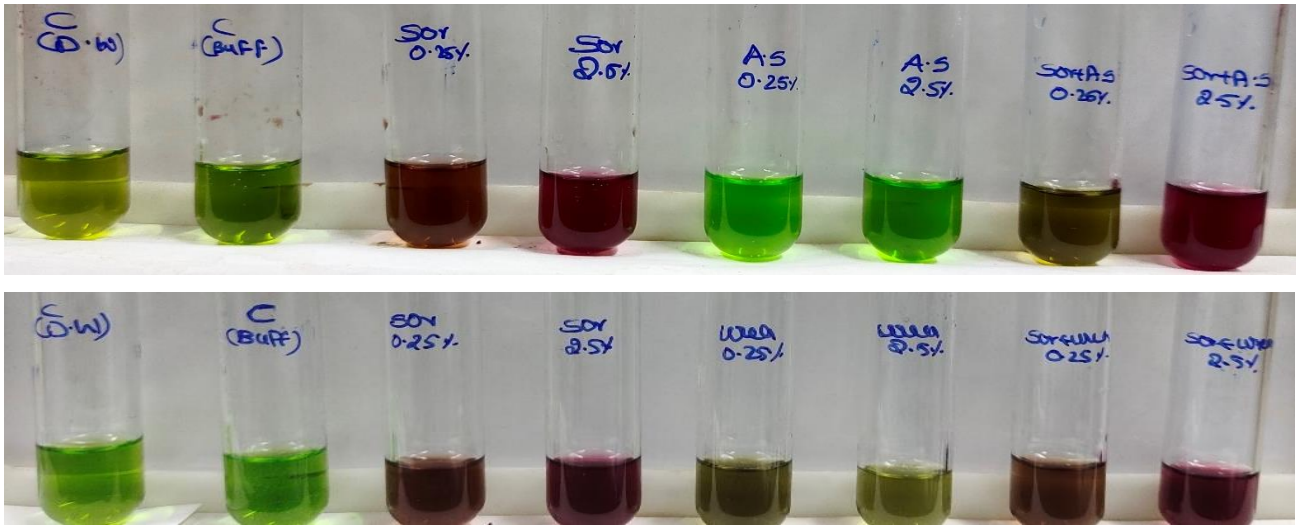


Fig:4.25 Effect of concentration of sorbitol along with urea and ammonium sulphate in buffalo milk filtrates that was obtained from acetone with 60µl of indicator on the intensity and hue of color

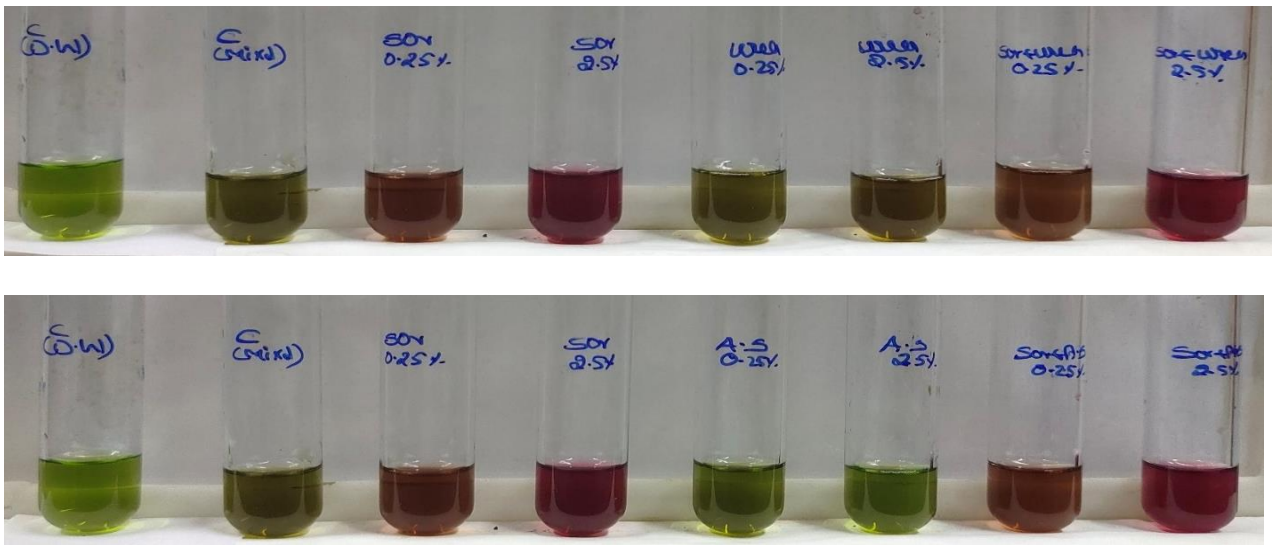


Fig:4.26 Effect of concentration of sorbitol along with urea and ammonium sulphate in mixed milk filtrates that was obtained from acetone with 60µl of indicator on the intensity and hue of color

It is evident from the results (Fig 4.24) discussed in the previous section that ammonium sulphate or urea did not affect the performance of the test in any adverse manner. From the results (Fig 4.24) it is clear that the limit of detection of sorbitol was 0.25% even in the presence of urea and ammonium sulphate and the change in color was akin to the change observed in control samples i.e., sorbitol spiked milk without any urea or ammonium sulphate spiking. These finding confirmed that standardized color based

method is workable in samples containing ammonium sulphate or urea. Similar observations were recorded in case of buffalo milk and mixed milk samples spiked with sorbitol in conjunction with urea or ammonium sulphate (Fig 4.25 and Fig 4.26).

CHAPTER-5

SUMMARY AND CONCLUSION

A study on the colorimetric method for the qualitative detection of sorbitol adulteration in milk has been conducted. In this study the standardization of mixed indicator to develop colour in milk samples containing varied concentrations of sorbitol and effect of common adulterants on the effectiveness of the standardized colour based qualitative method of sorbitol detection in milk was carried out. For this milk (cow, buffalo and mixed) samples were collected from the institute's cattle yard.

5.1 Sixty µl of the mixed indicator was found to be optimum to detect sorbitol as low as 0.1% in water.

5.2 Addition different volumes of mixed indicator (30µl, 60µl, 0.1ml and 0.2ml) to milk spiked with sorbitol, resulted into the change of color from green (control sample i.e., without any sorbitol) to pinkish. The color change was distinct to naked eyes in case of cow, buffalo milk sample spiked with sorbitol @ 5% and 2.5% in case of mixed milks at 60 µl indicator.

5.3 Detection of sorbitol in milk (cow and buffalo) after coagulation with acetone in case of control sample, the color was bright green. The level of sorbitol detection was improved a lot and now the addition of 0.5% of sorbitol was possible to detect with naked eyes.

5.4 Carbohydrates, urea and ammonium sulphate spiking did not affect the color change in sorbitol spiked samples and the results were same as in case of control and sorbitol spiked samples (5.3) visual limit of detection of sorbitol in milk (cow, buffalo, mixed) was 2.5%. However, the presence of neutralizers showed a considerable effect on the performance of the color based test both milk and filtrate obtained after precipitation with acetone.

CHAPTER-6

BIBIOLOGRAPHY

BIBIOGRAPHY

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